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266 *Current Neuropharmacology,* **2015**, *13*, **266-278**

Deciphering Subtype-Selective Modulations in TRPA1 Biosensor Channels

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Abstract: The transient receptor potential (TRP) proteins are a family of ion channels that act as cellular sensors. Several members of the TRP family are sensitive to oxidative stress mediators. Among them, TRPA1 is remarkably susceptible to various oxidants, and is known to mediate neuropathic pain and respiratory, vascular and gastrointestinal functions, making TRPA1 an attractive therapeutic target. Recent studies have revealed a number of modulators (both activators and inhibitors) that act on TRPA1. Endogenous mediators of oxidative stress and exogenous electrophiles activate TRPA1 through oxidative modification of cysteine residues. Non-electrophilic compounds also activate TRPA1. Certain non-electrophilic modulators may act on critical non-cysteine sites in TRPA1. However, a method to achieve selective modulation of TRPA1 by small molecules has not yet been established. More recently, we found that a novel *N*-nitrosamine compound activates TRPA1 by *S*-nitrosylation (the addition of a nitric oxide (NO) group to cysteine thiol), and does so with significant selectivity over other NO-sensitive TRP channels. It is proposed that this subtype selectivity is conferred through synergistic effects of electrophilic cysteine transnitrosylation and molecular recognition of the non-electrophilic moiety on the *N*-nitrosamine. In this review, we describe the molecular pharmacology of these TRPA1 modulators and discuss their modulatory mechanisms.

Keywords: Electrophile, nitric oxide, non-electrophilic compound, oxidative stress, transnitrosylation, TRP channel, TRPA1.

INTRODUCTION

In 1989, the transient receptor potential (TRP) protein was first identified as being encoded by the *trp* gene of *Drosophila* [1]. The TRP protein superfamily consists of a diverse group of calcium ion (Ca^{2+})-permeable non-selective cation channels, and is found in most living organisms [2-4]. Mammalian TRP channels are currently divided into TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystic kidney disease), TRPML (mucolipin) and TRPA (ankyrin) subfamilies, which consist of seven, six, eight, three, three and one members, respectively. TRP channels have a tetrameric subunit stoichiometry, and each subunit contains cytoplasmic N- and C-terminal regions, six transmembrane (TM) domains and a pore-forming region between TM5 and TM6. TRP channels are sensitive to a variety of stimuli, including receptor stimulation, temperature, plant-derived compounds, environmental irritants, osmotic pressure, mechanical stress, pH and voltage from the extracellular and intracellular milieu, and are involved in diverse physiological and pathological processes [4-16].

Several TRP channels appear to respond well to mediators of oxidative stress, such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and other electrophiles [17-20]. While oxidative damage to DNA, lipids and proteins is canonically known to cause cellular dysfunction, ROS and RNS are also increasingly recognized as cell signaling molecules [21, 22]. The first identified ROS-sensitive TRP channel, TRPM2, is activated by hydrogen peroxide (H_2O_2) and mediates several cellular responses, including cell death and chemokine production [23-26]. TRPM7, which can be modulated by both ROS and RNS, is an essential mediator of anoxic cell death [27, 28]. Some members of the TRPC and TRPV subfamily, including TRPC5 and TRPV1, are activated by H_2O_2, nitric oxide (NO) and reactive disulfides [29]. In addition, TRPA1 is remarkably activated by various oxidants, including ROS, RNS, reactive disulfides and other electrophiles [30-33].

TRPA1 proteins form a plasma membrane channel that contains many ankyrin repeats in its cytoplasmic N-terminal region [34, 35] and can form a tetrameric assembly [36] (Fig. **1**). TRPA1 is expressed in a subset of nociceptive C-fiber neurons, including the dorsal root, trigeminal and nodose ganglion neurons [37-39]. It is targeted by environmental irritants, such as allyl isothiocyanate (AITC) from mustard oil and wasabi, cinnamaldehyde from cinnamon oil, allicin from garlic, and acrolein present in tear gas or vehicle exhaust [40-44]. These environmental irritants are electrophiles [30, 31], and further studies using *Trpa1* knockout mice have shown that TRPA1 acts as a nociceptor for electrophilic environmental irritants to produce pain [42,

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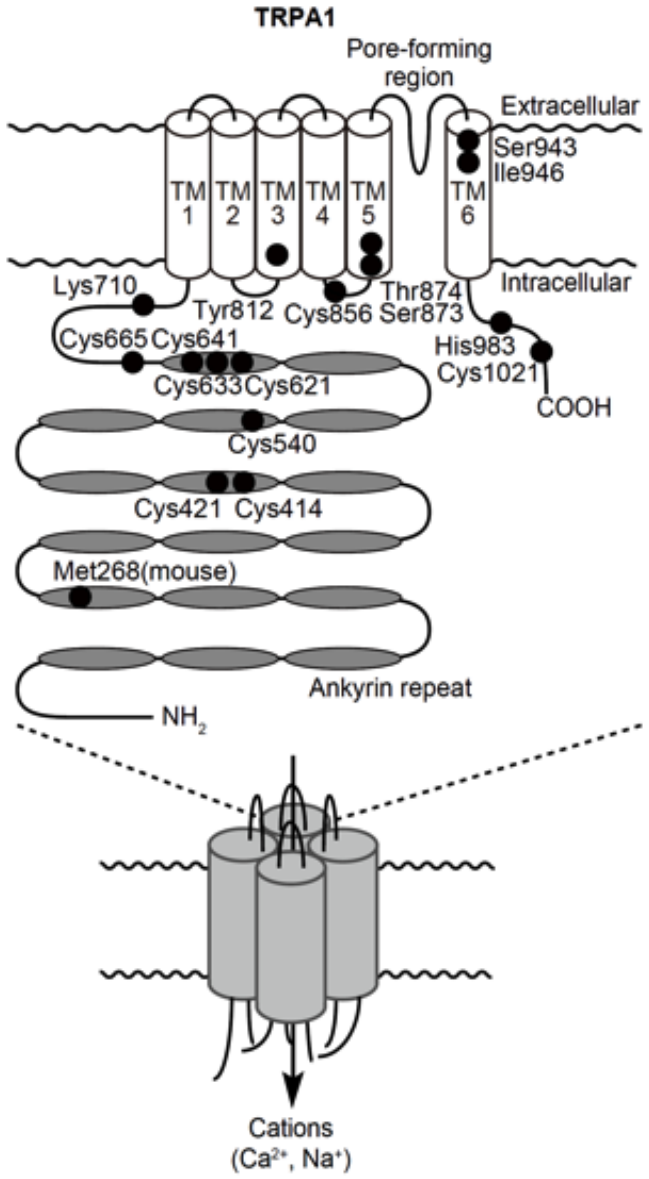

