

TRPA1 as a drug target—promise and challenges

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Abstract The transient receptor potential ankyrin 1 (TRPA1) channel is a nonselective cation channel belonging to the superfamily of transient receptor potential (TRP) channels. It is predominantly expressed in sensory neurons and serves as an irritant sensor for a plethora of electrophilic compounds. Recent studies suggest that TRPA1 is involved in pain, itch, and respiratory diseases, and TRPA1 antagonists have been actively pursued as therapeutic agents. Here, we review the recent progress, unsettled issues, and challenges in TRPA1 research and drug discovery.

Keywords TRPA1 · Drug target · Promise · Challenges

Introduction

Transient receptor potential ankyrin 1 (TRPA1) is one of the 28 members of the transient receptor potential (TRP) channel family and the sole member of the TRPA subfamily in mammals. Like all TRP channels, TRPA1 possess a tetrameric structure with a single pore present at the central axis. Each subunit contains six transmembrane alpha helices (labeled S1–S6) and intracellular N-terminal and C-terminal domains (see Fig. 1). The pore-forming selectivity filter is positioned between the S5 and S6 transmembrane helices. TRPA1 is unusual among mammalian TRP channels in having a very long ankyrin repeat within the N-terminal domain (14–18

ankyrin repeats depending on species). TRPV and TRPC channels also have N-terminal ankyrin repeats, although they are much shorter (three to six repeats). TRPA1 is permeable to both monovalent and divalent cations, and therefore, TRPA1 is capable of depolarizing the membrane and initiating Ca²⁺ signaling in the cells it is expressed.

Expression pattern

TRPA1 is highly expressed in small- and medium-sized peptidergic primary afferent somatosensory neurons present in sensory ganglia-containing nociceptors—the dorsal root ganglia (DRGs), the trigeminal ganglia (TGs), and the nodose ganglia (NGs) (Nagata et al. 2005). Depending on different reports, the fraction of DRG neurons expressing TRPA1 varies from 3.6 to 56.5 % (Story et al. 2003; Nagata et al. 2005; Bautista et al. 2006; Kwan et al. 2006; Niforatos et al. 2007), with the most commonly reported values being around 30 %. The capsaicin receptor TRPV1 appears to be co-expressed in most if not all TRPA1-expressing DRG neurons (Bautista et al. 2006; Anand et al. 2008). This finding is further supported by the observation that mustard oil-induced nocifensive behavior is eliminated in mice where the central terminals of TRPV1-expressing DRG neurons have been ablated by intrathecal injection of capsaicin (Shields et al. 2010). In addition to TRPV1, TRPA1-expressing nociceptors also express calcitonin gene-related peptide (CGRP), substance P, and the bradykinin receptor, which are key mediators/transmitters in nociceptive signaling (Jordt et al. 2004; Obata et al. 2005; Bautista et al. 2006).

TRPA1 expression outside of nociceptive neurons has been reported by many groups, though the results do not always have the same level of consistency as seen in DRG and TG neurons. Nonetheless, expression in such cells represents potential locations where selective TRPA1 antagonists might

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have on-target effects outside of pain. Hair cells in the inner ear were reported to express TRPA1 at both the RNA and protein level as determined by in situ hybridization and immunohistochemistry, respectively (Corey et al. 2004; Nagata et al. 2005). As such, TRPA1 was proposed to be a component of the hair cell tip-link mechanotransducer channel necessary for auditory transduction. However, further experiments with TRPA1 knockout (KO) mice demonstrated that TRPA1 appears not to contribute to hair cell transduction or auditory function in vivo (Bautista et al. 2006; Kwan et al. 2006). Sympathetic neurons such as those of the superior cervical ganglion (SCG) have been reported to express TRPA1 (Smith et al. 2004), though other groups have failed to detect significant levels of TRPA1 RNA in the SCG (Nagata et al. 2005; Munns et al. 2007). Myenteric neurons and enterochromaffin cells (as well as some nonneuronal epithelial cells) in the small and large intestine have also been proposed to express TRPA1 based on immunohistochemistry and RT-PCR (Anand et al. 2008; Nozawa et al. 2009; Poole et al. 2011; Kono et al. 2013). Furthermore, treatment of enterochromaffin cells with TRPA1 agonists induces serotonin release, and treatment of the isolated guinea pig ileum with allyl isothiocyanate (AITC) induces 5-HT₃-receptor-mediated gastrointestinal smooth muscle contractions. TRPA1 agonists have been further shown to delay gastric emptying in rats through this pathway (Doihara et al. 2009). However, it is not clear whether TRPA1 antagonists would have deleterious effects on gut motility.

Nonneuronal expression of TRPA1 has been reported by many groups. In the lung, besides its expression in innervating sensory fibers, TRPA1 has been detected in several nonneuronal cell types including lung fibroblasts, alveolar epithelial cells, and lung smooth muscle cells in both human and mouse (Mukhopadhyay et al. 2011; Nassini et al. 2012), though only very low levels of TRPA1 RNA in the mouse and human lung were detected in other reports (Jaquemar et al. 1999; Story et al. 2003; Jang et al. 2012) (author's unpublished data). In skin, TRPA1 has been found in several cell types including melanocytes, keratinocytes, and fibroblasts (Anand et al. 2008; Atoyian et al. 2009; Tsutsumi et al. 2010), and therefore, it has been hypothesized that TRPA1 plays a role in the regulation of keratinocyte differentiation and inflammation in the skin. TRPA1 has been found in epithelial cells lining the urinary tract (Streng et al. 2008; Gratzke et al. 2010) in addition to sensory fibers innervating the urinary tract (Andrade et al. 2006); therefore, TRPA1 may play a role in urinary micturition. It has also been found that TRPA1 is expressed in vascular endothelial (Gratzke et al. 2009; Qian et al. 2013) and in basal/subepithelial cells of the human prostate gland (Gratzke et al. 2010). Beta islet cells of the pancreas have been shown to express TRPA1, and activation of TRPA1 in these cells results in insulin release (Cao et al. 2012). Astrocytes in the brain appear to express TRPA1, and

astrocyte TRPA1 channels appear to play an important role in calcium homeostasis and the regulation of the GABA transporter GAT-3 (Shigetomi et al. 2012), albeit at relatively lower level than that of DRG neurons. As TRPA1 antagonists are developed for treating pain and other disorders, close examination of potential on-target side effects resulting from the expression of TRPA1 outside of the targeted tissue (e.g., DRG and TG) should be considered.

