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Tackling chronic pain and inflammation through the purinergic system

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Running title

Purines and pyrimidines in pain and inflammation

Abstract

The purinergic system is composed of purine and pyrimidine transmitters, of the enzymes that modulate the interconversion of nucleotides and nucleosides, of the membrane transporters that control their extracellular concentrations, and of the many receptor subtypes that are responsible for their cellular responses. The components of this system are ubiquitously localized in all tissues and organs, and their involvement in several physiological conditions has been clearly demonstrated. Moreover, extracellular purine and pyrimidine concentrations raise several folds under pathological conditions like tissue damage, ischemia, and inflammation, which suggest that this signaling system might contribute both to disease outcome and, possibly, to its tentative resolution. The complexity of this system has greatly impaired the clear identification of the mediators and receptors that are actually involved in a given pathology, also due to the often opposite roles played by the various receptor subtypes. Nevertheless, this knowledge is fundamental for the possible exploitation of these molecular entities as targets for the development of new pharmacological approaches. In this review, we aim at highlighting what is currently known on the role of the purinergic system in various pain conditions and during inflammatory processes. Although some confusion may arise from conflicting

results, literature data clearly show that targeting specific purinergic receptors may represent an innovative approach to various pain and inflammatory conditions, and that new purine-based drugs are now very close to reach the market with these indications.

Keywords

P1 receptors, P2X receptors, P2Y receptors, Adenosine, ATP, UTP, ectonucleotidases, membrane transporters

1. Introduction

The purinergic system represents one of the most complex, but yet partially unexplored signaling systems involved in both physiological and pathological conditions. Its intriguing complexity is due to several factors: i) purines and pyrimidine are ubiquitous molecules, which are involved in many biochemical processes spanning from the synthesis of nucleic acids, phosphorylation reactions and energy transfer to neurotransmission and cell-to-cell communication; ii) a close interconnection among the different signaling molecules is present, due to complex metabolic reactions catalyzed by various intracellular and extracellular enzymes; iii) each molecule activates a variety of membrane receptors, often leading to opposite functional effects on target cells or to the pleonastic activation of common downstream signaling pathways (see specific sections below); iv) extracellular concentrations of purines and pyrimidines are fine-tuned also by the activity of specific membrane transporters; v) physiological nanomolar/low micromolar extracellular purine and pyrimidine concentrations raise up to the high micromolar/millimolar ranges at sites of tissue damage, ischemia and/or under inflammatory conditions (Figures 1 and 2) [1, 2].

Such a complex organization provides a vast variety of different targets (i.e., receptor subtypes, enzymes, membrane transporters) which could be possibly modulated by selective pharmacological approaches, but also poses the difficulty of avoiding unwanted side effects and to selectively act at the desired site, either at cellular or tissue level. This is one of the main reason why, despite its well-known involvement in many pathological conditions, at present very few drugs acting on the purinergic system have reached the market [3]. In the last years, joint efforts of pharmacologists, biochemists, molecular biologists and pharmaceutical chemists have greatly advanced the research on innovative purine- and pyrimidines-based drugs for several diseases.

In this review, we shall summarize what is currently known on the role of the purinergic system in chronic pain and inflammatory conditions, and the possible future directions for the development of innovative analgesics and anti-inflammatory agents, with an eye also on non-conventional and traditional medicine approaches.

2. ROLE OF PURINES IN CHRONIC PAIN

2.1 Extracellular nucleotides in chronic pain

2.1.1 P2X ion-channel receptors and chronic pain

Pharmaceutical research continues to fail in addressing the increasing need for novel, effective, safe, and well-tolerated treatments for chronic pain and related conditions, despite decades

of innovation and efforts in understanding the cellular and molecular basis of pain transmission. As mentioned in the Introduction, since ATP is abundantly present in all tissues and cells, and it is over-released in different pathological environments, an increasing number of published papers is now pointing out its functional relevance in many disease processes, including pain. Therefore, the selective inhibition of ATP-gated cation channels, the P2X receptors (P2XRs, encompassing the P2X1,2,3,4,5,6,7R subtypes), has received significant focus from academic and pharmaceutical scientists in the search of innovative and effective "druggable" targets for the development of new analgesics.