Fig. (1). Predicted structural features of TRPA1 with putative position of critical residues involved in human TRPA1 modulation by compounds. TRPA1 subunit, which has six transmembrane (TM) domains, a pore-forming region between TM5 and TM6, and many ankyrin repeats (indicated as ovals) in the cytoplasmic N-terminal region [35], assembles into tetramers to form a cation channel. Collectively, indicated residues (filled circles) are reported to be important for TRPA1 activation or inhibition by several compounds [30-33, 76, 80, 91, 128, 130, 133].

45-48]. ROS, RNS and lipid peroxidation products also activate TRPA1, and can induce a TRPA1-mediated pain sensation [49-53]. In terms of disorders, it is known that the activation of TRPA1 by oxidative stress byproducts is reported to mediate both diabetic and anti-cancer medicine-induced neuropathic pain [54-57]. TRPA1 is also involved in neurogenic inflammation, respiratory irritation and coughing elicited by electrophiles [49, 51, 58-62]. Therefore, oxidative stress-sensitive TRPA1 has been proposed as a potential drug target for the treatment of neurological diseases.

In addition to the importance of TRPA1 in neurological diseases, TRPA1 activation also mediates vascular dilation [63, 64]. Furthermore, TRPA1 activation induces both serotonin release from enterochromaffin cells and cholecystokinin release from a mouse intestinal neuroendocrine cell line [65, 66]. TRPA1 also regulates respiration by sensing oxygen (O_2) availability [67, 68]. Thus, a better understanding of the modulatory mechanisms of TRPA1 by both inhibitors and activators is of high significance.

A number of TRPA1 modulators (activators and inhibitors) have been identified to date, including not only environmental electrophiles and oxidative stress mediators, but also non-electrophilic compounds [69, 70]. Some rodent models of neurological diseases respond positively to TRPA1 inhibitors [71-73], and some TRPA1 inhibitors have reached the clinical trial stage as novel analgesic drugs [74]. It is also reported that a novel TRPA1 agonist exerts both anti-constipation and anti-abdominal pain actions [75]. The mechanism of TRPA1 modulation by oxidative mediators is thought to involve oxidative modification of cysteine residues, unlike TRPA1 modulation by non-electrophilic compounds. TRPA1 modulation by certain non-electrophilic compounds appears to be dependent on different chemical moieties within these compounds. Furthermore, several critical sites have been identified in TRPA1 that seem to be important for some non-electrophilic compounds to bind and modulate TRPA1. Strikingly, the chemical structure seems to be important even for the activation of TRPA1 by specific electrophiles. A novel NO donor derived from the 7-azabenzobicyclo[2.2.1]heptane *N*-nitrosamines confer selectivity to modulatory action of NO on TRPA1 over the other NO-sensitive TRP channels [76]. This subtype selectivity may be conferred through synergistic effects of two chemical processes: cysteine transnitrosylation and molecular recognition of a non-electrophilic moiety of the novel *N*-nitrosamine. This review will attempt to explore current molecular pharmacological knowledge of TRPA1 modulation by small molecules.

OXIDATION SENSITIVITY OF TRPA1 CHANNEL

TRPA1 senses various oxidative stress mediators and environmental compounds (Fig. **2** and Table **1**). Cysteine residues within a protein are emerging as direct targets for the oxidant signal reaction [77, 78]. TRPA1 is not an exception. Its activation by oxidants is proposed to be mediated *via* oxidative modification of the free sulfhydryl group of cysteine residues, as described for the activation of TRPC5 and TRPV1 [29, 79].

For TRPA1, the oxidation sites have been identified (Fig. **1**). Simultaneous mutation of three cysteine residues within the cytoplasmic N-terminus of human TRPA1 (Cys621, Cys641 and Cys665) decreases TRPA1 channel activation by several exogenous cysteine-modifying electrophiles, such as isothiocyanates (e.g. AITC), α,β-unsaturated aldehyde compounds (e.g. acrolein, *N*-methylmaleimide and cinnamaldehyde), and diallyl disulfide [30]. Lys710 is also suggested to be involved in the activation of TRPA1 by AITC. Three cysteine residues in mouse TRPA1 (Cys415, Cys422, and Cys622, conserved in the human homolog as Cys414, Cys421, and Cys621) were