Modulation of TRPA1

Endogenous agonists

In order for TRPA1 to be regarded as a good target for pain and other disorders, it should be active in the context of some pathological state. The best-known activators of TRPA1, however, are a set of exogenous electrophilic agonists that form covalent adducts with cysteine and lysine residues contained within the N-terminal domain (Figs. 1 and 2). Examples of such covalent agonists are AITC from wasabi (Bandell et al. 2004; Jordt et al. 2004), cinnamaldehyde from cinnamon extracts (Bandell et al. 2004; Jordt et al. 2004), allicin from garlic extract (Bautista et al. 2005; Macpherson et al. 2005), acrolein in diesel exhaust (Bautista et al. 2006), and other noxious substances such as tear gases (Bessac et al. 2008). TRPA1 can therefore clearly function as a nociceptive chemosensor and may have evolved in part for this purpose. Endogenous agonists have also been found and have led to the idea that TRPA1 can be activated in the absence of exogenous agonists

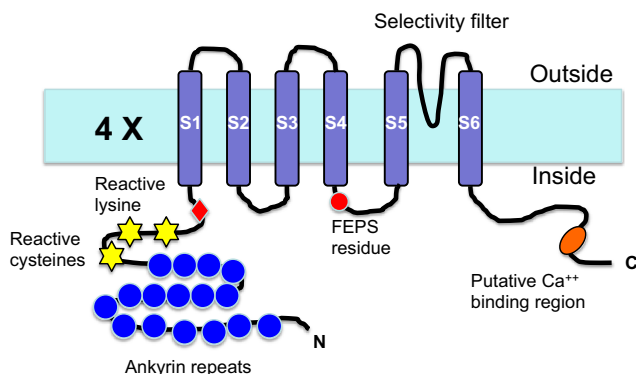


Fig. 1 Structure of the TRPA1 channel. The TRPA1 channel shares the overall architecture of voltage-gated ion channels. It is a homotetramer with each subunit containing six transmembrane helices and intracellular N- and C-termini (as shown). The transmembrane helices are labeled S1–S6 with S1–S4 representing the ancestral voltage-sensing domain (VSD) and S5–S6 forming the central pore and selectivity filter. The reactive lysine and cysteine residues are shown within the N-terminal domain, along with the N-terminal ankyrin repeats. Please note N855S, the residue mutated in familial episodic pain syndrome (FEPS), is shown on the intracellular end of S4 based on recent electron cryo-microscopy structure of TRPV1 (Liao et al. 2013) and comparison between TRPA1 and TRPV1 hydropathy plots, and the putative Ca⁺⁺ binding region is shown within the C-terminus

and potentially during certain pathological states. Examples of endogenous agonists are oxidized lipids such as 4-hydroxy-2-nonenal (4-HNE) (Taylor-Clark et al. 2008a; Trevisan et al. 2014a), 4-oxo-nonenal (Andersson et al. 2008), 5,6-eposyeicosatrienoic acid (5-6-EET) (Sisignano et al. 2012), 15-deoxy- Δ 12,14-prostaglandin J2 (Andersson et al. 2008; Cruz-Orengo et al. 2008), and 8-iso-prostaglandin A2 (Taylor-Clark et al. 2008b), nitrated lipids such as nitrooleic acid (9-OA-NO₂) (Taylor-Clark et al. 2009), general long-chain polyunsaturated fatty acids (Motter and Ahern 2012), and small endogenous reactive molecules such as H₂O₂ (Andersson et al. 2008). In this manner, TRPA1 may be viewed as a sensor for oxidative stress since endogenous ligands can accumulate and activate the channel directly, thereby contributing to inflammatory pain in a TRPA1-dependent manner. Oxidative stress has also been proposed to play an important role in several rodent models of neuropathic pain (Kim et al. 2004; Naik et al. 2006), and recently, TRPA1 has been found in some cases to play an important role in linking the presence of oxidative stress to inflammatory and neuropathic pain (Trevisan et al. 2013a; Trevisan et al. 2013b; Trevisan et al. 2014b). Additionally, methylglyoxal, a reactive metabolite that accumulates intracellularly in diabetes (Brownlee 2001; Nakayama et al. 2008) and chronic kidney disease (Nakayama et al. 2008), is able to directly activate TRPA1 resulting in a painful neuropathy in mice (Eberhardt et al. 2012; Andersson et al. 2013). It is therefore possible that methylglyoxal contributes to diabetic neuropathic pain and the neuropathic pain present in patients with chronic kidney disease. Another classes of potentially physiologically relevant

endogenous TRPA1 agonists are the small endogenous gasotransmitters NO (Takahashi et al. 2008) and H₂S (Andersson et al. 2012). While these have been proposed to activate TRPA1 in vivo, the concentrations necessary to induce TRPA1 activation are higher than would likely occur physiologically. Recently, nitroxyl anion (HNO), which can be formed via chemical reaction between NO and H₂S, has been shown to directly activate TRPA1 at physiologically relevant concentrations and may play an important role in local and systemic blood flow control (Eberhardt et al. 2014). This observation may open new therapeutic strategies for targeting cardiac failure but also indicates new potential adverse effects with TRPA1 blockade.

