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Design and pharmacological evaluation of PF-4840154, a non-electrophilic reference agonist of the TrpA1 channel

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ABSTRACT

TrpA1 is an ion channel involved in nociceptive and inflammatory pain. It is implicated in the detection of chemical irritants through covalent binding to a cysteine-rich intracellular region of the protein. While performing an HTS of the Pfizer chemical collection, a class of pyrimidines emerged as a non-reactive, non-covalently binding family of agonists of the rat and human TrpA1 channel. Given the issues identified with the reference agonist Mustard Oil (MO) in screening, a new, non-covalently binding agonist was optimized and proved to be a superior agent to MO for screening purposes. Compound **16a** (PF-4840154) is a potent, selective agonist of the rat and human TrpA1 channel and elicited TrpA1-mediated nocifensive behaviour in mouse.

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TrpA1 is a member of the Transient Receptor Potential (Trp) ion channel family and is involved in the perception of nociceptive and inflammatory pain. This pain can be triggered by mechanical stimuli, endogenous mediators such as Bradykinin[1] and 4-hydroxynonenal (4-HNE **1**),[2] peroxides and prostaglandins,[3] exogenous chemical substances including pungent natural compounds (Mustard Oil **2**) as well as environmental irritants such as formalin,[4] acrolein[5,6] and lachrymatory agents.[7] The mechanism of TrpA1 activation by noxious, electrophilic compounds has been linked to their covalent binding to the sulfide moiety of specific cysteines residues situated on the intracellular N-terminus of the TrpA1 protein.[8] The evaluation of TrpA1 as a drug target is complicated by interspecies differences; for instance, caffeine **3** is reported to be an agonist of the mouse TRPA1 channel, but an antagonist of the human channel[9] (Fig. 1).

As part of an ongoing program to assess the druggability of the TrpA1 channel, the Pfizer chemical collection was screened using a FLIPR-based assay at 10 μM concentration in an antagonist format, using Mustard Oil as the reference agonist. HTS triage indicated that a large percentage of hits were actually desensitizing agonists, notably electrophiles.[7] Mustard Oil itself desensitizes the TrpA1 channel[10] and thus behaves as a functional antagonist, in a fashion similar to the effect of capsaicin on the TrpV1 receptor.[11] As a reference agonist for in vitro assays, Mustard Oil suffers from serious drawbacks; it is toxic, volatile, has an unpleasant smell and is a potent lachrymator. Furthermore, in our hands its limited solubility and aqueous instability was linked to inconsistent screening data. While alternatives to Mustard Oil such as tear-gas derivatives have recently been described,[12] a non-reactive, non-volatile stable agonist would clearly enable the development of a safer and more robust screening assay.

Our goal was the identification of a suitable pharmacological tool, with potent agonist activity at both the rat and the human

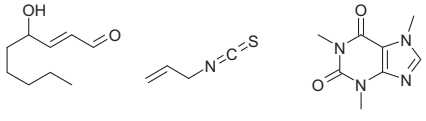

Figure 1. Structure of HNE **1**, Mustard Oil (MO) **2** and caffeine **3**.

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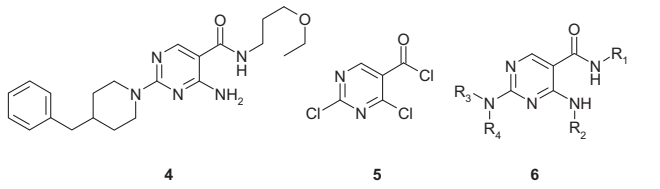


Figure 2. Structure of non-covalent hit **3**, versatile template **5** and target compounds **6**.

channel, appropriate solubility and some measure of selectivity against other drug targets to enable further assessment of the TrpA1 channel pharmacology.