P2XRs have a widespread tissue distribution. For example, on smooth muscle cells, they mediate the fast-excitatory potential that leads to depolarization and tissue contraction. In the central nervous system (CNS), P2XR activation allows calcium to enter neurons, thus evoking neuromodulatory responses. In primary afferent nerves, P2XRs are critical for the initiation of action potentials when they respond to ATP released from sensory cells. In immune cells, activation of P2X receptors triggers the release of pro-inflammatory cytokines such as interleukin 1β [4]. Not all P2XR subtypes have been found implicated in pain signaling pathways. Here we summarize the current knowledge on the most important nucleotide-activated ion channels involved in nociception.

P2X3Rs. Multiple purinergic pathways are involved in the pro-nociceptive actions of ATP. However, since their discovery, a fundamental role has been proposed for receptors containing P2X3 subunits (i.e. homotrimeric P2X3Rs and heterotrimeric P2X2/3Rs) in mediating the primary sensory effects of ATP.

P2X3Rs and P2X2/3Rs are crucially involved in both neuropathic and inflammatory pain [5, 6], also due to their specific localization on sensory neurons in nociceptive pathways. In fact, P2X3Rs and P2X2/3Rs are predominantly localized on small-to-medium diameter C- and Aδ fiber sensory neurons within the dorsal root ganglia (DRG) and cranial sensory ganglia, and on their peripheral nerve terminals in tissues including the skin, joints, and viscera [7]. P2X3Rs and P2X2/3Rs are also present on the central projections of primary sensory neurons within the dorsal horn of the spinal cord and in the brainstem, where they play a role in promoting the release of glutamate and substance P (SP) at this first sensory synapse [8]. Moreover, P2X3-containing ionotropic receptors are expressed in a large portion of unmyelinated and thinly myelinated primary afferent nerves that innervate essentially all tissues and organs [9]. ATP is released from many cell types in these receptive fields, as well as at the central terminals of activated afferents, and, as mentioned in the Introduction, its release is increased under conditions of injury, inflammation and stress. As a co-transmitter, ATP is co-released with γ-aminobutyric acid (GABA) by spinal interneurons, thus contributing to the

modulation of nociceptive pathways. Moreover, thermal hyperalgesia is mediated by spinal P2X3Rs via activation of N-methyl-D-aspartate (NMDA) receptors [10].

In addition, intrathecal administration of ATP produced long-lasting allodynia via P2X2/3Rs [11], and sensitization of P2X3Rs, rather than a change in ATP release, appeared to be responsible for the development and maintenance of neuropathic pain and related allodynia [6, 12].

It has also been postulated that P2X3R-dependent cytosolic phospholipase A2 (cPLA2) activity in primary sensory neurons is a key event in neuropathic pain, so that cPLA2 pathway might be a potential target for treating this type of pain [13]. Additionally, vascular endothelial growth factor (VEGF) is involved in neuropathic pain transmission mediated by P2X2/3Rs expressed by primary sensory neurons [14], and endothelin-1-induced hyperalgesia in rats was produced by its action on vascular endothelial cells, sensitizing them to release ATP, which in turn acted on P2X3Rs on nociceptors [15].

Surprisingly, besides the clear role for P2X3Rs and P2X2/3Rs in facilitating pain transmission, the intracerebroventricular (i.c.v.) administration of ATP and P2XR agonists produced mechanical and thermal antinociception in rats, leading to the hypothesis that supraspinal P2XRs might play an inhibitory role in pain transmission [16]. However, from then on, no further evidence supported this speculation.