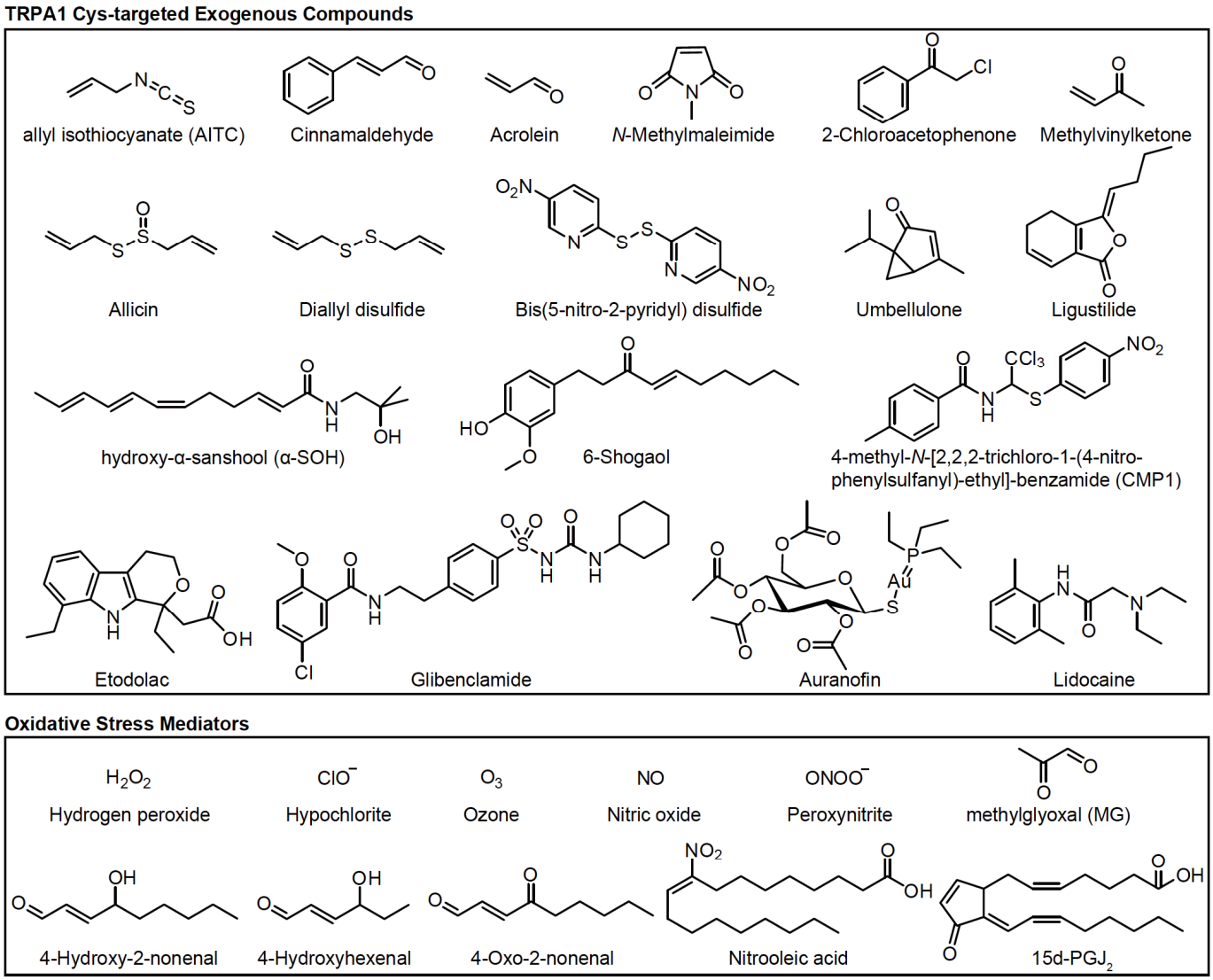

Fig. (2). Chemical structures of major TRPA1 Cys-targeted exogenous modulators and oxidative stress mediators.

independently identified as the target sites for AITC and cinnamaldehyde [31]. Intracellular Zn^{2+} also activates human TRPA1 by interacting with Cys641 and C-terminal Cys1021/His983 [80]. Systematic evaluation of TRP channels was performed using a series of reactive disulfides, such as bis(5-nitro-2-pyridyl) disulfide and diallyl disulfide [33]. These compounds possess different electron acceptor (oxidation) capacity (manifested as redox potential) and these studies revealed that only TRPA1 responds to the inert electrophile, diallyl disulfide. Thus, TRPA1 can sense inert oxidant O_2, and O_2 activation of TRPA1 is by oxidation of Cys633 and/or Cys856, located intracellularly within, respectively, the N-terminal region and the putative linker region between TM4 and TM5 [33]. In addition, TRPA1 cysteine residues seem also to be critical for TRPA1 activation by other exogenous compounds, including irritants (tear gases, such as 2-chloroacetophenone [81, 82], and α,β-unsaturated carbonyl-containing compounds, such as methylvinylketone [83, 84]), some plant constituents (umbellulone [85], ligustilide [86], hydroxy-α-sanshool (α-SOH) and 6-shogaol [87]), and others (the cyclooxygenase-2 inhibitor, etodolac [88]; the anti-diabetic drug, glibenclamide [89]; the gold-containing disease-modifying anti-rheumatic drug, auranofin [90]; CMP1 (4-methyl-*N*-[2,2,2-trichloro-1-(4-nitro-phenylsulfanyl)-ethyl]-benzamide) [91]; and lidocaine [92]). Therefore, TRPA1 is unarguably a receptor for oxidative exogenous electrophilic compounds.