Preliminary triage of non-electrophilic ligands of the TrpA1 channel identified compound **4** as a weak (6 μM) agonist of the human TrpA1 channel. The central core of **4** is a parallel chemistry-enabled template, as the commercially available 2,4-dichloropyrimidine-5-carbonyl chloride **5** is amenable to sequential functionalisation[13] to 2,4-diamino-pyrimidine-5-carboxylic acid amide derivatives **6** (Fig. 2).

In order to limit the very large number of potential targets, substituents were optimized sequentially. Preliminary data obtained by screening nearest neighbours of **4** showed that small alkyl groups such as cyclopropyl and isobutyl at R_2 were beneficial to agonist potency. Replacing the 4-benzyl piperidine with the more polar and synthetically enabled 4-benzylpiperazine designated **8** as a key intermediate for optimization of R_1 (Scheme 1).

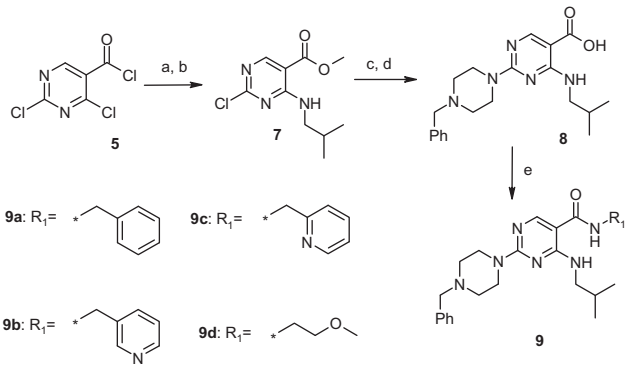
Pleasingly, **9a** (R_1 = benzyl) was readily identified as a moderately potent agonist of both the rat and human TrpA1 channels (Table 1). The 3-picolyl group (**9b**) was tolerated, but the 2-picolyl (**9c**) and methoxyethyl (**9d**) derivatives significantly lost potency. Keeping R_1 as benzyl and R_2 as either cyclopropyl or isobutyl, a range of piperazines was prepared from intermediates **12** and **13** (Scheme 2).

Amides **14a** and **14b** showed very different potencies, raising the possibility that the affinity of **14b** is due to the reaction between a sulfur atom from a cysteine residue of the protein and the fluoroaromatic ring, a reaction known to occur at room temperature.[14] Reductive aminations on derivative **15** showed that the cyclopropyl derivative was markedly less potent than its isobutyl analogue (**15a** vs **9a**). While *n*-butyl derivative **15b** displayed low activity, the cyclic derivative **15c** showed some potency at both the rat and human receptor (Table 1).

No attempts were made to separate the enantiomers of **16a**. Further profiling of PF-4840154 was initiated to establish its suitability as a reference agonist of the TrpA1 channel.

The compound was stable in aqueous solution, with a solubility (pH 7.2) of 3 μg/mL (6.4 μmol/L). This solubility, while limited, proved to be well above the top concentration (1.5 μmol/L) needed to use PF-4840154 as reference agonist in a screening assay.[15]

Before ascertaining that the in vitro activity of PF-4840154 at the TrpA1 channel was indeed translated into in vivo functional efficacy, the compound was screened on a range of targets to assess its selectivity. Significant blockade of the hERG channel[16] was observed (IC_{50} 580 nM). When tested on a broad range of over 100 proteins (Cerep Panel), micromolar activity on less than 10% of the target set was observed with weak inhibition of reference compound binding at the Sigma (1.1 μM) and the D_3 (1.9 μM) receptors, the chloride channel (1.5 μM) and the Dopamine (1.6 μM) and Noradrenaline (2.8 μM) transporters. In our in-house screens, PF-4840154 had no effect on established pain targets such as the hTrpV1, hTrpV4 and hTrpM8 channels, in either agonist or antagonist modes. This selectivity suggests that the pharmacology of the compound is likely to be TrpA1 mediated.