Concerning P2X3Rs activity in sensory ganglia, it is well known that they are highly expressed on trigeminal ganglion (TG) primary afferent neurons, suggesting that they may be targets for craniofacial pain therapies [17]. ATP, via P2X3Rs, induced the sensitization of TG nociceptors, and this in turn increased the sensitivity to ATP of surrounding satellite glial cells in chronic pain conditions [18]. Therefore, it is now widely accepted that ATP has a role in the sensitization of primary afferents at both peripheral and central terminals, and, as a consequence, in mechanisms underlying migraine headache [19]. From a molecular point of view, upregulation of nociceptive P2X3Rs on TG neurons by the migraine mediators calcitonin gene-related peptide (CGRP), nerve growth factor (NGF) and bradykinin (BK) has been reported [20-22], and anti-NGF treatment suppressed the responses evoked by P2X3R activation in an *in vivo* mouse model of TG pain [23]. It has also been reported that silencing C-terminal Src inhibitory kinase in TG neurons potentiated P2X3R responses, thus identifying another potential target for TG pain suppression [24]. Moreover, P2X3R activity on mouse TG sensory neurons was enhanced by the familial hemiplegic migraine type 1 (FHM-1) calcium channel mutation R192Q [25]; in fact, TG sensory neurons from FHM-1 knock-in mice exhibited a lower firing threshold and generated more action potentials in response to α,β-meATP, acting via P2X3Rs [26]. Therefore, the P2X3R pathway represents a promising candidate for the development of innovative antimigraine drug.

A very recent study showed that P2X3Rs in TG neurons are also involved in the development of chronic pain of the temporomandibular joints (TMJs) and masseter muscles associated with placement of an occlusal interference, since the administration of a P2X3R antagonist reversed mechanical hyperalgesia [27].

Recently, a microarray-based expression genomics study identified DRG-expressed genetic contributors to mechanical allodynia. Authors demonstrated that expression levels of the *Chrna6* gene, which encodes the α6 subunit of the nicotinic acetylcholine receptor (nAChR), are highly correlated to the development of allodynia, since mechanical allodynia associated with neuropathic and inflammatory injuries is significantly altered in α6 mutants. Furthermore, they showed that the role of *Chrna6* in allodynia is at least partially due to direct interaction of α6 nAChRs with P2X2/3Rs in DRG nociceptors, confirming the cross-communication among various signaling pathways involved in pain transmission [28]. All the above-mentioned evidence supports a therapeutic potential for the selective antagonism of P2X3Rs, and many research approaches have been utilized in the search of a selective inhibition of this receptor subtype: for example, the distribution of RNA and protein for this receptor subunit has been investigated, gene-targeting methods and novel selective antagonists have been administered to preclinical rodent models of pain, the ATP content has been analyzed in human pathological fluids and samples, in parallel with the tissue expression of P2X3Rs, and studies on the effects of ATP administration to healthy volunteers or to isolated tissue samples have been unsertaken [29]. The range of potential therapeutic opportunities covers essentially any condition in which a chronic dysregulation and sensitization of sensory neurons has been implicated.

So far, *in vivo* studies using selective pharmacological and gene-based tools support the hypothesis of a crucial role for P2X3Rs and P2X2/3Rs in chronic pain. TNP-ATP, a trinitrophenyl-substituted nucleotide (Table 1), is a very potent antagonist at both P2X3Rs and P2X2/3Rs, despite its short half-life *in vivo* [30]. A-317491 (synthesized by Abbott Laboratories), compound RO3 (synthesized by Roche, Palo Alto) and its derivative AF-353 are effective P2X3R and P2X2/3R antagonists. Systemic administration of both TNP-ATP and A-317491 reduced nociception in neuropathic pain models, demonstrating that peripheral and spinal P2X3Rs and P2X2/3Rs are involved in persistent chronic pain [31]. Moreover, P2X3Rs have been claimed to be useful targets for the treatment of pain in chronic prostatitis [32].

Results with P2X3R antagonists were corroborated by studies in mice lacking P2X3Rs, P2X2Rs or both receptor subunits [33], as well as in animals treated with P2X3R selective antisense [34] or short interfering RNA (siRNA) [35], which showed comparable results. Antisense oligonucleotides prevented the development of mechanical hyperalgesia and reverted hyperalgesia in

models of neuropathic (partial sciatic nerve ligation) and inflammatory (i.e., Complete Freund's Adjuvant, CFA,-induced) pain [36].

Concerning drugs already on the market, cyclooxygenase inhibitors reduced the expression of P2X3Rs in rat DRG, and hyperalgesia following chronic constriction injury (CCI) [37]. P2X3R-mediated signal transduction pathways of peripheral pain resulted to be attenuated by 17β-estradiol via estrogen receptor-α and GPR30 receptors [38, 39]. Finally, the anti-epileptic drug Pregabalin is also used for the treatment of neuropathic pain, and its action appears to be due to inhibition of P2X3R expression in the spinal dorsal horn [40].

