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Development of Novel Azabenzofuran TRPA1 Antagonists as *in vivo* Tools

Copeland, Katrina W.[a,*]; Boezio, Alessandro A. [a,*]; Cheung, Eugene[b]; Lee, Josie[c]; Olivieri, Philip[a]; Schenkel, Laurie B. [a]; Wan, Qian[a]; Wang, Weiya[d]; Wells, Mary C.[e]; Youngblood, Beth[d]; Gavva, Narender, R.[d]; Lehto, Sonya G.[d]; Geuns-Meyer, Stephanie[a]

*Corresponding authors. Tel 617-444-5350 and 617-444-5104, email kwoodin@amgen.com and aboezio@amgen.com

[a]Department of Medicinal Chemistry, Amgen Inc., 360 Binney St., Cambridge, MA 02142 USA

[b]Department of Pharmaceutics, Amgen Inc., 360 Binney St., Cambridge, MA 02142 USA

[c]Department of Neuroscience, Amgen Inc., 360 Binney St., Cambridge, MA 02142 USA

[d]Department of Neuroscience, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320 USA

[e]Department of Pharmacokinetics & Drug Metabolism, Amgen Inc., 360 Binney St., Cambridge, MA 02142 USA

Abstract

The transient receptor potential ankyrin 1 (TRPA1) channel is activated by noxious stimuli including chemical irritants and endogenous inflammatory mediators. Antagonists of this channel are currently being investigated for use as therapeutic agents for treating pain, airway disorders, and itch. A novel azabenzofuran series was developed that demonstrated *in vitro* inhibition of allyl isothiocyanate (AITC)-induced $^{45}Ca^{2+}$ uptake with nanomolar potencies against both human and rat TRPA1. From this series, compound **10** demonstrated *in vivo* target coverage in an AITC-induced flinching model in rats while providing unbound plasma concentrations up to 16-fold higher than the TRPA1 rat IC_{50}.

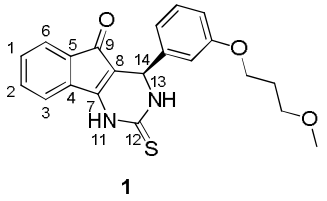
The transient receptor potential ankyrin 1 (TRPA1) channel is a nonselective cation channel that has been implicated in various physiological and sensory functions including nociception, and thermal, chemical, and mechanical sensation. It is primarily expressed in the sensory neurons of the dorsal root, trigeminal, and vagal ganglia, as well as in some non-neuronal tissues like skin and hair cells.[1] TRPA1 is activated by many pungent and reactive compounds including allyl isothiocyanate (AITC, a component of mustard oil), cinnamaldehyde, allicin (present in garlic), nicotine, and various tear gases.[2] These compounds covalently bind to the cysteine residues in the N-terminus of TRPA1 and allosterically open the channel.[2c,3] This opening increases intracellular Ca^{2+} concentrations, which initiates multiple downstream signaling processes, leading to a noxious or pain response.[4] Endogenous compounds that are products of tissue damage related to inflammation and oxidative stress (e.g., hydrogen peroxide,[5] reactive oxygen species (ROS),[5,6] 4-hydroxy-2-nonenal,[7] nitrooleic acid,[8] and cycylopentenone prostaglandins[9]) also activate TRPA1.[10] The level of TRPA1 expression increases in human sensory neurons following an inflammatory injury and in rodent models of inflammation and neuropathic pain.[11] Recently, a family suffering from Familial Episodic Pain Syndrome (FEPS), which causes episodes of debilitating upper body pain, was discovered to have a gain-of-function TRPA1 mutation.[12] In addition to playing a role in neurogenic inflammation and pain, TRPA1 functions as a mechanosensor; TRPA1 knock-out mice are less sensitive to mechanical stimuli as well as chemically-induced AITC flinching.[13]

The support from human genetics, the known roles for TRPA1 in causing pain and sensitization, and its localized expression have made TRPA1 antagonists an attractive target for novel analgesics and anti-inflammatory drugs. Antagonists may have further indications including itch[14] and respiratory diseases such as asthma and COPD.[15] Several groups have reported the identification of TRPA1 antagonists[16] and a few compounds entered into clinical trials.[17]

In previous studies with this target, we and others identified electrophilic TRPA1 antagonists that were presumed to covalently modify the TRPA1 protein and that exhibited cross-species differences in antagonist versus agonist behavior.[18] Unsatisfied with existing TRPA1 benchmark antagonists, our goal

has been to develop a tool compound that can provide high exposure *in vivo* (covering multiple fold in excess of the *in vitro* IC_{50}) to validate TRPA1 as a potential target for pain.