Calcium

Intracellular calcium can modulate TRPA1 by both directly activating the channel as well as enhancing activation by agonists (e.g., AITC, cinnamaldehyde, and icilin) (Doerner et al. 2007; Zurborg et al. 2007; Wang et al. 2008b) (Fig. 2). In fact, activation by icilin may require the presence of elevated intracellular calcium (Doerner et al. 2007). Additionally, calcium influx is also able to rapidly inactivate the channel. Inactivation may occur at significantly higher local intracellular calcium concentrations than is required for calcium-dependent enhancement of activation (Wang et al. 2008b). At the molecular level, the mechanisms for such actions are not entirely understood. Calmodulin (CaM) appears not to be involved based on experiments co-expressing a dominant-negative CaM mutant and CaM antagonists (Doerner et al. 2007; Zurborg et al. 2007). An EF-hand-like region was identified within the ankyrin repeat of the N-terminal domain, but mutagenesis to disrupt the EF-hand resulted in inconsistent results. Specifically, mutation of the proposed essential EF-hand residue (D468A) did not alter calcium sensitivity (Doerner et al. 2007; Zurborg et al. 2007); the mutation of a nearby residue (L474A) was found to eliminate calcium sensitivity by one group (Doerner et al. 2007), but this result could not be reproduced by another group (Wang et al. 2008b). Adding more complexity to the matter, a third group identified an separate domain within N-terminal ankyrin repeat region (outside the EF-hand-like region) as essential for calcium-dependent inactivation (Cordero-Morales et al. 2011), while a fourth group suggested that acidic residues in the C-terminal domain form a calcium binding site similar to the calcium binding site present in BK potassium channels (Sura et al. 2012) (Fig. 2).

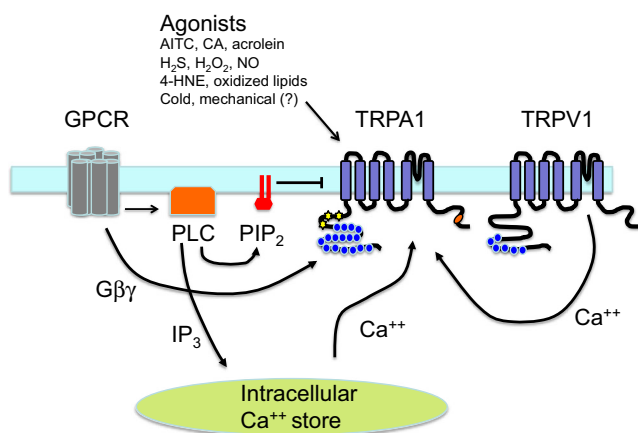


Fig. 2 Modulation of TRPA1. TRPA1 is modulated by in multiple ways and represents an integrator of nociceptive signals. Agonists bind to the channel (most but not all within the N-terminal domain) and result in direct activation. PIP₂ functions as an endogenous inhibitor which is removed by GPCR-dependent activation of PLC. PLC also liberates IP₃, resulting in release of Ca⁺⁺ from intracellular stores. Ca⁺⁺, either from stores or from influx through other channels such as TRPV1, positively modulates TRPA1 via an intracellular binding site which has not been fully elucidated. Cold and mechanical forces can also activate TRPA1 via mechanisms that are not well understood

Protons

Rodent DRG neurons are sensitive to low pH produced during tissue acidosis due to the presence of H⁺-sensitive ion channels such as TRPV1 and ASIC channels. Interestingly, it has

recently been shown that human TRPA1, but not rodent TRPA1 or even rhesus monkey TRPA1, can be directly activated by protons (de la Roche et al. 2013). Proton activation occurs over a pH range from 6.0 to 7.0 with a midpoint of around pH 6.5, which is within the physiological pH range that would be expected to occur during tissue acidosis. It is currently unclear whether this observed pH dependence of human TRPA1 plays an important role *in vivo*.

GPCR modulation

G protein-coupled receptors (GPCRs) such as the bradykinin receptor, the protease-activated receptor 2 (PAR2), the bile acid receptor TGR5, the thymic stromal lymphopoietin (TSLP) receptor, and the MAS-related GPCRs MrgprA3 and MrgprC11 have been shown to modulate the function of TRPA1 in cultured DRG neurons (Bandell et al. 2004; Dai et al. 2007; Wang et al. 2008a; Wilson et al. 2011; Wilson et al. 2013b; Lieu et al. 2014) (Fig. 2). Activation of these GPCRs results in the direct activation of TRPA1 and/or sensitization of TRPA1 to agonists such as AITC, cinnamaldehyde, and likely endogenous agonists. Activation of phospholipase C (PLC) appears to be required for such GPCR-mediated TRPA1 sensitization (with the exception of MrgprA3), though in the case of bradykinin receptor, protein kinase A (PKA) may play a role as well (Wang et al. 2008a). PLC likely sensitizes TRPA1 by removal of the inhibitory effect of PIP2 (Dai et al. 2007; Wang et al. 2008a) and by IP3-dependent release of calcium from intracellular stores (Jordt et al. 2004) (Fig. 2). Protein kinase C (PKC) appears not to play a role in bradykinin-dependent modulation of TRPA1 since neither GF109203X, a potent inhibitor of PKC, nor phorbol 12-myristate 13-acetate (PMA), a well-known PKC activator, alters bradykinin-mediated TRPA1 sensitization (Wang et al. 2008a). Nonetheless, PKC seems necessary for TGR5-dependent activation of TRPA1 (Lieu et al. 2014). MrgprA3 is activated by chloroquine and involved in histamine-independent itch. It signals through $G\beta\gamma$, as gallein, a small-molecule inhibitor of $G\beta\gamma$, markedly reduced chloroquine-evoked calcium influx in cultured DRG neurons, whereas U73122, a PLC inhibitor, had no effect (Wilson et al. 2011). A similar $G\beta\gamma$ dependence has been observed in the case of TGR5-dependent TRPA1 activation as well (Lieu et al. 2014).

