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PRIORITY REVIEW

Neuronal and non-neuronal TRPA1 as therapeutic targets for pain and headache relief

Luigi F. Iannone [a,b], Romina Nassini [b], Riccardo Patacchini[c], Pierangelo Geppetti [a,b], and Francesco De Logu [b]

[a]Headache Center and Clinical Pharmacology Unit, Careggi University Hospital, Florence, Italy; [b]Section of Clinical Pharmacology and Oncology, Department of Health Sciences, University of Florence, Florence, Italy; [c]Corporate Drug Development, Chiesi Farmaceutici S.p.A, Nuovo Centro Ricerche, Parma, Italy

ABSTRACT

The transient receptor potential ankyrin 1 (TRPA1), a member of the TRP superfamily of channels, has a major role in different types of pain. TRPA1 is primarily localized to a subpopulation of primary sensory neurons of the trigeminal, vagal, and dorsal root ganglia. This subset of nociceptors produces and releases the neuropeptide substance P (SP) and calcitonin gene-related peptide (CGRP), which mediate neurogenic inflammation. TRPA1 is characterized by unique sensitivity for an unprecedented number of reactive byproducts of oxidative, nitrative, and carbonylic stress and to be activated by several chemically heterogenous, exogenous, and endogenous compounds. Recent preclinical evidence has revealed that expression of TRPA1 is not limited to neurons, but its functional role has been reported in central and peripheral glial cells. In particular, Schwann cell TRPA1 was recently implicated in sustaining mechanical and thermal (cold) hypersensitivity in mouse models of macrophage-dependent and macrophage-independent inflammatory, neuropathic, cancer, and migraine pain. Some analgesics and herbal medicines/natural products widely used for the acute treatment of pain and headache have shown some inhibitory action at TRPA1. A series of high affinity and selective TRPA1 antagonists have been developed and are currently being tested in phase I and phase II clinical trials for different diseases with a prominent pain component.

Abbreviations: 4-HNE, 4-hydroxynonenal; ADH-2, alcohol dehydrogenase-2; AITC, allyl isothiocyanate; ANKTD, ankyrin-like protein with transmembrane domains protein 1; B2 receptor, bradykinin 2 receptor; CIPN, chemotherapeutic-induced peripheral neuropathy; CGRP, calcitonin gene related peptide; CRISPR, clustered regularly interspaced short palindromic repeats; CNS, central nervous system; COOH, carboxylic terminal; CpG, C-phosphate-G; DRG, dorsal root ganglia; EP, prostaglandins; GPCR, G-protein-coupled receptors; GTN, glyceryl trinitrate; MAPK, mitogen-activated protein kinase; M-CSF, macrophage-colony stimulating factor; NAPQI, N-Acetyl parabenzoquinone-imine; NGF, nerve growth factor; NH2, amino terminal; NKA, neurokinin A; NO, nitric oxide; NRS, numerical rating scale; PAR2, protease-activated receptor 2; PMA, periorbital mechanical allodynia; PLC, phospholipase C; PKC, protein kinase C; pSNL, partial sciatic nerve ligation; RCS, reactive carbonyl species; ROS, reactive oxygen species; RNS, nitrogen oxygen species; SP, substance P; TG, trigeminal ganglion; THC, Δ9-tetrahydrocannabinol; TrkA, neurotrophic receptor tyrosine kinase A; TRP, transient receptor potential; TRPC, TRP canonical; TRPM, TRP melastatin; TRPP, TRP polycystin; TRPM, TRP mucolipin; TRPA, TRP ankyrin; TRPV, TRP vanilloid; VG, vagal ganglion;

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Introduction

The transient receptor potential (TRP) channel superfamily encompasses a group of more than 56 heterogeneous ion channels with a role in thermo- and osmo-sensation, sight, taste, smell, hearing, touch, and pain [1]. Genetic studies have demonstrated the involvement of TRP channels in several biological processes, and TRP mutations (channelopathies) have been associated with a variety of human disorders (*e.g.* polycystic kidney disease, skeletal dysplasia, familial episodic pain syndrome, and other disorders) [2]. In mammals, the TRP superfamily comprises six subfamilies and 28 members of nonselective cation permeable channels. They exhibit different calcium/sodium permeability ratios [3,4] and share a similar architecture, with minor differences: six transmembrane domains (S1-S6) assembling as

CONTACT Pierangelo Geppetti geppetti@unifi.it

homo- or hetero-tetramers with a cation-pore loop between S5 and S6. The amino (NH_2) and carboxylic acid (COOH) termini are both localized in the cytosol [5]. The NH_2 terminus domain contains several ankyrin repeats. TRP subfamilies are distinguished in TRP canonical (TRPC), TRP melastatin (TRPM), TRP polycystin (TRPP), TRP mucolipin (TRPML), TRP vanilloid (TRPV), and TRP ankyrin (TRPA) channels [5]. Most subfamilies include several members (*e.g.* TRPM and TRPV1 subfamilies), while the TRPA subfamily consists of only one member, the TRPA1 [6]. TRPs are activated or modulated by several endogenous and exogenous physicochemical stimuli, including intracellular mediators [7].

TRP channels are expressed in cellular membranes, except for the nuclear envelope and mitochondria, of almost every excitable and non-excitable cell type. Some TRP channels are expressed in discrete subpopulations of primary sensory neurons, detecting noxious physical (thermal and mechanical) and chemical stimuli [8]. These channels have been collectively labeled as thermo-TRP (namely TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, and TRPA1), as they can be activated by a large range of temperatures, from noxious cold to noxious heat [9]. Thermo-TRPs constitute one of the largest groups of nociceptive ion channels involved in sensory transmission and have been proposed to contribute to transmission and modulation of pain signals in mammals [8,10,11]. Herein, we summarize data on the TRPA1 channel in models of neuropathic, cancer, and migraine pain, highlighting its role in neurons and glial cells.