Figure 1: Profile of Compound **1**



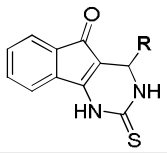
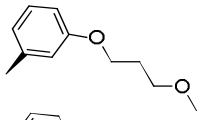
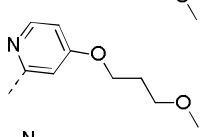
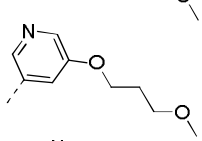
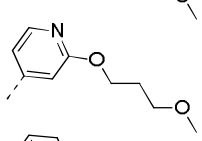
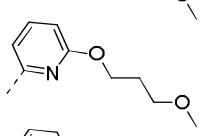
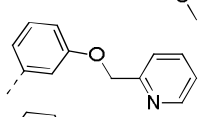
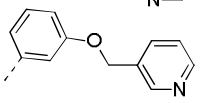
1

TRPA1 $^{45}Ca^{2+}$ IC_{50} rat (μM): 0.019
TRPA1 $^{45}Ca^{2+}$ IC_{50} human (μM): 0.026
cLogP: 2.47
Solubility, HCl / PBS / SIF (μg / mL) 5 / 4 / 12
HLM / RLM (μL / min mg) 150 / 230
Rat plasma protein binding (f_u): 0.010
Rat IV CL / Cl_u (L / h / kg): 2.9 / 290
%F: 10

A series of tricyclic 3,4-dihydropyrimidine-2-thione TRPA1 antagonists, represented by compound **1** (Figure 1), was recently reported.[19] The authors cited poor drug-like properties for this series of compounds, including low solubility, low metabolic stability, and potential toxicity of the thiourea moiety.[20] In-house profiling of this compound confirmed the low likelihood of achieving robust *in vivo* exposure (Figure 1).[21] Compound **1** displayed high turnover in human and rat microsomes, which translated to high *in vivo* clearance in rat (2.9 L/h/kg) with an associated unbound clearance (Cl_u) of 290 L/h/kg. Moreover, this clearance coupled with poor solubility led to poor oral bioavailability (%F = 10).

We sought to modify this lead to improve pharmacokinetic properties in order to achieve *in vivo* target coverage of TRPA1 toward understanding the therapeutic potential of this mechanism for further development. Our first strategy was to introduce polarity by including slightly basic heteroatoms to lower logP, with the expectation that oxidative clearance would be reduced and solubility improved. In addition, we sought to remove the ketone and thiourea moieties as toxicological liabilities have been reported for these functional groups[22] that may prevent further advancement.

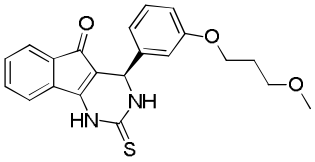
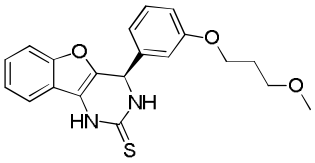
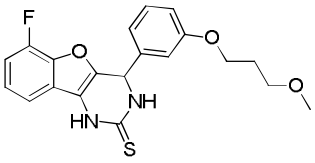
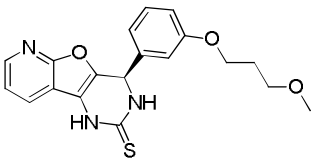
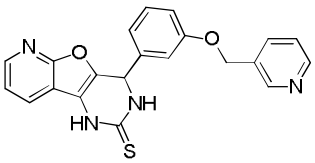
Table 1: Introduction of nitrogen atoms into the side chain