Mechanical sensitivity

Soon after its discovery, TRPA1 was proposed as a mechanosensor since its long ankyrin repeat could form a spring-like structure to sense mechanical forces (Howard and Bechstedt 2004; Sotomayor et al. 2005). In fact, it was brought forward early on as a candidate for the long sought-after tip-link mechanotransducer channel in hair cells of the inner ear (Corey et al. 2004). However, this idea was

dismissed due to normal hair cell function and auditory function in TRPA1 KO mice (Bautista et al. 2006; Kwan et al. 2006). Direct mechanical stimulation of DRG neurons evokes currents that can be characterized as rapidly adapting, intermediate-adapting, and slowly adapting. Analysis of such currents from DRGs obtained from either WT and TRPA1 KO mice has yielded inconsistent results with one group showing a complete loss of slow-adapting currents, but not intermediate- or rapidly adapting currents from IB4-negative DRG neurons (Vilceanu and Stucky 2010), and another group showing a small but significant effect on intermediate-adapting currents from small-diameter DRGs with no effect on slowly adapting currents. Further evidence for direct mechanosensitivity of TRPA1 has been obtained from TRPA1-expressing HEK293 cells where membrane shrinkage (via hypertonicity) and membrane curvature (via trinitrophenol) appear to activate TRPA1 (Hill and Schaefer 2007; Zhang et al. 2008). Most recently, it was shown that lipopolysaccharide (LPS), an abundant outer membrane glycolipid released by Gram-negative bacteria, activates endogenously expressed and heterologously expressed TRPA1 channels (Meseguer et al. 2014). The activation depends on the shape of membrane-anchoring lipid moiety of LPS, indicating that perturbation of the plasma membrane may underlie channel activation. Hypertonicity, trinitrophenol, and LPS may serve to sensitize TRPA1 channel despite that fact that their direct effects on TRPA1 are relatively small compared to those of electrophilic agonists (e.g., AITC). More research in this area will be needed to better understand whether TRPA1 is indeed acting as a direct mechanosensor.

In vitro skin–nerve preparation recordings indicate that TRPA1 gene KO and treatment with the TRPA1 antagonist HC-030031 can reduce mechanically induced action potential firing in dermal C-fibers (Kerstein et al. 2009; Kwan et al. 2009). A similar reduction in action potential frequency was observed in recordings of mechanosensitive visceral afferents from the colon (Brierley et al. 2009). Furthermore, the increased mechanical sensitivity observed in the skin–nerve preparation following CFA could be reversed by HC-030031 (Lennertz et al. 2012). *In vivo* electrophysiology recordings showed that A-967079 reduced the responsiveness of spinal wide dynamic range (WDR) and nociceptive-specific neurons to high-intensity mechanical stimuli in naïve and inflamed rats (McGarraughty et al. 2010). However, A-967079 only affected low-intensity mechanical stimuli-evoked responses in inflamed but not naïve rats.

Behavioral studies of acute mechanical nociception of TRPA1 KO mice have shown rather inconsistent results. Two early studies revealed no apparent deficit in acute mechanical nociception in KO mice (Bautista et al. 2006; Petrus et al. 2007), while deficits were detected in other studies (Kwan et al. 2006; Garrison and Stucky 2014; Minett et al.

2014). In particular, Minett et al. compared KO mouse strains of Nav1.3, Nav1.7, Nav1.8, Nav1.9, and TRPA1 and found that only TRPA1 KO mice had a defect in mechanical nociception as measured using the Randall-Selitto test applied to the paw (Minett et al. 2014). Interestingly, TRPA1 KO mice showed normal mechanical nociception using the same test applied to the tail (where both Nav1.7 and Nav1.8 KO mice displayed a clear defect). Finally, the deficit in acute mechanical sensitivity observed in TRPA1 KO mouse should be carefully interpreted since a recent study showed a significant reduction in intraepidermal nerve fiber density in TRPA1 KO compared to wild-type mice (Andersson et al. 2013).

Cold sensitivity

Activation of TRPA1 by cold (temperature <17 °C) has been shown by some groups (Bandell et al. 2004; Sawada et al. 2007; Karashima et al. 2009; del Camino et al. 2010; Lehto et al. 2013) but disputed by others (Jordt et al. 2004; Zurborg et al. 2007; Cordero-Morales et al. 2011). Efforts to resolve the discrepancy were confounded by differences in experimental procedures, including methodologies (Ca²⁺ imaging, whole-cell or single-channel recordings), ionic conditions (with or without extracellular Ca²⁺), and clones from different species (human, rat, and mouse). In a recent study, TRPA1 from four different mammalian species were characterized under the same experimental conditions (Chen et al. 2013). In Ca²⁺-influx assays as well as whole-cell and single-channel recordings, cold activates rat and mouse TRPA1, albeit with lower efficacy compared to AITC (~40 % open-probability relative to AITC). In contrast, neither human nor rhesus monkey TRPA1 could be activated by cold. These findings are in line of most of the literature data and suggest that previous discrepancies could be due to species differences. However, cold activation of human TRPA1 has been observed in whole-cell recordings by some groups (Bandell et al. 2004; Kremeyer et al. 2010), and human TRPA1 reconstituted into lipid bilayers is intrinsically cold-sensitive (Moparthi et al. 2014). Therefore, whether TRPA1 is a cold-sensitive channel is not formally settled. At behavioral level, at least five studies using TRPA1 KO mice produced conflicting results. Two studies found that TRPA1 KO mice retained normal responses in cold plate and acetone-evoked evaporative cooling tests and upon cold stimulation maintained normal c-fos expression (a marker for neural activity) (Bautista et al. 2006; Knowlton et al. 2010). A third study reported that female, but not male, KO mice had reduced cold sensitivity (Kwan et al. 2006). A fourth study reported that TRPA1 KO mice had no deficit in acute cold sensation but lost the jumping behavior when challenged with prolonged cold exposure (Karashima et al. 2009). The fifth study found no difference in cold sensitivity between wild-type and KO mice but found that cold enhanced the effects of AITC, thereby contributing to AITC-induced cold

allodynia (del Camino et al. 2010). In pharmacology studies using TRPA1 antagonists, A-967079 did not affect acute cold sensing in uninjured mice but attenuated cold allodynia in neuropathic rats (Chen et al. 2011a). Similarly, HC-030031 was shown to attenuate cold hyperalgesia in CFA (inflammatory), spared nerve injury (SNI, neuropathic), and paclitaxel-mediated cold hyperalgesia (del Camino et al. 2010; Materazzi et al. 2012). Additionally, TRPA1-specific antisense oligodeoxynucleotides were efficacious in attenuating cold allodynia in inflammatory and neuropathic pain models (Obata et al. 2005). Taken together, these results suggest that TRPA1 might not play a significant role in acute cold sensation but may be involved in cold allodynia under disease states where endogenous TRPA1 activation mechanisms are present.