The TRPA1 channel

TRPA1 was primarily identified in cultured lung fibroblast (first labeled as ankyrin-like protein with transmembrane domain protein 1, ANKTD) [12] and subsequently recognized as a TRP member due to structure homologies [13]. The TRPA1 gene (*trpa1*) is expressed on chromosome 8q13 (27 exons, 55,701 base pairs) and is present in many animal species, both vertebrates and invertebrates [14]. TRPA1 is non-selectively permeable to sodium and potassium, although with higher permeability to calcium [15]. Structurally, it shows a peculiar elongated ankyrin repeat region within the N-terminus (~16 ankyrin repeats), which connects transmembrane proteins to the cytoskeleton and is involved in protein interactions and channel trafficking [16] (Figure 1).

TRPA1 is co-expressed and localized with other TRP channels (i.e. TRPV1 and TRPV4) [13] in a subset of primary sensory neurons of the trigeminal (TG), vagal (VG), and dorsal root ganglia (DRG), particularly in thinly myelinated Aδ fibers and unmyelinated C fibers [17]. TRPA1 has also been detected in the central nervous system (CNS) (*e.g.* cortex, caudate nucleus, amygdala, and hypothalamus) where its role is still unknown [18]. Although TRPA1 is found in non-peptidergic neurons as it co-localized with the purinergic P2X3 receptor, isolectin B4, or the $Na_{1.8}$ channel [19,20], it is worth noting that at least a quarter of neurons expressing TRPA1 are peptidergic Aδ- and C-fiber nociceptors, which release the proinflammatory neuropeptides, substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP), from their peripheral and central endings [19]. The peripheral release of neuropeptides elicits neurogenic inflammation, which consists mainly of plasma protein extravasation in post-capillary venules (mediated by SP) and dilation of arterioles (mediated by CGRP) [21–23]. Preclinical and recent clinical evidence support the critical role of CGRP, but not SP, in neurogenic inflammation in humans and migraine-related pain [24].

TRPA1 and temperature

From the initial proposal of TRPA1 as a sensor of cold temperature [13], the channel role in the physiological sensing of temperature changes has been subject to debate, and experiments assessing cold thermo-sensation in non-human cell cultures remain uncertain [25]. Studies using purified human TRPA1 (hTRPA1) inserted into lipid-bilayers showed its intrinsic bidirectional (U-shaped) thermosensor activity that is modified by the redox state and ligands, with different TRPA1 channel conformations involved in cold and heat sensation [26,27]. Therefore, in mammals, TRPA1 may contribute to sensing warmth and uncomfortable heat in addition to noxious