TRPA1 is also modified *via* oxidative cysteine modification by endogenous oxidants. TRPA1 is activated by H_2O_2 [32, 50, 51, 93], hypochlorite [51], ozone [94] and the ROS generated by ultraviolet light [95]. In addition to ROS, TRPA1 is also activated by RNS such as NO [32, 53, 93] and peroxynitrite [93]. Functional characterization of site-directed mutants of TRPA1 collectively demonstrates that specific cytoplasmic N-terminal cysteine residues and a lysine residue (Cys421, Cys621, Cys641, Cys665 and Lys710 in human TRPA1) are the primary targets of ROS and RNS [32, 51, 53].

Table 1. Key features of selected modulators of TRPA1.

Class	Name	Effect	Other notes	References
TRPA1 Cys-targeted modulators (from Fig. 2 and 4)	Cinnamaldehyde	Activation (Bimodal)		[30, 31, (114)]
	Acrolein	Activation		[30]
	Umbellulone	Bimodal	Non-electrophilic moieties also contribute to TRPA1 activation.	[85]
	α-SOH	Activation	The *cis* C6 unsaturation is also important.	[87]
	CMP1	Activation (rat) Inhibition (human)	TM6 region is also critical.	[91]
	NO	Activation		[32, 53, 93]
	NNO-ABBH1	Activation	Transnitrosylation and molecular recognition synergistically contribute to selective activation.	[76]
Other modulators (from Fig. 3)	Icilin	Activation	Activation is not disrupted by Cys mutations.	[31]
	Flufenamic acid	Activation		[102]
	FTS	Activation	This activates Cys mutant.	[97]
	NPPB	Activation		[108]
	DHA	Activation	This may employ different mechanism compared with menthol.	[110]
	6-Paradol	Activation		[87]
	Capsiate	Activation	Activation is not disrupted by Cys mutations.	[112]
	Carvacrol	Activation	Activation is not disrupted by Cys mutations.	[51]
	Thymol	Bimodal		[113]
	Propofol	Bimodal (mouse) Activation (human)	This activates Cys mutant. This may employ different mechanism compared with menthol.	[118]
	Menthol	Bimodal (mouse) Activation (human)	The region from TM5 to TM6 is critical.	[113, 130]
	Nicotine	Bimodal		[116]
	Apomorphine	Bimodal	This selectively activates TRPA1, but no other sensory TRP channels.	[117]
	Camphor	Bimodal		[114]
	1,4-Cineol	Activation		[127]
	1,8-Cineol	Inhibition		[127]
	Borneol	Inhibition		[128]
	Caffeine	Activation (mouse) Inhibition (human)	N-terminal region is critical.	[132, 133]
	Isovelleral	Activation	This may have an electrophilic moiety, but activates Cys mutant.	[83]
	OC	Activation	This may have an electrophilic moiety, but activates Cys mutant.	[135]
Synthetic inhibitors (from Fig. 3)	HC-030031	Inhibition		[46]
	AMG5445	Activation (rat) Inhibition (human)		[120]

In addition to ROS and RNS, other endogenous electrophilic mediators of oxidative stress modulate TRPA1 activity. Lipid peroxidation products such as 4-hydroxy-2-nonenal, 4-hydroxyhexenal, 4-oxo-2-nonenal, nitrooleic acid and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) activate TRPA1 channels through oxidative modification of the cysteine residues [32, 49, 50, 96-99]. Labeling experiments using biotinylated 15d-PGJ_2 demonstrated that Cys621 mediates the binding of 15d-PGJ_2 to human TRPA1 [32]. Another electrophilic dicarbonyl compound, methylglyoxal (MG), which is believed to be associated with the development of diabetic neuropathy, also activates TRPA1 by hemithioacetal formation [54, 55]. Taken together, we can surmise that endogenous electrophilic products activate TRPA1 channels by cysteine oxidation.

To understand the modulatory mechanism of TRPA1 channel activity, protein structural-functional analysis is important. Recently, the structure of the capsaicin-sensitive polymodal receptor, TRPV1, was revealed at 3.4 Å resolution [100, 101]. With regard to TRPA1, its structure has been published at 16 Å resolution with docked molecular models [36]. It is suggested that Cys414 and Cys421 in the N-terminal ankyrin repeats from one subunit and Cys621 located on an adjacent subunit form a ligand-binding pocket between the subunits. It is possible that covalent modification of cysteine residues around this pocket could alter the interaction between the subunits and the domains within each subunit, promoting conformational changes and leading to channel activation [36].

MODULATION OF TRPA1 BY OTHER ACTIVATORS AND INHIBITORS

As discussed above, oxidative stress mediators and environmental electrophiles activate TRPA1, but it has also been demonstrated that various other activators and inhibitors modulate TRPA1 (Fig. **3** and Table **1**).

Icilin, 2-aminoethyl diphenylborinate and carvacrol are compounds with no obvious reactivity towards cysteine residues and activate TRPA1 in a way that is not disrupted by cysteine mutations [30, 31, 51]. TRPA1 is also activated by non-reactive compounds including non-steroidal anti-inflammatory drugs, such as flufenamic acid [102]; general anesthetics, such as isoflurane [103]; farnesyl thiosalicylic acid (FTS) [97]; and others [104-107]. The chloride channel blocker, NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), activates TRPA1, and a structure-activity relationship study using a group of NPPB analogs indicates that its phenylalkane, carboxylic and nitro groups are critical for its activation of TRPA1 [108]. From mutagenesis studies, NPPB and FTS are suggested to have similar molecular mechanisms of action at TRPA1. Thymol, 2,6-diisopropylphenol (propofol) and related simple alkyl phenols also activate TRPA1, and investigation with a series of alkyl phenol analogs indicates that bulky carbon substituents and high calculated log*P* values are correlated with an increased ability to activate TRPA1 [109]. TRPA1 is also activated by polyunsaturated fatty acids, which should contain at least three double bonds and 18 carbon atoms, such as docosahexaenoic acid (DHA) [110]. Arachidonic acid and its derivatives also activate TRPA1 independently of cysteine oxidation [111]. 6-Paradol and 6-gingerol activate TRPA1, whereas the non-TRPA1 agonist capsaicin does not, suggesting that a phenol core of these compounds is not sufficient to confer TRPA1 activation [40, 87]. Moreover, capsiate, a non-pungent capsaicin analog, also activates TRPA1 through a mechanism distinct from cysteine and histidine modification [112]. Therefore, TRPA1 activation by non-reactive compounds is dependent on their chemical structures rather than cysteine oxidation.