Intriguingly, TRPA1 knockout mice showed higher tolerance to heat, higher heat threshold of cutaneous C-mechano-heat-sensitive fibers, and less heat-induced CGRP release (Hoffmann et al. 2013). Also HC-030031 was found to decrease heat hyperalgesia in the paclitaxel model of chemotherapy-induced neuropathic pain (Chen et al. 2011b). These effects might be due to TRPA1 sensitization, instead of direct activation by heat. Unlike their counterparts in invertebrate and ancestral vertebrates, mammalian TRPA1 channels are not activated by heat directly (Chen et al. 2013).

Physiological and pathological role of TRPA1

Pain

Several lines of evidence suggest that TRPA1 is involved in pain sensation. TRPA1 is expressed in sensory neurons and co-localized with pain markers such as TRPV1 and bradykinin receptors (Obata et al. 2005; Bautista et al. 2006). Its expression is increased in animal models of inflammatory and neuropathic pain and in human avulsion-injured DRG neurons (Obata et al. 2005; Anand et al. 2008). TRPA1 agonists evoke neurotransmitter release, pain, and inflammation in rodents and humans (Namer et al. 2005; Bautista et al. 2006; Kwan et al. 2006), and endogenous agonists such as 4-HNE are elevated under human pathologies (Morquette et al. 2006). Gene KO attenuates agonist sensitivity, and antagonist treatment attenuates pain in several animal models (Bautista et al. 2006; Kwan et al. 2006; McNamara et al. 2007; Petrus et al. 2007; Eid et al. 2008; Chen et al. 2011a). Recently, a TRPA1 gain of function mutation was linked to human congenital pain condition called familial episodic pain syndrome (FEPS) (Kremeyer et al. 2010). Based on data in the literature, TRPA1 has been suggested to play a role in many sensory modalities, including chemical nociception, mechanical nociception, and cold nociception.

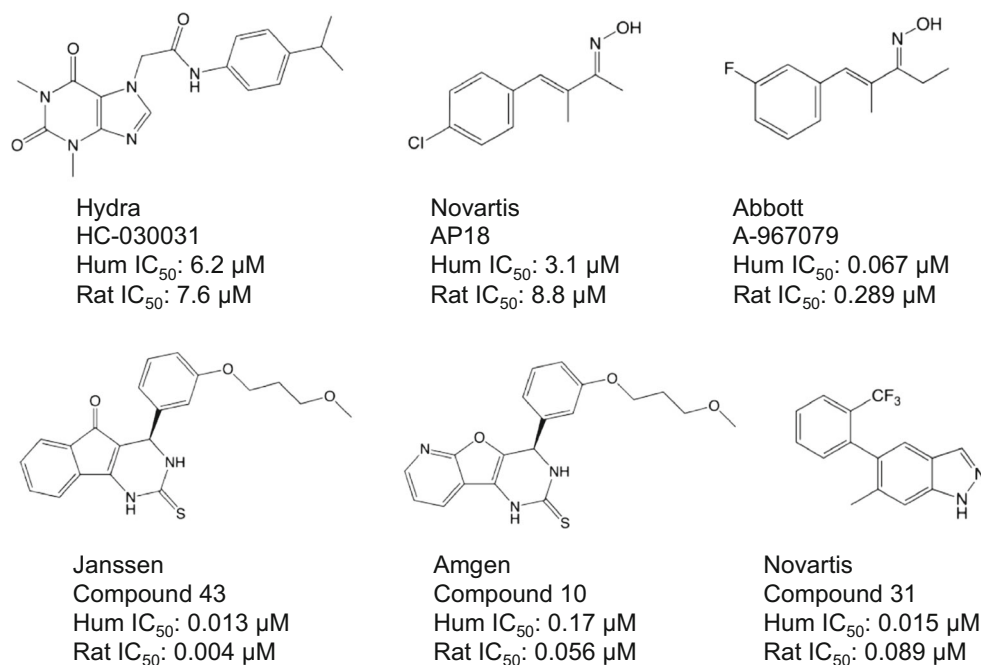
Among various sensory modalities, the best-established and least controversial is chemical nociception. TRPA1

detects a variety of exogenous noxious chemicals such as AITC, cinnamaldehyde, allicin, and acrolein. The response of DRG neurons to these compounds and the nocifensive behaviors they elicit have been rigorously demonstrated to be TRPA1-dependent after TRPA1 KO mice became available in 2006 (Bautista et al. 2006; Kwan et al. 2006). The same level of rigor has been applied to identify endogenous agonists as well, clearly establishing that endogenous agonist-dependent DRG calcium influx and nocifensive behaviors are TRPA1 dependent. Less clear, however, is the role that these endogenous activators play in inflammatory and/or neuropathic pain states. For example, while injection of the glucose metabolite methylglyoxal (MG) results in a TRPA1-dependent sensory neuropathy in mice (Eberhardt et al. 2012; Andersson et al. 2013) and while MG is elevated in patients with diabetic neuropathy, it has not yet been demonstrated that MG or TRPA1 plays a causative role of any kind in diabetic neuropathy. Similarly, while many other endogenous activators of TRPA1 (e.g., 4-HNE and acrolein) have been found to be sufficient in causing pain behavior in rodents, there is currently no evidence that these molecules play a causative role in any form of inflammatory or neuropathic pain.