	Cmpd	TRPA1 $^{45}Ca^{2+}$ IC_{50} rat (μM)[a]	TRPA1 $^{45}Ca^{2+}$ IC_{50} human (μM)	cLogP	Solubility, HCl / PBS / SIF[b] (μg / mL)	HLM / RLM (μL / min mg)[c]
	**1** **1 (rac)**	0.019 0.028	0.026 0.030	2.47 2.47	5 / 4 / 12 1 / 10 / 23	150 / 230 -- / --[d]
	**2**	>2[e]	>2[e]	1.47	500 / 62 / 39	43 / 220
	**3**	0.53	1.2	1.47	120 / 2.8 / 14	90 / 200
	**4**	>40	>40	1.87	22 / 0 / 0	82 / 350
	**5**	1.0	0.97	1.87	17 / 36 / 35	160 / 230
	**6**	0.36	0.71	2.47	140 / 1 / 15	150 / 250
	**7**	0.23	0.30	2.47	1 / 10 / 200	180 / 310

[a]Antagonism was measured using a $^{45}Ca^{2+}$ fluorimetry assay in both rat and human TRPA1 expressed cells. No agonism was observed for all compounds, $^{45}Ca^{2+}$ EC_{50} > 40 μM. [b]HCl: 0.01 N hydrogen chloride solution in water; PBS: phosphate buffered saline, pH 7.4; SIF: fasted state simulated intestinal fluid, pH 6.8 containing 5 mM sodium taurochol, 1.5 mM lecithin, 2.9 mM KH_2PO_4, 0.22 M KCl. [c]*In vitro* (HLM = human liver microsomes; RLM = rat liver microsomes). [d]HLM/RLM data not obtained for the racemate. [e]No IC_{50} value obtained; instead single point $^{45}Ca^{2+}$ % Inhibition at 2 μM was 1% in humans and showed 0% in rat.

Our first tactic toward lowering the logP was to introduce nitrogen atoms into the chiral aromatic ether side chain of **1** (Table 1).[23] First, nitrogen was positioned around the side-chain aryl ring, but these permutations led to significant losses in potencies (compounds **2**-**5**). Compound **2** showed significant improvement in solubility and **3** showed a slight improvement in both solubility and human microsomal stability compared to **1**, but poor potency prevented further progression of these compounds. Replacement of the 3-methoxypropoxy side chain with pyridine rings was better tolerated and compounds **6** & **7** showed TRPA1 antagonism. Both these compounds showed improved solubility, but were unstable in liver microsomes.

Table 2: Modification of the core

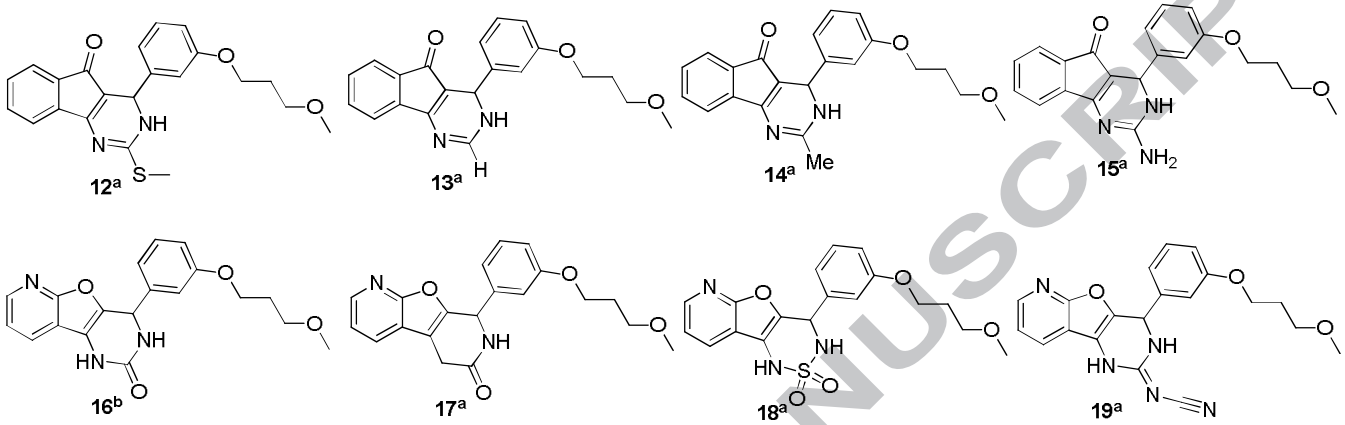
	Cmpd	$^{45}Ca^{2+}$ IC_{50} rat (μM)[a]	$^{45}Ca^{2+}$ IC_{50} human (μM)	cLogP	Solubility, HCl / PBS / SIF (μg / mL)[b]	HLM / RLM (μL / min mg)[c]
	**1** **1 (rac)**	0.019 0.028	0.026 0.030	2.47 2.47	5 / 4 / 12 1 / 10 / 23	150 / 230 – / –[d]
	**8**	0.23	0.59	2.97	1 / 1/ 23	90 / 350
	**9**	0.14	0.20	3.34	14 / 17 / 66	70 / 140
	**10**	0.045	0.17	2.20	28 / 21 / 46	30 / 90
	**11**	1.3	0.94	2.20	200 / 15 / 21	100 / 140