There are several protean-type non-electrophilic compounds that bimodally modulate TRPA1. Menthol and its derivatives [113], camphor [114, 115], nicotine [116], apomorphine [117], and propofol [118] all activate TRPA1 at low concentrations but show inhibitory effects at high concentrations. Bimodal modulation of TRPA1 is also a feature of certain cysteine-reactive compounds, such as cinnamaldehyde [114], AITC [119], umbellulone [85] and ligustilide [86].

Inhibitors of TRPA1 have been developed, exemplified by the synthetic inhibitors HC-030031, Chembridge-5861528 (a derivative of HC-030031), AP-18, A-967079 (a derivative of AP-18), AMG5445 and AZ868 [46, 48, 71, 72, 120-124]. Another, ADM_09, is an antagonist of TRPA1 with a putative dual-binding mode of action, which involves the synergic combination of Ca^{2+}-mediated binding of the carnosine group and disulfide-formation by its lipoic acid group [125]. Notably, some structurally related compounds have different effects on TRPA1 activity. Oxime derivatives related to AP-18 possess both activatory and/or inhibitory activity at TRPA1 [126]. Camphor and 1,8-cineol are naturally occurring inhibitors of human TRPA1, but 1,4-cineol is an activator [127]. Borneol is a more effective natural inhibitor than camphor and 1,8-cineol, and the hydroxyl group of borneol is suggested to contribute to its inhibitory action [128].

Several compounds have species-specific modulatory effects on TRPA1. AMG5445 inhibits human TRPA1, but activates rat TRPA1 [120]. The pharmacological profile of the human and rhesus monkey TRPA1 is relatively distinct from mouse and rat TRPA1 [129]. Importantly, findings of species-specific effects have helped to identify the critical region that determines TRPA1 modulation (Fig. **1**). Menthol is known to be a bimodal modulator of mouse TRPA1, whereas it does not inhibit human TRPA1, and *Drosophila* TRPA1 is insensitive to menthol [130]. Chimera and mutagenesis studies indicate that specific residues within TM5 (notably Ser876 and Thr877 of mouse TRPA1, corresponding to Ser873 and Thr874 of human TRPA1) are critical for menthol responsiveness. Furthermore, the region from TM5 to TM6 in mouse and human TRPA1 is the critical domain determining the inhibitory effects of menthol. The same two residues (Ser and Thr within TM5) are also critical for the sensitivity of TRPA1 to AMG5445, AP-18 and A-967079 [130, 131]. Ser873, Thr874 and Tyr812 residues of human TRPA1 are critical to the inhibitory effects of borneol, but not to camphor or 1,8-cineol [128]. Like menthol, propofol and lidocaine show bimodal effects on mouse TRPA1, but only work as activators of human TRPA1 [92, 118]. Although the species-specific differences above were linked to the TM5 and TM6 region, the residues within TM5 are not involved in the action of propofol and lidocaine. DHA sensitivity is limited to human and mouse TRPA1: *Drosophila* TRPA1 does not respond to DHA [110]. Neither the cytoplasmic N-terminal region nor TM5 of TRPA1 are directly involved in DHA sensing. These compounds (propofol, lidocaine and DHA) probably employ different or additional mechanisms to modulate TRPA1 as compared with menthol.

Caffeine, which is not a reactive chemical reagent, activates mouse TRPA1, but suppresses human TRPA1 [132]. A Met268Pro mutation in the N-terminal cytoplasmic region of mouse TRPA1 converts this residue to the human form and consequently changes caffeine action from activation to suppression [133]. An electrophilic compound CMP1, a structural analog of AMG5445, inhibits human