TRPA1 has also been proposed to play a role in mechanical allodynia following induction of inflammatory and neuropathic pain, indicating that TRPA1 might be a potential target for novel chronic pain drugs. Early reports on the phenotype of the TRPA1 KO mouse indicated that there is little if any defect in mechanical allodynia following either CFA-induced inflammatory pain or nerve injury-induced mechanical allodynia (Kwan et al. 2006; Petrus et al. 2007). However, more recent TRPA1 KO experiments appear to show a major

defect in CFA-induced inflammatory pain in the TRPA1 KO mouse, however, especially in older mice (Garrison and Stucky 2014). Also, while Petrus et al. 2007 failed to find a defect in the CFA model in the TRPA1 KO mouse, they were able to show that the TRPA1 blocker AP-18 was able to reverse CFA-induced inflammatory pain in WT mice but not in TRPA1 KO mice, indicating that indeed TRPA1 contributes to CFA-induced mechanical allodynia and perhaps some form of compensation occurs in the TRPA1 KO mouse allowing a TRPA1-independent CFA-response in these animals. Other TRPA1 antagonists such as HC-030031 have shown similar attenuation of mechanical allodynia in the CFA model (Petrus et al. 2007; Eid 2009). Intrathecally administered Chembridge-5861528 attenuated mechanical hypersensitivity in spinal nerve ligation model and capsaicin-induced secondary hyperalgesia model (Wei et al. 2011). However, some newer generation TRPA1 antagonists with more robust exposure (free plasma concentration vs. *in vitro* IC₅₀) (Fig. 3) were not effective in these models despite showing robust inhibition of AITC-induced nocifensive behavior. For example, A-967079 at IC₈₅ coverage did not attenuate mechanical hyperalgesia in CFA, chronic constriction injury, or spinal nerve ligation models (Chen et al. 2011a). AMG0902 at an even higher drug exposure (fourfold over IC₉₀ coverage) had only a modest effect on mechanical allodynia in CFA model and no effect in the spinal nerve ligation (SNL) model (Lehto et al. 2013). On the other hand, potent inhibitors from Glenmark Pharmaceutical Ltd. as well as the recently reported potent inhibitor from Novartis (Novartis compound 31 in Fig. 3) did appear to show efficacy in the CFA model (Rooney et al. 2014).

Fig. 3 Examples of TRPA1 antagonists. HC-030031, AP18, and A-967079 are currently the most commonly used TRPA1 tool antagonists for studying TRPA1 biology *in vivo* and *in vitro*. A newer generation of TRPA1 antagonists (*lower row*) have recently been developed that offer better potency on the rodent TRPA1 channels as well as significantly improved PK properties compared to earlier generation compounds



Several groups have demonstrated a role for TRPA1 in rodent models of chemotherapy-induced painful neuropathy (CIPN) and painful diabetic neuropathic (PDN). These two forms of neuropathic pain are of particular interest since they can be directly studied in early proof-of-concept clinical trials in humans. As shown using either TRPA1 KO mice or the TRPA1 antagonist HC-030031, TRPA1 appears to contribute significantly to paclitaxel-induced mechanical allodynia (Materazzi et al. 2012) as well as bortezomib- and oxaliplatin-induced mechanical allodynia (Trevisan et al. 2013a) in mice. Strikingly, block of TRPA1 during the 5-h period encompassing the time of bortezomib dosing appears to completely prevent the development of neuropathic pain in this model (Trevisan et al. 2013a). In the case of DPN, no published data exists showing whether or not the development of diabetic neuropathic pain is impaired in TRPA1 KO mice. However, intrathecal injections of the TRPA1 antagonist CHEM-5861528 have been found to reverse mechanical allodynia in streptozotocin-induced diabetic rats (Wei et al. 2009; Wei et al. 2010) and may prevent diabetes-induced loss of nociceptor nerve endings in the skin (Koivisto et al. 2012). More recently, Glenmark Pharmaceuticals Ltd. reported that GRC 17536, a peripheral acting TRPA1 antagonist, exhibited efficacy in a phase 2a proof-of-concept study in patients with painful diabetic neuropathy.

TRPA1 is present in nociceptive fibers innervating internal organs (e.g., colon and pancreas) and implicated in visceral pain and inflammation. When activated during inflammation, these fibers carry visceral pain signals and also release neuropeptides such as substance P and CGRP, resulting in neurogenic inflammation. Desensitization of TRPV1 receptor-expressing C-fibers using treatment with either resiniferatoxin (RTX) or capsaicin results in significantly reduced severity of dextran-sulfate-sodium (DSS)-induced colitis in mice (Kihara et al. 2003; Engel et al. 2012), indicating that TRPV1-positive nociceptor fibers are involved in this model of colitis. Furthermore, DSS-induced colitis was found to be ameliorated in both CGRP KO mice and substance P KO mice (Engel et al. 2012). TRPV1 itself does not appear to play a role in either DSS-induced colitis or trinitrobenzene-sulfonic-acid (TNBS)-induced colitis as these models are not altered in the TRPV1 KO mouse (Engel et al. 2011; Engel et al. 2012). However, both DSS-induced and TNBS-induced colitis were significantly reduced in TRPA1 KO mice or WT mice treated with the TRPA1 antagonist HC-030031. Similar data have been obtained in the case of caerulein-induced pancreatitis, where TRPA1 antagonists and genetic removal of TRPA1 (TRPA1 KO mouse) have been shown to reduce the extent of inflammation and pain in this model (Ceppa et al. 2010; Schwartz et al. 2011; Schwartz et al. 2013).

Spinal activation of TRPA1 may contribute to pain and represents a target for pharmacological intervention (Wei et al. 2011). However, a recent study suggests that spinal

TRPA1 activation may underlie the antinociceptive effect of acetaminophen (Anderson et al. 2011). It was shown that reactive metabolites of acetaminophen can directly activate TRPA1 at the central terminals of primary sensory neurons, causing inactivation of voltage-gated Ca^{2+} and Na^{+} channels and thereby reducing neurotransmitter release and neuronal excitability. The antinociceptive effect of spinal TRPA1 activation does not appear limited to covalent TRPA1 agonists, as Δ^9 -tetrahydrocannabinol, a noncovalent agonist, is also antinociceptive in the hot plate test. Thus, spinal activation of TRPA1 can be either nociceptive or antinociceptive, perhaps depending on the degree of TRPA1 activation. Therefore, it is possible that both antagonists and agonists of TRPA1 may have utility for pain relief.