[a]Antagonisum was measured using a $^{45}Ca^{2+}$ fluorimetry assay in both rat and human TRPA1 expressed cells. No agonism was observed for all compounds, $^{45}Ca^{2+}$ EC_{50} > 40 μM. [b]HCl: 0.01 N hydrogen chloride solution in water; PBS: phosphate buffered saline, pH 7.4; SIF: fasted state simulated intestinal fluid, pH 6.8 containing 5 mM sodium taurochol, 1.5 mM lecithin, 2.9 mM KH_2PO_4, 0.22 M KCl. [c]*In vitro* (HLM = human liver microsomes; RLM = rat liver microsomes. [d]HLM/RLM data not obtained for the racemate.

Modifying the core instead led to more dramatic changes in the physical-chemical properties of this series (Table 2).[24] Replacing the carbonyl of indenone **1** by incorporating the oxygen into the ring resulting in a benzofuran (compound **8**), led to a significant loss in potency on rat and human TRPA1, and no marked change in solubility or microsomal stability. Addition of a fluorine atom adjacent to the oxygen (compound **9**) led to an increase in both potency and microsomal stability. Finally, the replacement of the fluorine in **9** with a nitrogen (compound **10**) created a new azabenzofuran core with significantly restored potency, especially against rat TRPA1. As anticipated, the installation of an ionizable nitrogen reduced the clogP, which led to an improvement in both solubility and microsomal stability of the compound.

When the 3-(pyridin-2-ylmethoxy)phenyl side chain of **7** was combined with the new azabenzofuran core, the solubility was further increased in acidic media but a loss of potency was also observed (compound **11**).

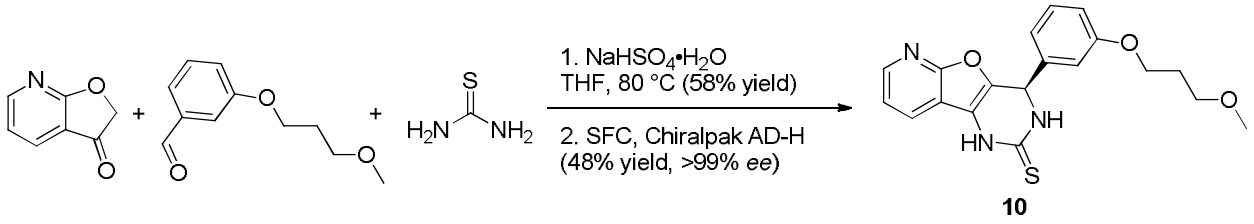
Figure 2: Inactive replacements for the thiourea moiety



[a] Compounds had either TRPA1 $^{45}Ca^{2+}$ IC_{50} > 40 μM or inhibition <20% at 2 μM. Compounds 16-19 did not show agonism, $^{45}Ca^{2+}$ EC_{50} > 40 μM; Compounds 12-15 were not tested. [b] TRPA1 $^{45}Ca^{2+}$ IC_{50} rat was 6.3 μM for this compound; no agonsim observed.