Taken together, these data highlight P2X3R as the principal actor connecting ATP release with chronic neuronal sensitization, with no clear evidence for its involvement in the acute sensation of noxious stimuli. Thus, the hypothesis that blocking P2X3Rs and/or P2X2/3Rs has a potential therapeutic role in the management of chronic pain conditions is now reasonable [41].

In this respect, in the last decade, medicinal chemistry has made a breakthrough in proposing selective P2X3R and P2X2/3R antagonists with good potency, selectivity and bioavailability to be tested in humans, which are currently under development by several pharmaceutical companies. For example, the selective P2X3R antagonist AF-219 from Afferent Pharmaceuticals is the first compound tested in clinical trials. Phase I clinical studies in healthy volunteers indicated good safety and tolerability, and subsequent Phase II studies are currently in progress on chronic cough, joint pain and visceral pain [42]. A second compound, AF-130, has recently entered Phase I clinical testing (ClinicalTrials.gov Identifier: NCT02652936) and will be next evaluated in a number of pain-related conditions. Clinical trials for other P2X3R antagonists are in progress, but unfortunately so far all the tested compounds failed in advanced clinical phases, due to significant side effects (see Table 2).

P2X4Rs. Several findings indicate that chronic neuropathic pain leads to the activation of spinal microglia, but until recently its causal role in neuropathic pain remained an open question. Only by elucidating the role of P2X4Rs, activated microglia has been directly implicated in the pathogenesis of chronic pain. In fact, the identification of P2X4Rs in the spinal cord as a requirement for neuropathic pain first came from a pharmacological investigation of pain behavior after nerve injury using the P2XRs antagonists TNP-ATP and PPADS [43]. Following this initial discovery, a number of papers addressed the pro-nociceptive role of spinal microglial P2X4Rs and underlined the mechanisms involved in their activation in neuropathic pain conditions [44-46].

In particular, literature data showed that P2X4R-stimulated microglia released brain-derived neurotrophic factor (BDNF) as a crucial signaling factor to lamina I neurons, causing a collapse of their transmembrane anion gradient with subsequent neuronal hyperexcitability [47]. Investigation

on the cellular mechanisms by which microglial P2X4R activation could trigger the release of BDNF showed that stimulation of P2X4Rs causes Ca^{2+} flux and p38-MAPK activation, which in turn promoted the synthesis and release of BDNF [48].

The generation of P2X4R knockout mice showed a remarkable reduction of tactile allodynia and a complete absence of mechanical hypersensitivity after spinal nerve injury in comparison with wild-type animals [49]. The development of genetically modified mice has been an important step to directly assess the role of microglial P2X4Rs in chronic pain, since these findings indicated that microglial P2X4R stimulation is not only necessary but also sufficient to cause tactile allodynia. The up-regulation of P2X4R expression on microglial cells is clearly a key process in neuropathic pain, and studies have shown that the extracellular matrix protein fibronectin is involved [50]. Indeed, intrathecal delivery of ATP-stimulated microglia to the rat lumbar spinal cord revealed that microglia treated with fibronectin could induce allodynia more effectively than control microglia. Blockade of the fibronectin receptor attenuated nerve injury-induced P2X4R up-regulation and allodynia [51]. In parallel, the intrathecal delivery of fibronectin increased P2X4R expression and induced allodynia, a behavior that was not evoked in P2X4R-deficient mice. Moreover, fibronectin failed to induce up-regulation of P2X4R expression in microglial cells lacking Lyn tyrosine kinase, a member of the Src family kinases [52]. It was also reported that activating both toll-like receptors and NOD2 (another pattern-recognition receptor) in cultured microglia increased the expression of P2X4R at the mRNA level [53], thus suggesting the involvement of these receptors in the regulation of P2X4Rs. Mechanical allodynia and upregulation of P2X4Rs in spinal microglia is also a feature of experimental autoimmune neuritis [54].

Recent papers showed that interferon regulatory factor-5 (IRF-5), which is induced in spinal microglia after peripheral nerve injury, is responsible for direct transcriptional control of P2X4Rs [55]. Dexmedetomidine, a selective α_2-adrenoceptor agonist which has been used as analgesic, reduced pain in a spared nerve injury (SNI) rat model of neuropathic pain, in parallel to a reduced expression of P2X4Rs and BDNF in microglia of the spinal dorsal horn [56].