Respiratory diseases

TRPA1 is expressed in primary sensory neurons innervating the airways where it acts as a chemosensor for airway irritants such as acrolein, ozone, isocyanate, tear gas, and chlorine (Andre et al. 2008; Bessac et al. 2008; Bessac et al. 2009). TRPA1 activation leads to pain and reflex (sneezing, cough, and respiratory depression and avoidance), therefore serves as a protection to limit or eliminate irritant exposure. TRPA1 can also be activated chronically in chronic cough, asthma, chronic obstructive pulmonary disease (COPD), and rhinitis, where endogenous TRPA1 ligands (e.g., 4HNE, H_2O_2) and pro-inflammatory mediators (e.g., bradykinin and nerve growth factors) are elevated (Dolovich et al. 1970; Winterbourn and Kettle 2000). These ligands activate TRPA1 directly (e.g., 4HNE and H_2O_2), indirectly (bradykinin), or by increasing TRPA1 surface expression (nerve growth factor). Reciprocally, heightened TRPA1 function leads to neurotransmitter release and promotes neurogenic inflammation (Andre et al. 2008; Caceres et al. 2009).

The role of TRPA1 in airway pathologies has been corroborated by studies using the TRPA1 KO mouse and TRPA1 antagonists. In wild-type mice, airway exposure to hypochlorite or H_2O_2 evoked respiratory depression as manifested by a reduction in breathing frequency and increase in end expiratory pause, both of which were attenuated in TRPA1 KO mice (Bessac et al. 2008). In an ovalbumin-induced mouse asthma model, gene KO and treatment with HC-030031 reduced the induction of cytokines, chemokines, neurotransmitters, as well as leukocyte infiltration and airway hyperactivity (Caceres et al. 2009). In the guinea pig, acrolein, crotonaldehyde, and extracts from cigarette smoke induced neurotransmitter release, tracheal plasma extravasation, and bronchi contraction. These effects could be attenuated by HC-030031 (Andre et al. 2008). These results are certainly encouraging, although it is worth noting that chronic respiratory diseases are multifaceted disorders, often involving airway damage and tissue remodeling. It remains to be

demonstrated whether TRPA1 antagonists are capable of reversing disease progression and outperforming current standard of care such as anti-inflammatory drugs and bronchodilators.

Itch

Recent studies suggest that TRPA1 is involved in mediating histamine-independent itch (Wilson et al. 2011; Wilson et al. 2013a). The antimalarial drug chloroquine and the endogenous pruritogen BAM8-22 induce itch through Mas-related G protein-coupled receptors (MrgprA3 and MrgprC11, respectively), and their effect is mediated through TRPA1. In lesioned skin of human atopic dermatitis patients, TRPA1 expression is increased in dermal afferent fibers, dermal cells, and mast cells (Oh et al. 2013). In a mouse model of atopic dermatitis generated by overexpression of IL-13, TRPA1 expression is also increased in afferent fibers, dorsal root ganglia, and mast cells in a manner similar to that observed in human patients (Oh et al. 2013). Furthermore, HC-030031 and genetic deletion of mast cells attenuated itch-scratching behaviors. In oxazolone-induced contact dermatitis models, TRPA1 KO and HC-030031 decreased pro-inflammatory cytokines, T cell infiltration, dermatitis score, and edema, indicating that TRPA1 may play a central role in inflammation and pruritus (Liu et al. 2013).

Antagonist tools

An increasing number of TRPA1 antagonists have been disclosed, and only a few examples are listed here (Fig. 3). HC-030031, a xanthine alkaloid discovered by Hydra Bioscience, has been the most widely used in the literature as a tool for studying TRPA1-mediated biology (McNamara et al. 2007; Eid et al. 2008). HC-030031 inhibits human and rat TRPA1 with IC_{50} of 6.2 and 7.6 μ M, respectively (McNamara et al. 2007; Bianchi et al. 2012). It is selective against several TRP channels ($IC_{50} > 10$ or 20 μ M). However, when tested in radioligand binding assays at 10 μ M concentration, HC-030031 showed activity against several proteins including sodium channels (40 %) and sigma receptors (37 %) (Chen et al. 2011a). In addition, HC-030031 has high clearance (27 ml/kg/min), low exposure ($C_{max} = 355$ ng/ml following a 12-mg/kg dose), a short half-life (32 min), and high plasma protein binding (90 %) in rats (Rech et al. 2010). With such poor pharmacokinetic (PK) properties, it is unlikely that HC-030031 can reach unbound concentrations above the TRPA1 IC_{50} even at very high doses. Nonetheless, it exhibited efficacy in CFA, SNL, and other pain models (McNamara et al. 2007; Petrus et al. 2007; Eid et al. 2008). The efficacy of HC-030031 has not been conclusively attributed to the block of TRPA1 by showing, for example, that efficacy is lost in the TRPA1 KO mouse, and therefore, results obtained with

this low-potency compound should be interpreted with caution.

Recently, Abbott, Amgen, AstraZenica, Glenmark, Hydra Bioscience, Janssen, Merck, and other companies have disclosed additional TRPA1 antagonists with improved potency, selectivity, and pharmaceutical properties (Fig. 3). Pharmacokinetic and efficacy data have been described for A-967079 (Abbott), Compound 10 (Amgen), and Compound 31 (Novartis) (Chen et al. 2011a; Copeland et al. 2014; Rooney et al. 2014). Compound 10, an azabenzofuran analog, inhibits human and rat TRPA1 with IC_{50} of 0.17 and 0.045 μ M, respectively, and has favorable PK properties allowing robust unbound plasma exposure (14-fold over rat IC_{50} when dosed at 100 mg/kg). This compound was highly efficacious in the AITC-flinch model, but its efficacy in other pain models was not reported (Copeland et al. 2014). This new generation of TRPA1 antagonists will be valuable in exploring TRPA1 function and therapeutic utility and will hopefully replace the use of older TRPA1 tool compounds.