Scheme 1. Synthetic methodology for the variation of R_1. Reagents and conditions: (a) methanol (1.2 equiv), Et_3N (1.2 equiv), DCM, 0 °C, 2 h, 57%; (b) isobutylamine (1.05 equiv), Et_3N (1.05 equiv), DCM, 0 °C, 16 h, 82%; (c) 4-benzylpiperazine (1.1 equiv), Et_3N (1.1 equiv), acetonitrile, rt 1 h, 97%; (d) NaOH (20 equiv), MeOH–water (50:50), reflux, 1 h, 99%; (e) amine R_1-NH_2 (1 equiv), Et_3N (1 equiv), HATU (1 equiv), DMF, 6 h, rt.

Table 1
Agonist activity[a] at the rat and human TrpA1 channels[15]

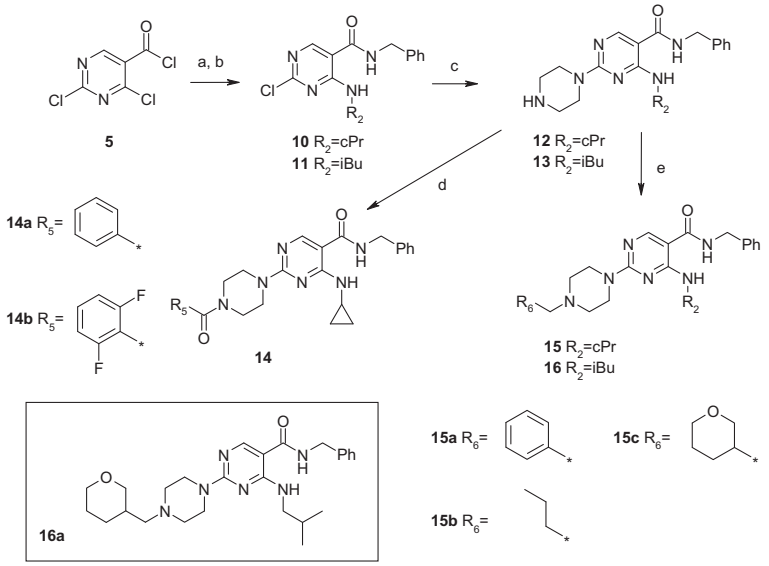
Compound	rTrpA1 EC_{50}, nM	hTrpA1 EC_{50}, nM
2 (MO)	5813 ± 246 (*n* = 330)	1120 ± 165 (*n* = 10)
9a	175	79
9b	215	49
9c	2250	na
9d	581	na
14a	5600	na
14b	186	27
15a	468	na
15b	30% (10 μM)	na
15c	848	109
16a	97 ± 4.7 (*n* = 136)	23 ± 0.64 (*n* = 773)

[a] Values are geometric means of a minimum of two experiments, unless stated otherwise.

Having established the selectivity of PF-4840154 in vitro, we needed to establish its functional activity at the TrpA1 channel in vivo.

The mouse Formalin test is a well established pain model in which intraplantar (*i.pl.*) injection of the TrpA1 activator formalin elicits paw licking behaviour.[4] In a similar experiment, we examined the effects of intraplantar (*i.pl.*) injections of PF-4840154 on licking behaviour in TrpA1 WT and KO mice. The *i.pl.* injections of PF-4840154 caused a significant increase in the time spent licking of the treated paws as compared to vehicle (*n* = 4). PF-4840154-evoked nocifensive behaviours were significantly less in TrpA1 KO mice. Furthermore, pre-treatment with the selective TrpA1 antagonist HC-030031[4] significantly reduced the time spent licking the injected paws in PF-4840154-treated animals.[15] Thus both genetic and pharmacological disruption of the TrpA1 channel blocked the effects of PF-4840154 in this model, demonstrating the functional agonism of PF-4840154 at the TrpA1 channel (Fig. 3).

In conclusion, having conducted an HTS we identified a novel class of non-covalent TrpA1 agonists that was successfully utilized as a replacement for the well established yet problematic reference agonist Mustard Oil. PF-4840184 proved to be a potent and selective agonist of the human and rat TrpA1 channel in vitro, and a potent functional agonist of the mouse TrpA1 channel in vivo. This compound is adequately soluble at pH 7.2, stable in aqueous solution, non-volatile, and proved to be vastly superior to Mustard Oil as a new reference agonist for the HTS of compound collections.