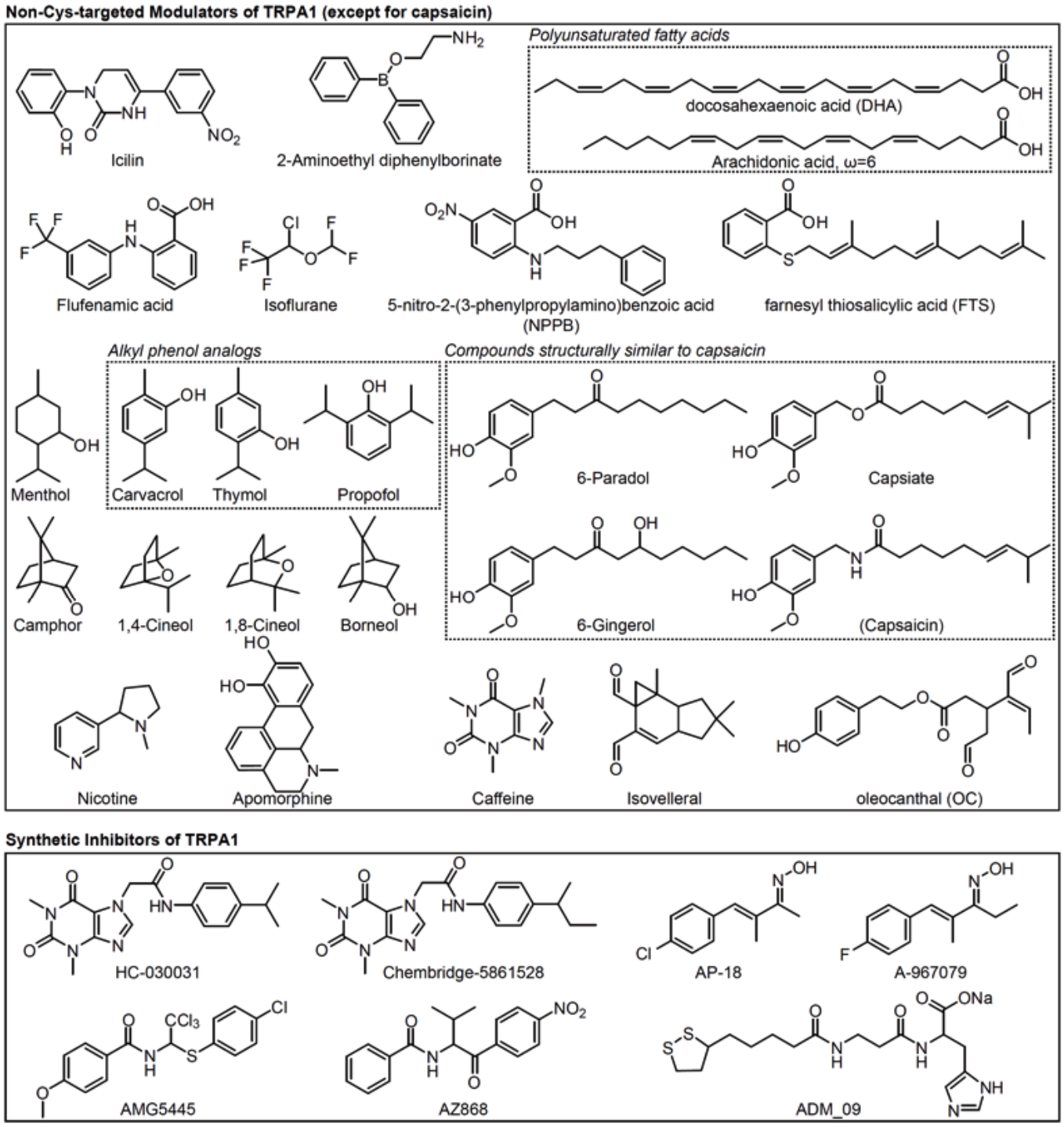

Fig. (3). Chemical structures of TRPA1 modulators other than Cys-targeted activators. Major non-Cys-targeted modulators and synthetic inhibitors of TRPA1 are shown.

TRPA1 and activates rat TRPA1 *via* modification of human Cys621 and rat Cys622, respectively [91, 134]. The specific mutations Ala946Ser and Met949Ile in the upper portion of the TM6 region of rat TRPA1 change the effect of CMP1 from activatory to inhibitory. Therefore, these studies demonstrate that specific regions and residues within TRPA1 determine the TRPA1 modulatory activity of non-electrophilic compounds, and that the key domains/residues vary between compounds. Furthermore, while direct physical interaction of non-electrophilic compounds with TRPA1 is likely to be critical for modulation, it is unclear whether or not these critical sites are involved in binding.

There have been other studies regarding chemical structure-specific TRPA1 activators. Isovelleral, a fungal natural product which contains an α,β-unsaturated aldehyde

moiety, activates TRPA1 independently of cysteine oxidation [83]. A major compound in extra-virgin olive oil, oleocanthal (OC), is an electrophile that does not require cysteine residues to activate TRPA1 [135]. A structure-activity relationship study using synthetic OC analogs indicated that OC requires both aldehyde groups to activate TRPA1. TRPA1 cysteine mutants show diminished responses to α-SOH, but further study with synthetic analogs of α-SOH indicated that the configuration of the *cis* C6 unsaturation in the alkylamides is also a determinant of the compound effect on TRPA1 [87]. The mouse Cys622Ser TRPA1 mutant is still sensitive to umbellulone, albeit less so than wild type TRPA1 [85]. Dihydroumbellulone, which lacks electrophilic properties because of reduction of the enone moiety, retains residual TRPA1-activating capacity. Zhong *et al* suggest that umbellulone is a mechanistically hybrid activator, apparently combining covalent interaction at a reactive cysteine with noncovalent interaction with a second site on TRPA1 [85]. Thus, chemical structure recognition by TRPA1, a clearly distinct mechanism from cysteine oxidation, is supposed to be important even for TRPA1 activation by some specific electrophiles.

SUBTYPE SELECTIVE *S*-NITROSYLATION BY A NOVEL NITROSAMINE

Protein *S*-nitrosylation, the covalent attachment of an NO moiety to the sulfur atom of cysteine residues to form *S*-nitrosothiol, regulates various protein functions to mediate NO bioactivity [136]. Receptor-activated (TRPC5, TRPC1 and TRPC4) and thermosensor (TRPV1, TRPV3, TRPV4 and TRPA1) TRP channels are activated by exogenous NO-releasing donors through *S*-nitrosylation [29, 32], but with very limited TRP subtype selectivity. Recently, this problem was partly solved with our finding that the 7-azabenzobicyclo [2.2.1]heptane (ABBH) *N*-nitrosamine selectively *S*-nitrosylates TRPA1 through transnitrosylation without releasing NO [76].