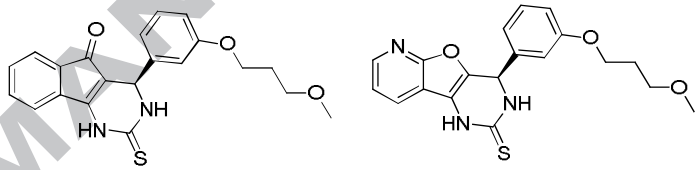
In addition to improving the physical-chemical properties, we also sought to replace the thiourea in either the indenone or azabenzofuran core, depending on synthetic ease (Figure 2). Methylating the thiourea (compound **12**) led to loss of activity on TRPA1. Replacing the cyclic thiourea with dihydropyrimidine **13**, methyl dihydropyrimidine **14**, and amino dihydropyrimidine **15** also led to loss of activity. In the azabenzofuran core, the substitution of the thiourea for a urea to produce **16** led to a dramatic loss in potency on TRPA1.[25] Converting the urea to a lactam to form compound **17** also did not show any antagonism. The thiourea bioisosteres[26] sulfamide **18** and 2-cyanoguanidine **19** were also showed no antagonism of TRPA1. Thus our preliminary screening results showed that the thiourea as essential for maintaining potency on TRPA1. The importance of the thiourea may perhaps be attributed to the potential for covalent binding with cysteine residues present in the protein.[27]

Scheme 1: Synthesis of **10** via the Biginelli reaction



The azabenzofuran thioureas, as exemplified by **10**, were synthesized using a modification of the three-component Biginelli reaction reported by Gijsen *et al.* (Scheme 1).[28] Replacing the hydrochloric acid as previously reported with sodium bisulfate in the coupling increased the yields from ~10% to above 50%. The most potent racemic products were then separated using supercritical fluid chromatography (SFC, Chiralpak AD-H column) to provide each enantiomer with >99% *ee*.

Table 3. *In Vitro* and *In Vivo* ADME Properties of Compound **1** and **10**



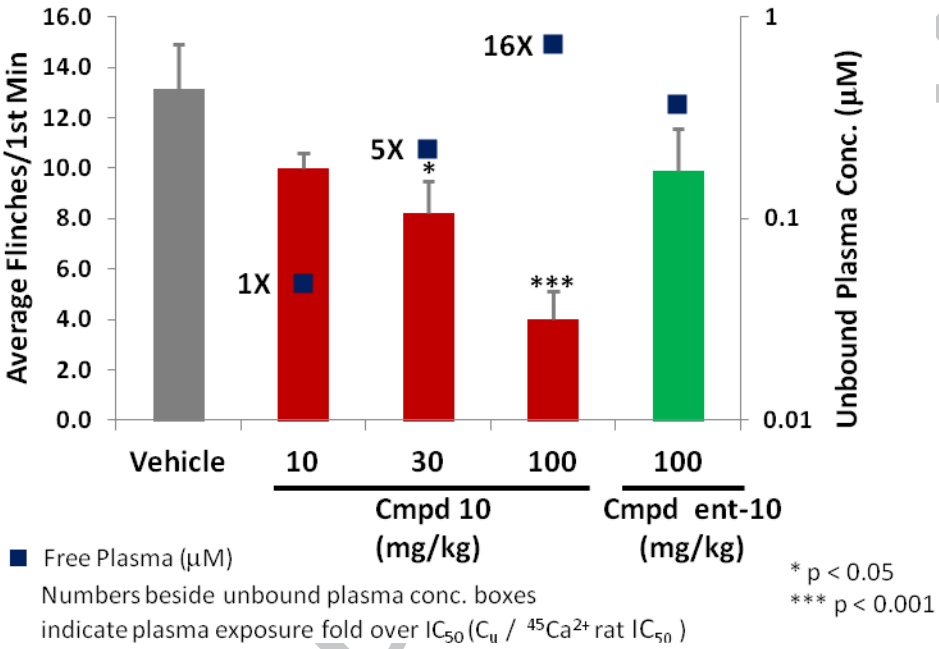
	1	**10**
RLM (µL / min mg)	230	90
Rat plasma protein binding (f_u)[a]	0.010	0.085
Rat[b] *iv* CL / CL_u (L / h / kg)	2.9 / 290	3.0 / 36
Vss (L / kg) / $T_{1/2}$ (h)	4.7 / 2.1	4.0 / 1.2
Unbound rat Cmax (µM)[c,d] / % F at 10 mg/kg	0.005 / 10	--
at 30 mg/kg	--	0.082 / 13
at 100 mg/kg	--	0.612 / 79