Scheme 2. Reagents and conditions: (a) benzylamine (1.05 equiv), Et_3N (1.05 equiv), DCM, −10 °C, 2 h 86%; (b) cyclopropylamine or isobutylamine (1.05 equiv), Et_3N (1.05 equiv), DCM, 0 °C, 16 h, 75–82%; (c) piperazine (10 equiv), acetonitrile, rt, 1 h, 93–96%, amine (4-benzylpiperazine) (1.1 equiv), Et_3N (1.1 equiv), acetonitrile, rt 1 h, 97%; (d) acid (1 equiv), Et_3N (1 equiv), HATU (1 equiv), DMF, 6 h, rt; (e) aldehyde (1.5 equiv), $NaBH(OAc)_3$ (2 equiv), DCM, AcOH 1%, 0 °C → rt, 16 h, 78–89% with tetrahydro-pyran-3-carbaldehyde.

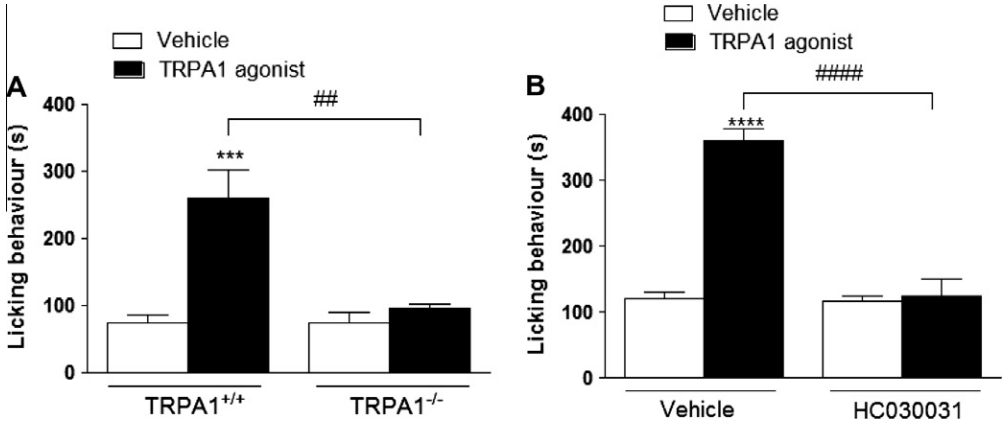


Figure 3. The in vivo effect of PF-4840184 is mediated by TrpA1. (A) Total time (s) spent licking the TRPA1 agonist (**16a**, PF-4840154, 30 nmol) or vehicle injected paws of female $TRPA1^{+/+}$ or $TRPA1^{-/-}$ mice ($n = 4$) over 1hr period. In $TrpA1^{+/+}$ mice, PF-4840154 elicits robust licking behaviour upon *i.pl.* administration, but has no significant effect on $TrpA1^{-/-}$ animals. (B) Total time (s) spent licking the TRPA1 agonist (**16a**, PF-4840154, 30 nmol) or vehicle injected paws of female CD1 mice pretreated with TrpA1 antagonist HC030031 (100 mg/kg) or vehicle (10% DMSO in saline) over 1 h period ($n = 4$). Pre-treatment with the selective TrpA1 antagonist HC-030031 abolishes the effect of PF-4840154 on licking behaviour. All data are represented as mean ± SEM and analysed by One-Way ANOVA followed by Bonferroni's test (A) $^{***}p$ <0.001 versus vehicle-treated paws and ##p <0.01 versus TRPA1 agonist-treated paws, (B) $^{****}p$ <0.001 versus vehicle-treated paws and ####p <0.0001 TRPA1-agonist treated paws of mice pre-treated with HC030031.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.035.

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