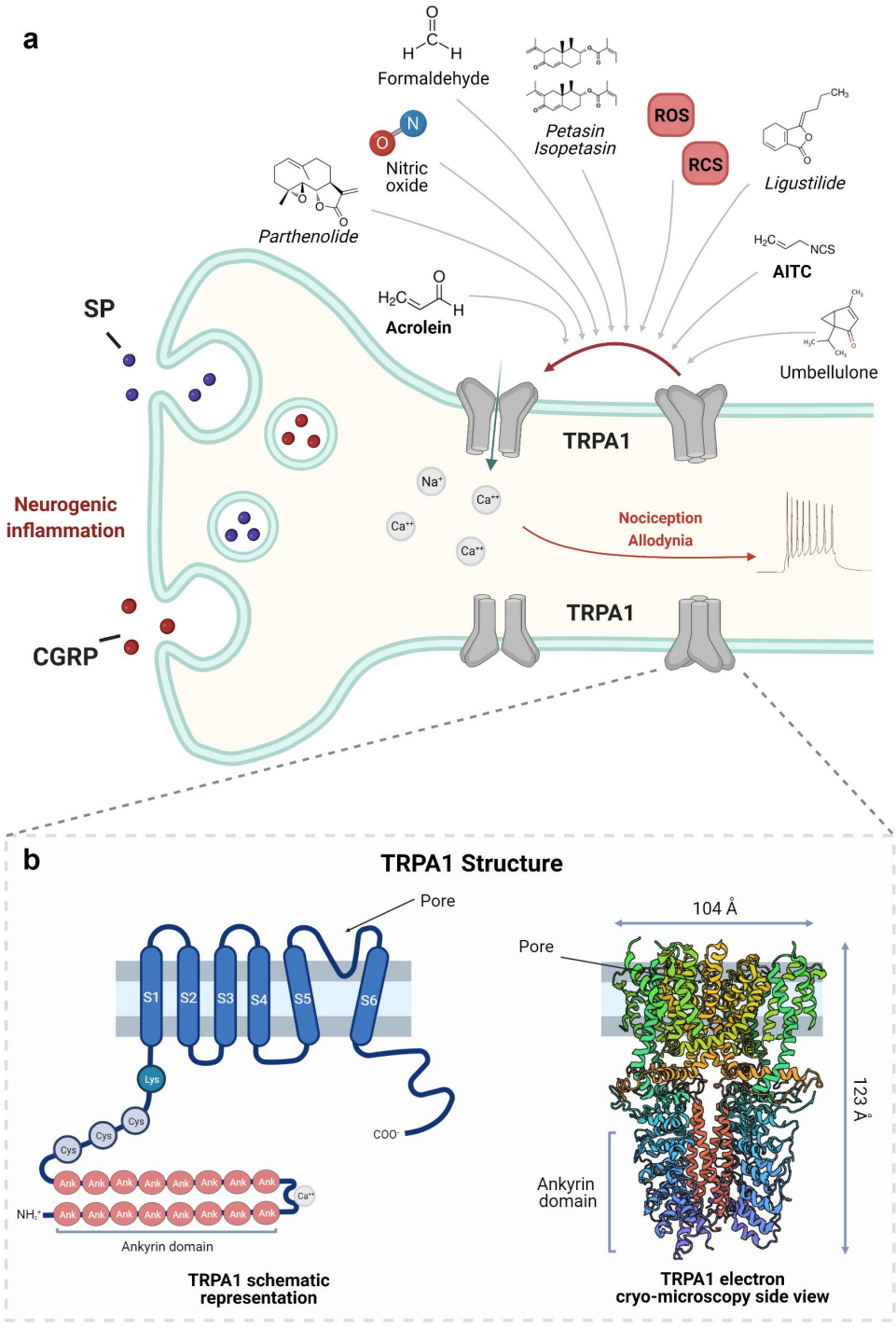

Figure 1. (a) Targeting of the TRPA1 ion channel by a series of endogenous and exogenous agonists with relevance in pain. The TRPA1 activation signals acute nociception and allodynia to the brain and elicits CGRP and SP release from peripheral fibers to produce neurogenic inflammation. Partial agonists and desensitizing agents of the channel are reported in italics. Commonly used stimulants of TRPA1 are in bold. (b) Schematic representation of TRPA1 ion channel structure and as determined by single particle electron cryo-microscopy. Structure is based on atomic coordinates in the Protein Data Bank (https://www.rcsb.org). *Image created with Biorender.com. AITC, allyl isothiocyanate; ANK, ankyrin; CGRP; calcitonin gene related peptide; Cys, cysteine; Lys, lysine; NO, nitric oxide; RCS, reactive carbonyl species; ROS, reactive oxygen species; SP, substance P; TRPA1, Transient receptor potential ankyrin.*

cold. Accordingly, a series of findings indicate that TRPA1 can be heat-sensitive in some mammalian somatosensory neurons (in a triad with TRPV1 and TRPM3) and that TRPA1 is implicated in thermal sensation, including heat (in cooperation with TRPV1 and TRPM3) [28]. However, hTRPA1 overexpressed in HEK293 (human embryonic kidney) cells failed to respond to cold stimulus (5°C)

thus suggesting a possible not intrinsic activation [29]. Considering that cooling can increase calcium ion influx and that hTRPA1 can be directly activated by calcium ions, an indirect activation by background calcium influx could also be hypothesized [25,30].

Comparative analyses of TRPV1 and TRPA1 among various species (including humans) revealed changes underlying different sensory perceptions. Whereas TRPV1-dependent sensitivity to capsaicin shows remarkable variations across a wide variety of vertebrate species, responses to heat appear to be conserved [31,32]. On the other hand, TRPA1 maintains a general and homogenous chemical sensitivity to electrophilic compounds but its sensitivity to thermal stimuli shows a puzzling scenario [31,32]. Further insights on the TRPA1 role for mechanical pain and thermal sensitivity have been reported by epigenetic studies, exploring changes in DNA methylation in the TRPA1 promoter. A genome-wide methylation analysis on monozygotic twins found that methylation of a C-phosphate-G (CpG) dinucleotide in the promoter of TRPA1 may have an impact on gene expression and skin thermal sensitivity, with methylation inversely associated with the threshold for heat-induced pain [33]. However, the study did not report any measure of TRPA1 expression in nociceptors [33]. Interestingly, the same hypermethylated CpG site was found in healthy adults with a low threshold for pressure pain, suggesting a correlation between TRPA1 methylation status and mechanical pain sensitivity [34]. Using multiple linear regression, females showed a marginally and not significant, higher methylation status of the promoter combined with higher pressure pain sensitivities compared with males [34].

Importantly, the expression of TRPA1 is not limited to neurons, as it is also found in extraneuronal tissues, including human keratinocytes and melanocytes [35], rat vascular endothelial cells, astrocytes, hair cells of the inner ear, epithelial cells in the lung, small intestine, pancreas, bladder, and skin fibroblasts [36]. In the context of the present discussion, the identification of TRPA1 in non-neuronal cells of the central and peripheral nervous system, and specifically in glial cells of the Schwann cell/oligodendrocyte lineage [37,38], has been instrumental for understanding the role of TRPA1 in the regulation of neuroinflammation and chronic pain.

TRPA1 agonists and antagonists

Numerous compounds with different chemical structures have been shown to activate TRPA1 *via* non-covalent or covalent binding to thiol moieties of specific cysteine residues. The chemical reactivity of these agonists, which are strong electrophiles, results in covalent binding that modifies the channel conformation in an activated state. These molecules include exogenous environmental irritants [39], laboratory chemicals, and some components of cigarette smoke (*e.g.* acrolein, crotonaldehyde, and formaldehyde) [40,41], general anesthetics (*e.g.* lidocaine and propofol) [42], as well as pungent plant ingredients (*e.g.* mustard oil, allicin, and isothiocyanates), often found in alimentary sources [43–45]. Endogenous products that target the channel by a covalent binding are commonly found at sites of tissue injury or inflammation, and include byproducts of oxidative, nitrative stress [reactive oxygen (ROS), nitrogen (RNS), and carbonyl (RCS) species, including hydrogen peroxide, peroxy-nitrite, acrolein, 4-hydroxynonenal (4-HNE), and others] [46–48] (Figure 1). It is important to underline that TRPA1 is targeted by changes in redox status and is exquisitely sensitive to modifications of cysteine residues, such as electrophilic reaction, oxidation, and S-nitrosylation of sulfhydryl groups [48]. TRPA1-deficient mice lack specific somatosensory or behavioral responses to acrolein, formalin, or 4-HNE [49]. Agonists that do not bind TRPA1 covalently include a chemically heterogeneous series of compounds of herbal origin [allyl isothiocyanate (AITC), Δ9-tetrahydrocannabinol (THC), carvacrol, thymol] [50,51] and several drugs, such as non-steroidal anti-inflammatory drugs (*i.e.* ibuprofen and diclofenac) [39], clotrimazole, and nifedipine [52] (see Table 1).

In addition to a direct activating mechanism, TRPA1 can be stimulated or sensitized through intracellular G protein-coupled signaling pathways. Stimulation of G-protein-coupled receptors (GPCRs), including bradykinin 2 (B2) [53],

Table 1. Exogenous and endogenous compounds identified as TRPA1 agonists.