[a] f_u: unbound fraction; separation method = equilibrium dialysis. [b] *In vivo* experiments with male Sprague-Dawley rats (n = 3); *iv*, 0.5 mg/kg (DMSO). [c] All dose levels were run *po* in 2% HPMC, 1% Tween 80 in H_2O, pH 2.2 with MSA. [d]The 10 and 30 mg/kg groups of animals were fasted during PK studies, the 100 mg/kg were fed.

The rat *in vivo* clearance and steady state volume of distribution were very similiar for **1** and **10** (Table 3). The unbound clearance (CL_u) of azabenzofuran **10** was approximately 8-fold better than **1** as a result of its higher free fraction and comparable clearance. The improved solubility of **10** relative to **1** allowed compound dosing at 100 mg/kg, which likely saturated the primary elimination mechanisms based on the nonlinear increase in bioavailability between the 30 and 100 mg/kg doses (13% compared to 79%). The incorporation of polarity in the azabenzofuran replacement of the indenone core resulted in a slight loss of potency, which was well made up for by the reduction in unbound clearance and increase in solubility.

Since this change led to a dramatic improvement in unbound plasma concentration (C_u) for **10**, 14-fold over the rat IC_{50} at the 100 mg/kg dose, this compound was advanced into an AITC-induced flinching model.

Figure 3: AITC (0.1%)-Induced Flinching in Rats



Compound **10**,[29] as well as its inactive enantiomer **ent-10**,[30] which served as a negative control, were then evaluated in a target-coverage model using AITC-induced flinching in rats (Figure 3).[31] AITC is an agonist of TRPA1 that covalently modifies key cysteine and lysine residues in the N-terminus of the channel, and activates the C-mechano-heat nociceptors to release neuropeptides leading to inflammatory edema, hyperalgesia and pain.[32] Male Harlan Sprague-Dawley rats[33] were dosed orally with either vehicle (2% HPMC/1% Tween-80), the TRPA1 antagonist **10**, or **ent-10**. After 1 hour, each animal's left ventral hind paw was injected with the agonist AITC (0.1%). The nocifensive behaviors (including flinching and licking) were then observed and recorded during the first minute post injection. The unbound plasma concentrations (C_u) for the 10, 30, and 100 mg/kg doses were 0.048 ± 0.010 (n=8), 0.22 ± 0.04 (n=6), and 0.74 ± 0.14 (n=8) µM, respectively, covering the *in vitro* rat TRPA1 IC_{50} at approximately 1X, 5X, and 16X, respectively. Significant reversal of AITC-induced flinches was observed at 30 and 100 mg/kg with

compound **10**.[34] As expected, the inactive enantiomer **ent-10** showed no blockade of AITC-induced flinches at 100 mg/kg with an exposure (C_u = 0.37 ± 0.12 μM) in a range where **10** was efficacious, supporting the likely on-mechanism effect of **10**.

In conclusion, we have described a novel azabenzofuran thiourea core that displays good *in vitro* antagonism of the TRPA1 channel. The genesis of the series came from modification of Compound **1** in an attempt to improve solubility and clearance by lowering the logP and introducing ionizable atoms. Although we were unable to replace the thiourea, we identified a compound (**10**) that provides an unbound plasma concentration 16-fold in excess of the *in vitro* IC_{50} and demonstrates significant effect in an on-target biochemical challenge study (AITC-induced flinching). Further investigations with this tool compound in various pain models will be disclosed in due course.

Acknowledgements

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[21] Data reported in Figure 1 obtained internally.

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[23] Compounds were screened for antagonism using a $^{45}Ca^{2+}$ fluorimetry assay in both rat and human TRPA1 expressed cells. See reference 18 for experimental details on the fluorimetry assay used for this program.