Although protein *S*-nitrosylation is widely accepted, questions regarding target selectivity of *S*-nitrosylation signaling are incompletely understood [137]. NO is produced *in vivo* by only three NO synthase (NOS) isoforms [138], and NO is reactive and diffusible within cells. Binding of NOS to targets or their adaptors have been demonstrated to localize nitrosylation reactions, but there are many *S*-nitrosylated proteins (>1,000) [136, 139, 140]. Recent studies have identified protein-protein transnitrosylation, the transfer of the NO group from one protein to another in the absence of apparent NO release, is a potentially important targeting pathway [140-142]. Transnitrosylation has been reported between specific proteins, exemplified by transnitrosylation of X-linked inhibitor of apoptosis by SNO-caspase-3 in apoptotic cell death [143-146]. A binding interaction between the two proteins is also required for transnitrosylation, because a binding-deficient mutant of one protein abrogates this protein-protein transnitrosylation [143, 145].

To develop transnitrosylation-based subtype-selective activators of TRP channels, it is necessary to first identify a synthetic NO donor that has only the transnitrosylative reactivity. However, SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine) and NOR3 ((±)-*(E)*-4-ethyl-2-[*(E)*-hydroxyimino]-5-nitro-3-hexenamide) are NO-releasing donors. In addition, it is suggested that nitroglycerin also releases NO by its metabolism [147]. *S*-Nitrosoglutathione is known to be a biological transnitrosylating agent, but also releases NO [148, 149]. In contrast, the ABBH *N*-nitrosamines constitute a new class of NO donors that, at physiological pH and temperature, transnitrosylate thiols to generate *S*-nitrosothiols without releasing NO [150-152]. Surprisingly, our intracellular Ca^{2+} imaging measurements have demonstrated that *N*-nitroso-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane (NNO-ABBH1) induces robust Ca^{2+} influx *via* recombinant human TRPA1 channels, but not *via* other SNAP-activated TRP channels, suggesting that NNO-ABBH1 selectively *S*-nitrosylates TRPA1 [76] (Fig. **4**). A modified labeling assay to biochemically identify protein *S*-nitrosothiol [153] showed that SNAP *S*-nitrosylates both TRPA1 and TRPV1, but NNO-ABBH1 *S*-nitrosylates only TRPA1. TRPA1 activation by NNO-ABBH1 is suppressed by specific cysteine mutations but not by NO scavenging, indicating that transnitrosylation underlies the activation of TRPA1 by NNO-ABBH1. This is supported by a positive correlation of N–NO bond reactivity and TRPA1-activating potency in a congeneric series of ABBH *N*-nitrosamines. Cys540, Cys641, and Cys665 of human TRPA1 are involved in its modification by NNO-ABBH1. Cys641 and Cys665 are also required for responsiveness to SNAP [32, 53], indicating that Cys540 may be a unique target for NNO-ABBH1.

To further explore this structure-activity relationship, we developed several non-electrophilic analogs of NNO-ABBH1: *N*-H (NH-ABBH), *N*-formyl (NCHO-ABBH), and *N*-methyl (NMe-ABBH) (Fig. **4**). These also activated TRPA1 but less potently than NNO-ABBH1. They also did not cause *S*-nitrosylation of TRPA1 and their activity was not affected by cysteine mutation of TRPA1, confirming that oxidative modification of cysteine residues is not critical for their mechanism of action. Importantly, the dose-response relationship for NMe-ABBH-induced recombinant TRPA1 activation was shifted to the left in the presence of SNAP (10 μM), which by itself cannot significantly activate TRPA1 [76]. This result supports the idea that TRPA1 activation by these non-electrophilic analogs may be subject to positive synergistic interactions between nitrosylation and molecular recognition, indicating that NNO-ABBH1 may be a hybrid activator. It is reported that a non-electrophilic TRPA1 activator flufenamic acid synergistically potentiates the activation of TRPA1 by AITC [102]. Also, umbellulone has been proposed to activate TRPA1 by combining covalent interaction at a reactive cystein with noncovalent interaction with a second site on TRPA1 [85]. It is important to note that TRPC5 and TRPV1 failed to respond to 300 μM NMe-ABBH, suggesting that TRPC5 and TRPV1, unlike TRPA1, may lack the molecular recognition sites for the non-electrophilic moiety of NNO-ABBH1 [76]. Thus, molecular recognition of chemical groups other than NO may explain the subtype-selective activation of TRPA1 by these compounds.

Despite evidence of synergistic effects between cysteine transnitrosylation and molecular recognition of the non-