Exogenous Agonists	Endogenous Agonists
Natural compounds	***ROS***
Cinnamaldehyde, allicin, dyallil disulfide, allyl/benzyl-isothiocyanate, thymol. carvacrol, delta-9-tetrahydrocannabinol	Superoxide, hypochlorite, hydrogen peroxide
Environmental irritants	***RNS***
Acrolein, acetaldehyde, crotonaldehyde, formaldehyde, toluene diisocyanate, nicotine, tear gases, heavy metals	Nitric oxide, proxynitrite
Drugs and metabolites	***Phospholipid's metabolites***
Anaestetic gases (propofol, isoflurane, desflurane, sevuflurane), lidocaine, diclofenac, indomethacin, ketoprofen, clotrimazole, dihydropyridines, glibenclamide, apomorphine, niflumic, flufenamic,	Nitrooleic acid, 4-HNE, 4-HHE, 4-ONE, malondialdehyde
	Prostaglandins and isoprostanes
	15-deoxy-delta-PGJ2, PGA2, PGA1, 8-isoprostane-PGA2
	Other
	Hydrogen sulfide

protease-activated receptor 2 (PAR2) [54], MAS-related (MrgprA3 and MrgprC11) [55], prostaglandins (EP) [56,57], and bile acid (TGR5) [58] receptor, can activate/sensitize the TRPA1 channel. The TGR5 receptor enhances TRPA1 activity directly through protein kinase C (PKC) activation, whereas EP, B2, and PAR2 receptors target TRPA1 by phospholipase C (PLC)-dependent increase in cytoplasmic calcium levels [53,54,56,57]. Increased intracellular calcium ions can directly activate/potentiate the channel, probably through calcium-binding with an EF-hand domain, frequently observed in calcium-interacting proteins [59,60]. TRPA1 can also be activated/sensitized *via* stimulation of TRPV1 or of other calcium permeable channels [47]. Activation of other GPCRs (*e.g.* MrgprA3 or MrgprC11) promotes the release of the G beta-gamma complex (Gβγ), which acts as the cytoplasmic activator of TRPA1 [55] (Figure 2). Furthermore, increased levels of nerve growth factor (NGF) activate the neurotrophic receptor tyrosine kinase A (TrkA) that enhances phosphorylated p38 mitogen-activated protein kinase (MAPK), regulating TRPA1 expression.

Finally, a variety of TRPA1 antagonists or partial agonists have been identified so far, including natural compounds contained in camphor, basil, wormwood, rosemary, lemon eucalyptus, and sage [51,61], and drugs, such as gadolinium, amiloride, gentamicin, and ruthenium red [62]. Some of these compounds have been shown to attenuate chemical, thermal (cold), and mechanical hypersensitivity in rodent models of inflammatory, cancer, and neuropathic pain [63].

The role of TRPA1 in pain

Nociceptive and neuropathic pain

Nociceptive pain, which can be subcategorized in somatic and visceral pain [64], results from tissue injury and inflammation. Prostaglandins (PGs) exert a major role in inflammatory pain, but oxidative stress may also contribute [65]. The underlying mechanisms of neuropathic pain, caused by damage or disease affecting the somatosensory nervous system [64,66], implicate the participation of central and peripheral glial cells and oxidative stress [67–69]. Response to tissue and nerve injury may evolve into a chronic pain status, with prolonged allodynia and hyperalgesia, typically represented by hypersensitivity to mechanical and thermal stimuli [64]. In addition to the central mechanisms, hypersensitivity is driven by overactivity of peripheral nociceptors *via* sensitization of their nerve terminals [70].

TRPA1 has been identified as a major component in mediating prolonged hypersensitivity to thermal, chemical, and mechanical stimuli in models of nociceptive and neuropathic pain. The first evidence of the involvement of TRPA1 in inflammatory nociception was reported in a study that showed a reduction in nociceptive response induced by formalin in rat and mouse paws following pharmacological antagonism or gene deletion of the channel [71]. Furthermore, acute inhibition of TRPA1 reduced cold and mechanical hypersensitivity related to persistent inflammation in animal models induced by carrageenan and complete Freund's adjuvant [72–74]. TRPA1 has also been shown to contribute to peripheral and central neuropathic pain. In a rodent model of diabetic neuropathy elicited by streptozotocin,