[24] Racemic compounds that showed potency were further separated using chiral chromatography and were further tested as individual enantiomers. Assignment of stereochemistry was made in analogy to Compound **1** , see reference 19. As was observed by Gijsen *et al.*, one enantiomer in these new compounds was potent in the $^{45}Ca^{2+}$ assay, and the other enantiomer showed minimal TRPA1 antagonism.

[25] This trend was similarly observed in the indenone core as reported by Gijsen *et al.* in reference 19.

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[30] Data for **ent-10**: $^{45}Ca^{2+}$ human IC_{50}: >40 mM; $^{45}Ca^{2+}$ rat IC_{50}: undefined (failed to achieve proper hill fit to generate IC_{50} curve).

[31]For experimental details on the AITC mechanical challenge model used for this program see: de Oliveira, C.; Garami, A.; Lehto, S.; Pakai, E.; Tekus, V.; Pohoczky, K.; Youngblood, B.; Wang, W.; Kort, M.; Kym, P.; Pinter, E.; Gavva, N.; Romanovsky, A.. *Journal of Neuroscience*, **2014**, *34* , 4445.

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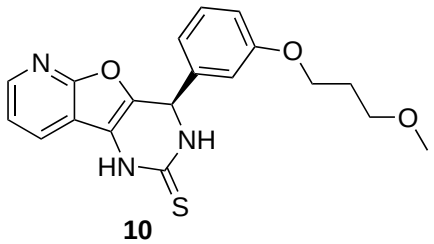
[34] No significant effects on open-field activity were observed with this compound.

Supporting Information:

A mixture of 3-(3-methoxypropoxy)benzaldehyde (7.19 g, 37.0 mmol), sodium hydrogensulfate hydrate (0.102 g, 0.740 mmol), furo[2,3-b]pyridin-3(2H)-one (5.0 g, 37.0 mmol) and thiourea (5.63 g, 74.0 mmol) in 100.0 mL of THF was heated at 80°C for 48 h until complete by LCMS. The reaction mixture was then cooled to rt and diluted with water (300 mL) and extracted with EtOAc (2 × 200 mL). The organic extracts were washed with brine (1 × 150 mL) and dried over Na_2SO_4. The solution was filtered and concentrated in vacuo to give a yellow solid. The crude material was absorbed onto a plug of silica gel and purified via column chromatography (Biotage) using a 350 g column eluting with 0 % to 30% DCM :EtOAc to provide 4-(3-(3-methoxypropoxy)phenyl)-3,4-dihydropyrido[3',2':4,5]furo[3,2-d]pyrimidine-2(1H)-thione (7.95 g, 21.52 mmol, 58% yield) as yellow solid. The compound was then separated into its enantiomers via prep-scale supercritical fluid chromatography using a Chiralpak AD-H 2 x 15 cm, 5 micron column with an isocratic 45% carbon dioxide / 55% methanaol with 0.2% diethyl amine solvent system at 60 mL/min, to yield *R*-4-(3-(3-methoxypropoxy)phenyl)-3,4-dihydropyrido[3',2':4,5]furo[3,2-d]pyrimidine-2(1H)-thione (**10**, 3.8 g, 48% yield). MS ESI *m/z*

= 370.0 (M+H). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.93 (quin, *J*=6.33 Hz, 2 H) 3.23 (s, 3 H) 3.45 (t, *J*=6.26 Hz, 2 H) 4.00 (t, *J*=6.36 Hz, 2 H) 5.97 (s, 1 H) 6.79 - 6.98 (m, 3 H) 7.31 (t, *J*=8.02 Hz, 1 H) 7.35 - 7.45 (m, 1 H) 8.13 - 8.39 (m, 2 H) 9.32 (s, 1 H) 10.88 - 11.21 (m, 1 H). $[\alpha]_D^{20}$ = -1.38° (*c* 1.0, EtOH)



10

TRPA1 $^{45}Ca^{2+}$ IC_{50} rat (μM): 0.045
TRPA1 $^{45}Ca^{2+}$ IC_{50} human (μM): 0.17

Compound **10** provides an unbound plasma concentration 16-fold in excess of the *in vitro* TRPA1 IC_{50} and demonstrates significant effect in an on-target biochemical challenge study (allyl isothiocyanate-induced flinching).