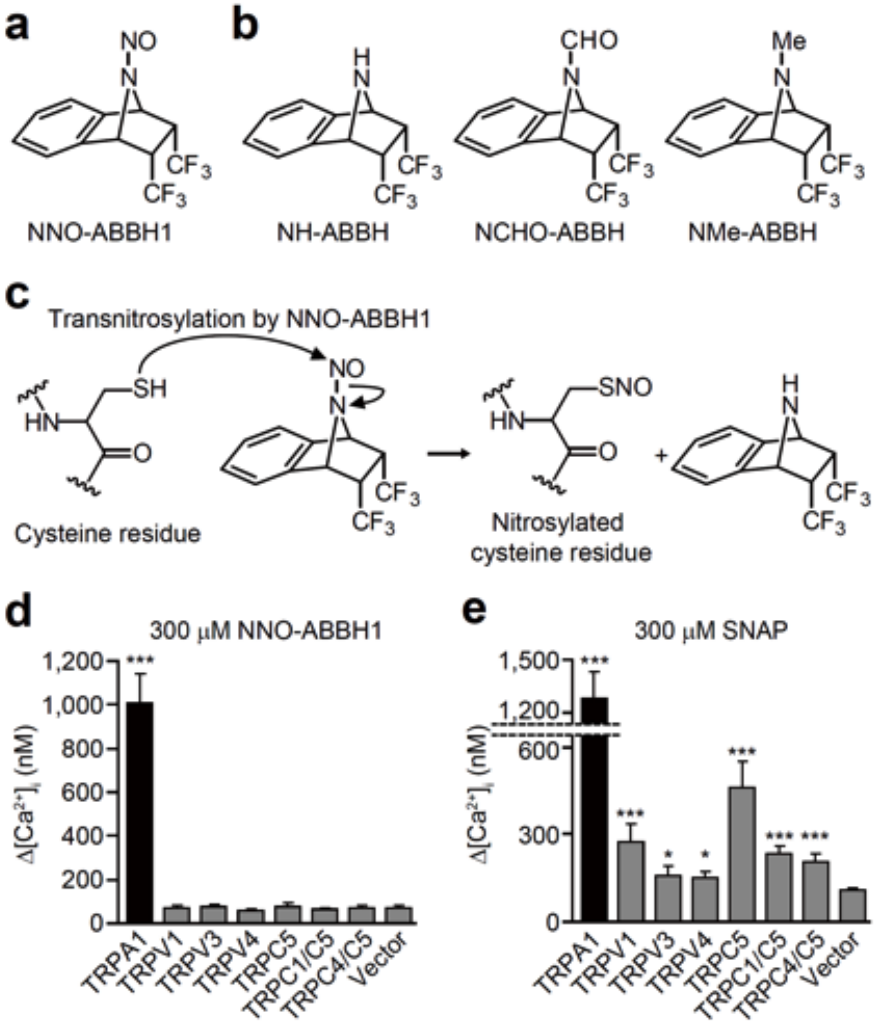

Fig. (4). Selective *S*-nitrosylation of human TRPA1 by a novel *N*-nitrosamine. Chemical structures of NNO-ABBH1 (**a**) and non-electrophilic analogs (**b**). (**c**) The chemical mechanism underlying the transnitrosylating action of NNO-ABBH1 on protein thiol group. (**d**) Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements using fura-2. Maximum rises in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) evoked by 300 μM NNO-ABBH1 in HEK 293T cells expressing TRPA1, TRPV1, TRPV3, TRPV4, TRPC5, TRPC1/TRPC5, TRPC4/TRPC5, or vector. (n = 47–107). $^{***}P <$ 0.001 compared to vector. (**e**) $\Delta[Ca^{2+}]_i$ evoked by 300 μM SNAP in HEK 293T cells expressing TRPA1, TRPV1, TRPV3, TRPV4, TRPC5, TRPC1/TRPC5, TRPC4/TRPC5, or vector (n = 22–111). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to vector. Reproduced from Fig. **1** of [76] with permission.

electrophilic moiety, it remains unclear how the transnitrosylation site and the non-electrophilic molecular recognition site converge in TRPA1. Also, it is unknown whether NNO-ABBH1 and other non-electrophilic analogs have bimodal and/or species-specific effects on TRPA1. Further detailed studies into TRPA1 modulation by ABBH *N*-nitrosamines will provide a basis for developing new drugs selectively targeting *S*-nitrosylation of TRPA1. In addition, these studies will present the opportunity for developing selective transnitrosylating modulators of other proteins. The findings of the selective activation of TRPA1 by NNO-ABBH1 or its non-electrophilic derivatives suggest that the ABBH skeleton imbues target protein selectivity *via* molecular recognition. Thus, designing and evaluation of various derivatives of ABBH could possibly be a strategy to find a derivative, which has an inhibitory effect specific on TRPA1 activity.

CONCLUSION

Because TRPA1 mediates neuropathic pain, vascular dilation and other functions, it has the potential to be an excellent drug target. Therefore, it is important to understand the mechanisms of both activation and inhibition of TRPA1 by small molecules. Recent studies have revealed that TRPA1 modulation by electrophiles is through cysteine oxidation, and that molecular recognition of chemical structures is a key determinant of TRPA1 modulation not only by non-electrophilic compounds, but also by some specific electrophiles. A novel ABBH *N*-nitrosamine induces selective *S*-nitrosylation of TRPA1 probably through synergistic processes of cysteine oxidation and molecular recognition of non-electrophilic moiety. However, molecular bases of TRPA1 modulation by non-electrophilic compounds are very poorly understood. Further studies are required to

delineate the entire mechanism. Similarly, further research is needed to define in detail the molecular mechanisms by which chemical ligands induce the activation of other TRP channels, such as TRPV1 and TRPM8 [10, 154]. This might support our understanding of TRPA1 mechanisms. TRPA1 channel activity is also modulated by Ca^{2+}, receptor stimulation, pH, osmotic pressure and temperature [40, 60, 69, 155-160], so a better understanding of the complexities of its modulation is critical to the development of novel TRPA1-specific drugs. It will also improve our appreciation of the physiological and pathological functions of TRPA1.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank N. Ogawa for helpful discussions. Fig. **4** was reproduced with permission from Fig. **1** in Daisuke Kozai, Yoji Kabasawa, Maximilian Ebert, Shigeki Kiyonaka, Firman, Yuko Otani, Tomohiro Numata, Nobuaki Takahashi, Yasuo Mori, and Tomohiko Ohwada, Transnitrosylation Directs TRPA1 Selectivity in *N*-Nitrosamine Activators, *Mol. Pharmacol January* **2014**; *85*: 175-185.

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Received: July 15, 2014 Revised: September 10, 2014 Accepted: October 07, 2014