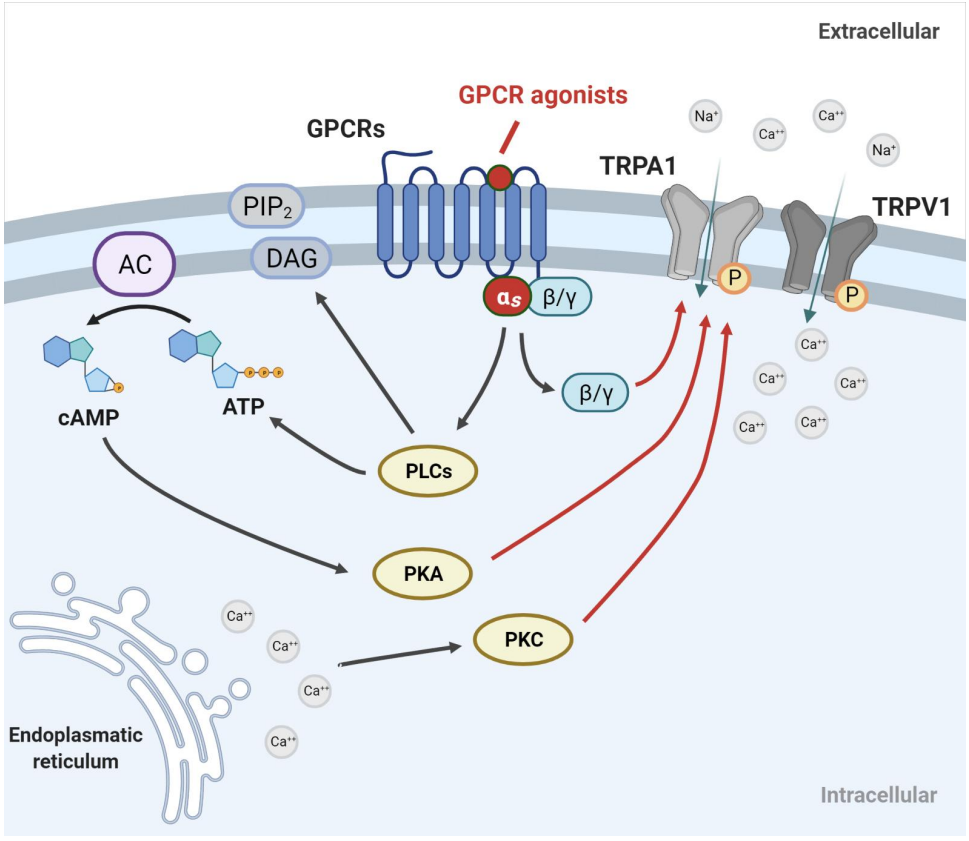

Figure 2. Intracellular modulation of TRPA1. G protein-coupled receptors (GPCRs) including bradykinin receptor 2 (B2), prostaglandins receptors (EP), MAS-related GPCRs (MrgprC11 and MrgprA3), protease-activated receptor 2 (PAR2), and bile acid receptor 5 (TGR5), through their second messenger signaling cascades regulate TRPA1 function. TRPA1 can also be activated or sensitized by mechanisms that increase cytoplasmic calcium levels (*e.g.* TRPV1 or other calcium permeable channels activation). *Image created with Biorender.com. AC, adenyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; GPCR, G protein–coupled receptor; PIP2, phosphatidyl inositol-4,5-bisphosphate; PLC, phospholipase C; PKA, protein kinase A; PKC, Protein kinase C; TRPA1, Transient receptor potential ankyrin; TRPV1, transient receptor potential vanilloid.*

a TRPA1 antagonist reduced both mechanical allodynia and hypersensitivity [75,76]. The oxidative stress and glucose metabolism byproducts, 4-HNE [46] and methylglyoxal [77], which are usually increased in diabetes, stimulate TRPA1, thus sustaining hyperalgesia.

A common and debilitating adverse event of anticancer agents includes the chemotherapeutic-induced peripheral neuropathy (CIPN), characterized by spontaneous pain, paresthesia, and long-lasting mechanical and cold hypersensitivity [78]. CIPN has been replicated in mouse models using thalidomide (and its derivatives) [79], oxaliplatin, paclitaxel, or bortezomib [80–83], producing prolonged mechanical and cold hypersensitivity. Both sensory abnormalities can be transiently reverted by TRPAl antagonists (only in part for paclitaxel) or ROS scavenger. The anticancer and toxic effects of chemotherapeutics are associated with their ability to generate an oxidative burst [84]. Notably, CIPN can be permanently prevented in TRPA1-deleted mice or when TRPA1 antagonists are continuously given when chemotherapeutics are administered and the ensuing oxidative insult is produced [81]. TRPA1 can also be directly gated by other chemotherapeutic agents (*e.g.* dacarbazine, exemestane, letrozole, and anastrozole), leading to painfully more transient mechanical hypersensitivity [85,86].

Mechanical hypersensitivity that follows nerve damage induced by moderate mechanical trauma (partial sciatic nerve ligation, pSNL) in mice is substantially mediated by TRPA1 [37,87]. Mechanical allodynia attenuates after systemic and/or intrathecal injection of TRPA1 antagonists in a spinal nerve ligation model [88]. Further

evidence has been supported by pharmacological antagonism and genetic deletion of the channel. Both interventions markedly reduced the mechanical allodynia evoked by the constriction of the infraorbital nerve (a model of type-II trigeminal neuralgia) in mice [89]. However, subsequent papers showed that the relationship between TRPA1 and chronic pain is more complex as additional cellular and molecular pathways are implicated. The identification of TRPA1 in glial cells of the Schwann cell/oligodendrocyte lineage [37,38] has moved the initial cellular site of the TRPA1 signaling pathway from neurons to glial cells. In a mouse model of brain damage by ischemia, a major role has been proposed for TRPA1 expressed by oligodendrocytes, which promotes a myelinated cell damage by uncontrolled calcium overload [38]. pSNL in mice elicits neuroinflammation in the nerve trunk and sustained mechanical allodynia. In this model, we reported that Schwann cells express a functional TRPA1 that is targeted by oxidative burst generated by invading hematogenous macrophages (typical of the Wallerian degeneration). Activated Schwann cell TRPA1, *via* a calcium-dependent NADPH oxidase 1 (NOX1) stimulation, promotes a bidirectional amplification of oxidative stress. On the one hand, Schwann cell TRPA1 releases ROS that, in a feed-forward manner, maintains the influx of macrophages inside the injured nerve trunk. On the other hand, ROS target the neuronal TRPA1 to signal a sustained allodynia [37].

In a model of alcohol-evoked peripheral polyneuropathy [90], Schwann cell alcohol dehydrogenase-2 (ADH-2) converts oral ethanol into acetaldehyde, which targets Schwann cell TRPA1 to generate ROS and 4-HNE, which, in turn, activate TRPA1 in nociceptors [90]. In this case, the Schwann cell-TRPA1 pathway is activated independently from macrophages. In mouse models of neuropathic pain, the prominent role of TRPA1 has been extensively reported, but in rats the ability of TRPA1 deletion to attenuate mechanical hypersensitivity has not been replicated in models of peripheral severe nerve damage (chronic constriction injury) or CIPN where TRPA1 was deleted by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology [91].

The prominent role of TRPA1 in different rodent models of pain seems to have a counterpart in humans, as a familial episodic pain syndrome has been associated with a gain-of-function mutation of TRPA1 [92]. Selective TRPA1 antagonists have been tested in preclinical studies but few clinical trials have been conducted with TRPA1 antagonists in pain diseases, as discussed below.