Beside small molecules, monoclonal antibodies (mAbs) have been generated using a cell immunogen approach (Lee et al. 2014). The most potent mAb, 2B10, inhibited activation by AITC (IC_{50} , 260 nM), cold (IC_{50} , 90 nM), and hypertonicity (IC_{50} , 350 nM). 2B10 also had similar effect on mouse TRPA1, and therefore, it likely targets a domain conserved in human and mouse channels, probably in the extracellular pore loop. Similar to pore-blocking antibodies for other ion channels, the inhibition of TRPA1 plateaued at 70 %. 2B10 might be a potentially useful tool if it can get access to relevant tissues.

Species differences

The electrophile sensitivity of TRPA1 is conserved across species (e.g., fruit fly, lizard, snake, chicken, mouse, and humans), indicating electrophile detection is a core function of TRPA1. On the other hand, TRPA1 also exhibits evolutionary divergence in temperature sensitivities, from being heat-sensitive in invertebrates/ancestral vertebrates (fruit fly, rattlesnake, lizard, and frog) (Viswanath et al. 2003; Hamada et al. 2008; Kwon et al. 2008; Gracheva et al. 2010; Kang et al. 2012), to being cold-sensitive in rodents (Story et al. 2003; Chen et al. 2013), and to being temperature-insensitive in primates (Jordt et al. 2004; Chen et al. 2013). The evolutionary divergence in thermo-sensitivity indicates the likelihood that TRPA1 from different species (e.g., human and rats) may have different functions. At the amino acid level, human and rat TRPA1 are poorly conserved (79 % identical, compared to 94 % for TRPM8 and 86 % for TRPV1 for examples), which may lead to qualitative or quantitative differences in sensitivity to ligands. Additionally, other cross-species variations (metabolic mechanisms, transduction pathways, and animal physiology) can lead to species difference of TRPA1 function.

Species difference in response to pharmacological agents has been well demonstrated for TRPA1 (Bianchi et al. 2012). For example, thioaminols and caffeine activate rodent channels but block the human channel (Klionsky et al. 2007; Chen et al. 2008; Nagatomo et al. 2010). Menthol activates the human channel at high concentration but blocks rodent channels (Xiao et al. 2008). In high throughput screening and medicinal chemistry efforts, we and others have found that a majority of human TRPA1 antagonists have reduced potency, or act as agonists, on the rodent TRPA1 channels. Such compounds could not be advanced in the drug discovery process due to the need to use rodents as preclinical species. Recently, we showed that monkey and human TRPA1 share similar pharmacology and therefore monkey could potentially serve as a surrogate species (Bianchi et al. 2012). However, the practical use of monkeys is hindered by high cost, low throughput, and the limited number of monkey disease models available. The generation of humanized transgenic animals (i.e., knocking-in of human TRPA1) could be a valuable alternative path forward. In all, species difference is a serious problem and requires creative solutions.

Potential adverse effects

Although TRPA1 is predominantly expressed in sensory neurons, it is also expressed in other tissues. Therefore, targeting TRPA1 could have unintended adverse effects. TRPA1 KO mice appear normal in appearance, reproduction, and auditory function (Bautista et al. 2006; Kwan et al. 2006), although physical hyperactivity was recently reported (Bodkin et al. 2014). Unlike TRPV1 or TRPM8, TRPA1 is not involved in body temperature regulation at basal level or under cold challenge (Chen et al. 2011a; de Oliveira et al. 2014), and therefore, TRPA1 antagonists should be devoid of the adverse effects associated with TRPV1 antagonists (hyperthermia) or TRPM8 antagonists (hyperthermia).

One potential safety concern for TRPA1 antagonists is the deficit in sensing environmental irritants. Activation of TRPA1 by acrolein, ozone, and chlorine leads to pain, mucus secretion, sneezing, cough, and respiratory depression (Bautista et al. 2006; Andre et al. 2008; Bessac et al. 2008; Kwan et al. 2009). These nociceptive and behavioral responses help limit or eliminate irritant exposure, therefore protecting the body from damage. As an example, chlorine is widely used as a domestic (disinfection) and industry product with an annual consumption of 15 million tons in the USA. While most individuals can react quickly to avoid severe exposure, injuries do occur in the form of pulmonary edema, restrictive lung diseases, and obstructive disease. Airway exposure of chlorine results in reduced breathing frequency and increased end expiratory pause in wild-type mice, but such protective reflexes are absent in KO mice (Bessac et al. 2008). Will TRPA1 antagonists cause deficits in sensing and

avoiding irritants in humans? This question should be addressed during clinical development.

Concluding remarks

In the TRP family and ion channel superfamily as a whole, TRPA1 distinguishes itself by its covalent activation mechanism and its role in sensing a wide range of noxious chemicals. TRPA1 is also one of the few ion channels implicated in pain by human genetics. A large body of evidence supports the therapeutic utility of TRPA1 antagonists for pain, respiratory, itch, and other diseases. Additionally, TRPA1 antagonists appear not to cause significant safety concerns thus far. As such, TRPA1 has replaced TRPV1 as one of the most sought-after therapeutic targets for novel pain drugs. In September 2014, Glenmark announced that GRC 17536, a peripherally acting selective TRPA1 antagonist, exhibited efficacy in a phase 2a proof-of-concept study in patients with painful diabetic neuropathy. Despite this clear progress, there are many unanswered questions. Is TRPA1 a receptor for noxious cold? Does it play a role in mechosensation? What are the endogenous ligands? Do TRPA1 KO mice tell the full story? Do TRPA1 have cross-species difference in physiological and pathological function? What are the best therapeutic indications for TRPA1 antagonists? Do TRPA1 antagonists cause deficits in sensing noxious irritants? Recognizing and addressing these questions will not only reveal the physiological function of TRPA1 but also pave the way for developing new therapies.

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