Cancer pain

Cancer-related pain is defined as pain caused by the primary cancer itself, metastases, or its treatment (chronic post-cancer treatment pain) [66]. Cancer pain is frequently experienced by patients as it affects more than half of the patients undergoing anticancer therapy or presenting advanced disease [93]. Despite the dramatic impact of pain in cancer, the exact mechanism underlying these probably unrelated conditions (cancer, metastasis, and their therapy) is poorly understood and, consequently, patients are undertreated. The first indirect evidence relating TRPA1, and cancer pain was shown by inoculating human squamous cell carcinoma cells in the mouth cavity and in the right-hind paws of mice [94]. Treatment with anti-NGF, which reduced tactile allodynia and oral pain (increasing chewing time), simultaneously decreased the expression of TRPA1 and TRPV1 in trigeminal ganglia [94]. More direct evidence of oxidative stress and the TRPA1 role in mechanical and cold allodynia was obtained in a mouse model of metastatic cancer pain evoked by injection of melanoma cells in the hind paw [95]. NOX and hydrogen peroxide levels were increased in the hind paw skin 2 weeks after inoculation. Genetic deletion or pharmacological antagonism of TRPA1 or the use of antioxidants (α-lipoic acid) attenuated mechanical and cold allodynia [95]. It is worth noting that ROS released from infiltrating macrophages may target TRPA1-expressing melanoma cells to amplify the oxidative stress signal that affects tumor cell survival and proliferation [96]. These findings have been replicated using breast tumor cells in a model of bone metastasis pain. NOX and hydrogen peroxide levels were increased in the sciatic nerve and hind paw skin samples, and these mice developed a mechanical

and cold allodynia reversed by TRPA1 antagonist or α-lipoic acid [97]. TRPA1 antagonists reduced mechanical and heat hypersensitivity in mice administered with dacarbazine in combination with melanoma cells inoculated into the hind paw [86] or mechanical and cold allodynia with breast carcinoma cell line inoculated into the tibia [98]. Thus, TRPA1 seems to contribute to pain developed during cancer and may represent a potential target for cancer pain treatment.

The cellular and molecular mechanisms responsible for TRPA1 involvement in cancer pain have been investigated in more detail in murine models where melanoma or lung carcinoma cells were injected into the hind paw [99]. For the first time, the key role of macrophages in these models of cancer pain was identified. However, unlike the pSNL model, allodynia and neuroinflammation were sustained by expansion of endoneurial resident macrophages and not invasion of hematogenic macrophages. Targeted deletion of TRPA1 in Schwann cells revealed that cancer-dependent macrophage expansion was promoted by Schwann cell TRPA1, which targeted by oxidative stress (from cancer cells or expanded macrophages), releases the macrophage chemo-attractant macrophage-colony stimulating factor (M-CSF). Thus, a feed-forward mechanism implicating M-CSF, resident macrophages, oxidative stress, and Schwann cell TRPA1, operating throughout the entire nerve trunk, sustains neuroinflammation and signals cancer-evoked pain [99].

Migraine

The underlying pathway responsible for migraine pain and how a large variety of heterogeneous stimuli trigger headache pain remains poorly known. However, the successful development of drugs that antagonize the CGRP pathway in acute (gepants) and prophylactic (anti-CGRP monoclonal antibodies, anti-CGRP mAbs) treatments has underlined the crucial role of CGRP in the headache phase of migraine attacks [100–102].

TRPA1+ ve terminals of both C- and Aδ-fibers frequently co-localized with CGRP have been documented in the superficial lamina of the trigeminal *nucleus caudalis* and perivascular tissue of cranial vessels [103]. Therefore, neuronal activation may lead to neuropeptide release from the central and peripheral terminals of trigeminal nociceptors. Neurogenic inflammation may occur in cranial tissues innervated by the trigeminal nerve, including in the dura mater [21]. Migraine-provoking substances in humans (*i.e.* CGRP, pituitary adenylate cyclase-activating polypeptide, prostaglandin E_2, prostacyclin, and histamine) are also able to initiate periorbital mechanical allodynia (PMA) in mice by acting on peripheral terminals of trigeminal afferents [43]. As cutaneous allodynia is frequently reported by patients with migraine attacks [104], PMA has been proposed as a possible migraine model in mice and rats [105].

Meningeal vasodilation has also been caused by intranasal administration of TRPA1 agonists, whereas a selective antagonist of the TRPA1 receptor and a topical CGRP antagonist ($CGRP_{8-37}$) prevented the increases in blood flow [21]. Systemic administration of a headache provoking substance, glyceryl trinitrate (GTN) elicits in migraineurs an early, mild-to-moderate headache, followed by delayed, more severe migraine-like attacks [105]. In rodents, GTN causes an immediate and transient vasodilation and a delayed and sustained mechanical allodynia in the cutaneous periorbital area [106,107]. A TRPA1 antagonist reduced pain biomarkers elicited by GTN and mechanical allodynia produced by the constriction of the infraorbital nerve in rats [108]. The underlying mechanism of the TRPA1 implication in GTN-evoked periorbital mechanical allodynia was recently better clarified. The following series of events was proposed. Nitric oxide (NO), a known TRPA1 agonist [109], generated from GTN by aldehyde dehydrogenase-2 is required to initiate the allodynia, but is not sufficient for its maintenance [105]. In fact, NO-mediated stimulation of TRPA1 in the soma of trigeminal nociceptors increases intracellular calcium, which activates NOX1 and NOX2 to release ROS. ROS promotes a feed-forward ROS/TRPA1-dependent pathway that sustains allodynia. From the stimulated neuron soma, an antidromic action potential propagates to the terminal nerve fibers from which CGRP is released, thus contributing to PMA [105].

Umbellulone, the major constituent of the *Umbellularia californica* (California bay laurel), is

known to provoke migraine and cluster headache in susceptible individuals [110]. This irritant molecule has been identified as a TRPA1 agonist *in vitro* and can cause nociceptive behavior, vasodilation in the nasal mucosa, and release of CGRP from meningeal tissue in rats. All *in vivo* responses evoked by umbellulone were abolished in mice with TRPA1 deletion [110]. The hypothesis that migraine provoking agents, such as chlorine, cigarette smoke, formaldehyde, and others, may act in humans by stimulating TRPA1 is strengthened by these findings.

A recent study [111] showed results that explain the cellular and molecular mechanisms by which CGRP released from trigeminal fibers causes migraine pain by an unexpected pathway that involves TRPA1. CGRP released by subcutaneous injection of capsaicin in mouse periorbital area targets the CGRP receptor (CLR/RAMP1) on surrounding Schwann cells to elicit intracellular events associated with the internalization of the agonist-receptor complex in endosomes. In this manner, CGRP causes a cAMP-dependent formation of NO that gating TRPA1 on Schwann cell releases ROS. On the one hand, ROS by continuous Schwann cell TRPA1 targeting amplifies neuroinflammation and, on the other hand, targeting neuronal TRPA1 signal mechanical allodynia [111] (Figure 3).

Some analgesics and herbal medicines/natural products widely used for the acute treatment of headache attacks seem to act *via* TRPA1. The highly reactive metabolite of acetaminophen N-Acetyl-parabenzoquinone-imine (NAPQI), responsible for hepato- and nephron-toxic effects of the drug, target TRPA1, promoting a mild neurogenic inflammatory response [112]. CYP450 monooxygenase activity may produce NAPQI

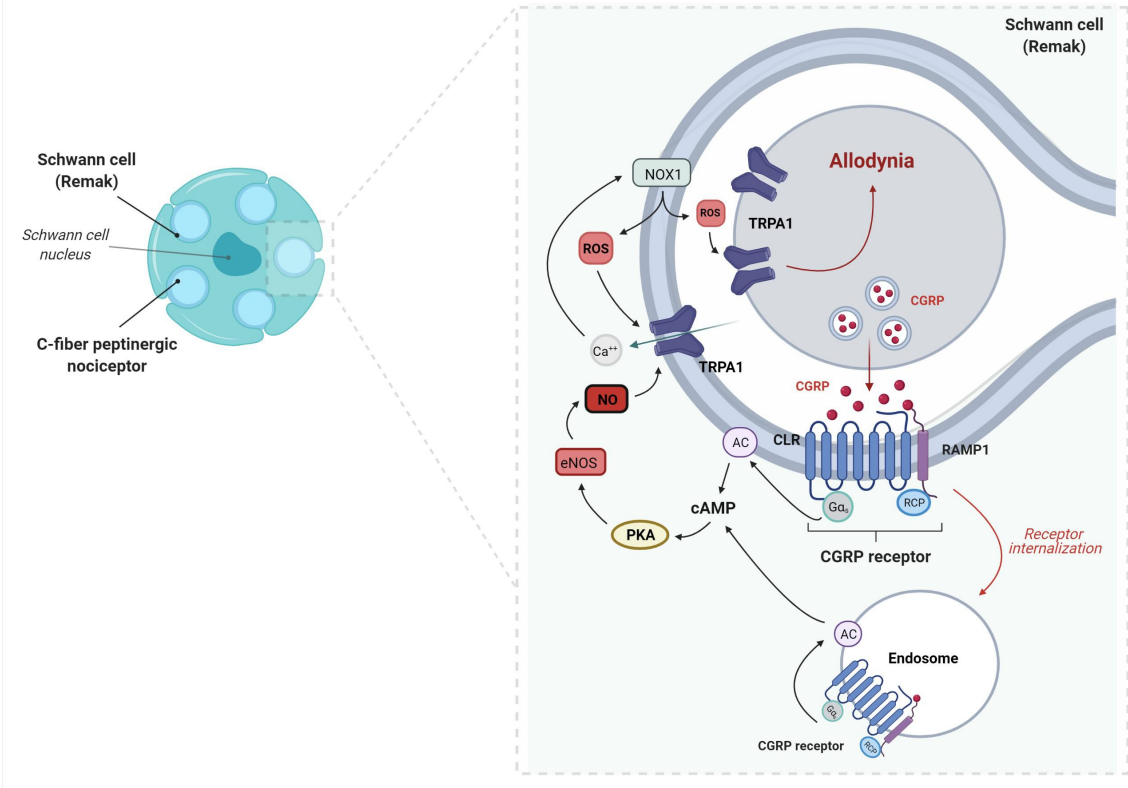

Figure 3. The mechanism of CGRP released from peptidergic trigeminal fibers to elicit mechanical allodynia. CGRP targets the CGRP receptor complex in adjacent Schwann cells (possibly of the Remak subtype) that, following internalization in endosomes, promotes a series of intracellular events that eventually targets Schwann cell TRPA1 to initiate a feed-forward pathway that amplifies the oxidative stress signal. On one hand, ROS by continuous Schwann cell TRPA1 targeting amplify and prolong neuroinflammation and on the other hand by targeting the neuronal TRPA1 sustain mechanical allodynia. *Image created with Biorender.com. AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; CLR, calcitonin receptor-like receptor; CGRP; calcitonin gene related peptide; eNOS, endothelial nitric-oxide synthase; NO, nitric oxide; NOX1, NAD(p)H oxidase 1; PKA, protein kinase A; RCP, receptor component protein; ROS, reactive oxygen species;RAMP1, receptor activity modifying protein 1; TRPA1, transient receptor potential ankyrin 1.*

and parabenzoquinone (*p*-BQ) in the spinal cord, thus locally activating TRPA1 and causing a prolonged desensitization of voltage-gated calcium and sodium currents in primary sensory neurons. The effect of spinal and systemic administration of acetaminophen was absent in TRPA1 deleted mice, suggesting that NAPQI and *p*-BQ produce a TRPA1-mediated spinal antinociception in [113]. Pyrazolone derivatives (including dipyrone, antipyrine, aminopyrine, and propyphenazone) have been used for decades for the acute relief of migraine attacks, although the specific mechanism of their antimigraine and analgesic action remains unknown [114]. Dipyrone and propyphenazone have been found to selectively antagonize TRPA1 *in vitro* and *in vivo* and to attenuate nociception and allodynia in animal models of neuropathic and inflammatory pain, independently from prostaglandin production and *via* a TRPA1-mediated mechanism [115].

Regarding herbal products, *Tanacetum parthenium* (feverfew) and *Petasites hybridus Gaertn* (butterbur) have been used for centuries to treat migraine and other pain conditions. Some preparations containing parthenolide (a feverfew constituent) and butterbur (in particular, its components, petasin and isopetasin) are or have been used for migraine prophylaxis [116]. In rodents, parthenolide and isopetasin behave as TRPA1 partial agonists [61,117] with an initial activation followed by prolonged concentration- and dose-dependent specific TRPA1 desensitization and nonspecific desensitization of peptidergic nociceptors, which express the channel [61]. In this manner, nociceptor nerve fibers became unresponsive to any stimulus, and unable to release CGRP from their terminals, including those present in the trigeminovascular system. Finally, a compound contained in plants largely used in traditional medicine, ligustilide, has been identified as a TRPA1 partial agonist, with a certain degree of inhibitory activity on mustard oil activated currents in the dural [118].

Animal models of migraine pain do not completely and satisfactorily recapitulate the human disease. However, recent evidence that small-molecule receptor antagonists and monoclonal antibodies against CGRP or its receptor are beneficial for the acute treatment and the prophylaxis of migraine [119] indicate this peptide neurotransmitter, released from terminals of trigeminal neurons, as a major mediator of migraine pain. Therefore, the administration of CGRP or agents that release CGRP from trigeminal nerve terminals in a typical anatomical area of migraine pain (that is, periorbital area) could be a suitable mouse migraine model [37,89,111].

Clinical status of TRPA1 antagonists

In the last decade, several selective and potent TRPA1 antagonists have been developed [120], and five of them have been tested in clinical trials for the treatment of pain or asthma [121,122]. A few phase II clinical studies have been previously conducted, and recently new phase II has been started. However, at present, no TRPA1 antagonist has been analyzed in phase III trials. In particular, two TRPA1 antagonists (ODM-108 by Orion Pharma and GDC-0334 by Genentech/Roche) have been tested in phase I trials for potential use in neuropathic pain (NCT02432664) and asthma (NCT03381144), respectively, but the development was discontinued due to complex pharmacodynamic features for ODM-108 and for the termination of molecule development for GDC-0034 due to toxicity in preclinical species [123]. Glenmark pharmaceutical reported in a 2014 press release positive results of the TRPA1 antagonist ISC 17536 (formerly known as GRC17536) in a proof-of-concept phase IIa trial enrolling patient with chronic painful diabetic peripheral neuropathy. The successive publication reported that the study did not meet the primary end point (change from baseline to end of treatment in the mean 24-h average pain intensity score) [124,125]. However, in a subpopulation of patients with preserved small nerve fiber function, defined by quantitative sensory testing, statistically significant and clinically meaningful improvement in pain were observed [124,125]. There was no evidence of CNS or other drug related adverse events [124,125]. However, its pharmacokinetic/bioavailability is challenging, being a lipophilic, large, and poorly soluble molecule. Recently, the phase IIb trial to assess dose-range effect of GRC17536 has started recruitment across multiple centers in India [CTRI/2021/08/035410]. The

primary outcome measure is the mean 24-h average pain intensity change from baseline. No results have been reported yet.

The compound CB-189625, TRPA1 antagonist, was tested in an open-label, dose-escalation, phase I clinical trial for acute surgical pain by a partnership of Cubist Pharmaceuticals and Hydra Biosciences. However, the study was discontinued due to pharmacokinetic issues (*i.e.* low solubility) [4]. Hydra Biosciences owns another TRPA1 antagonist, the HX-100 compound, tested for painful diabetic neuropathy and allergic asthma. A phase I trial was completed in 2016 but no results have been reported so far.

After the acquisition of the TRPA1 antagonist program from Hydra Biosciences in 2018 from Eli-Lilly started the clinical testing of a TRPA1 highly selective, small-molecule antagonist (LY3526318, previously known as HX-260) in two randomized, placebo controlled, phase 2 clinical trials for the treatment of chronic low back pain [NCT05086289] [126] and diabetic peripheral neuropathic pain [NCT05177094] [127]. The primary outcome measure for both studies is the change from baseline for average pain intensity measured by the numeric rating scale (NRS). This drug has already been studied in two phase I studies between 2020 and 2021; a third phase I study was terminated due to COVID-19 pandemic [128]. Nowadays, TRPA1 small-molecule patents have been filed by several pharmaceutical companies, highlighting the therapeutic potential of the TRPA1 antagonism.

Conclusions

The current understanding of the molecular mechanisms underlying central and peripheral nociceptive pathways is still limited, and pain management remains a major unmet medical need. The identification of TRPA1 channels in nociceptors, where they sense noxious stimuli, has provided new insights on the neural processing of pain signals. However, the recent discovery of the presence of TRPA1 in glial cells of the central and peripheral nervous system has greatly expanded the ways in which the channel contributes to process pain. Notably, the Schwann cell TRPA1/NOX1 pathway that amplifies the oxidative burden within the nerve trunk has been identified as a major contributing factor to sustain neuroinflammation and the ensuing chronic pain.

Disclosure statement

P.G. received personal fees from Allergan, Eli Lilly, Novartis, Amgen, TEVA; Grants from Amgen, TEVA, Eli-Lilly, Allergan, Chiesi; Scientific Advisory Board, Endosome Therapeutics; Founder and shareholder FloNext srl, Spinoff of the University of Florence. R.N. and F.D.L. are founding scientists of FloNext srl. R.P. is fully employed at Chiesi Farmaceutici SpA, Parma, Italy. No disclosures have been reported by the other author

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ORCID

Luigi F. Iannone http://orcid.org/0000-0003-3598-0195
Romina Nassini http://orcid.org/0000-0002-9223-8395
Pierangelo Geppetti http://orcid.org/0000-0003-3797-8371
Francesco De Logu http://orcid.org/0000-0001-8360-6929

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