Also in the case of P2X4Rs, studies with selective and non-selective antagonists were a useful tool to validate the receptor as a potential target for treating chronic pain. A series of benzofuro-1,4-diazepin-2-ones were reported to be effective P2X4R antagonists in a Bayer Health Care, AG patent [57]. The microglia-to-neuron P2X4R–BDNF–K^+–Cl^- cotransporter pathway could be a therapeutic target for preventing hyperalgesia [58]. Also, morphine tolerance resulted to be attenuated by inhibition of microglial P2X4Rs receptors [59]. Antidepressants have been claimed to be effective in relieving neuropathic pain; among these, paroxetine was an effective antagonist of P2X4Rs in transfected cells, and preliminary clinical studies showed that it was successful in inhibiting chronic

pain [60]. Carbamazepine derivatives have been recently shown to have potent P2X4R blocking activities as well, thus offering a promising development for the treatment of neuropathic pain [61].

Interestingly, a recent study investigated the effect of hyperbaric oxygen (HBO) treatment at various stages following CCI of the sciatic nerve. Early HBO treatment produced a persistent antinociceptive effect and inhibited the CCI-induced increase in the expression of P2X4Rs [62].

Finally, a very recent study identified NP-1815-PX (Table 1) as a novel selective antagonist at P2X4Rs, with high potency and selectivity compared to other P2XR subtypes. *In vivo* assays for both acute and chronic pain showed that the intrathecal administration of NP-1815-PX induced an anti-allodynic effect in mice with traumatic nerve damage without affecting acute nociceptive pain and motor function. Furthermore, intrathecal NP-1815-PX suppressed the induction of mechanical allodynia in a mouse model of herpetic pain [63].

Surprisingly, recent evidence has shown that the role of microglia in pain is sexually dimorphic. In fact, despite similar microglia proliferation in the dorsal horn in both sexes, females do not upregulate P2X4Rs and utilize a microglia-independent pathway to mediate pain hypersensitivity. On the other hand, adaptive immune cells, possibly T cells, seem to mediate pain hypersensitivity in female mice only [64]. Moreover, unpublished data presented at the 16th IASP® World Congress on Pain (September 26th-30th 2016, Yokohama, Japan) revealed that blocking P2X4R-BDNF signaling reverses SNI-induced pain hypersensitivity in males only, and *P2rx4* gene expression was not upregulated in female mice subjected to peripheral nerve injury (data presented by M. Salter within the Topical Workshop "Distinct Forms and Phases of Neuroinflammation in Chronic Pain"; abstract title: "Sexual dimorphism in immune-neuronal signaling in pain hypersensitivity"). This profound sex difference highlights the importance of including subjects of both sexes in preclinical pain research.

Unfortunately, at the moment no clinical trials are ongoing to test the efficacy or P2X4R antagonists in chronic pain.

P2X7Rs. Among the P2XR family, P2X7Rs are considered the most unusual with respect to their functional and molecular characteristics. For example, homomeric P2X7Rs require 10-fold higher concentrations of ATP (>100 μM) than other receptor subtypes to be activated, with prolonged agonist exposure inducing the formation of large cytolytic pores in the cell membrane [65]. P2X7R subunits were initially cloned from rat and human macrophages, and they are mainly expressed by cells of the immune system including lymphocytes and peripheral macrophages [66]. In the rat CNS, functional P2X7Rs are expressed by microglia and astrocytes, while their presence on neurons is still controversial, even though evidence reports their expression by some neuronal populations (see 3.1.1)

[67]. The specific distribution of P2X7Rs has led to a large interest in drug development research, where they are mostly targeted to treat inflammatory diseases (see 3.1.1). Nevertheless, although mainly involved in inflammation processes, a role for P2X7Rs in chronic pain has also been proposed [68].

In fact, the pharmacological blockade of P2X7Rs reduced neuropathic pain in different experimental models [69]. Several P2X7R antagonists have been used so far, including: oxidized ATP (oATP), Brilliant Blue G, the tyrosine derivatives KN-62 and KN-04, cyclic imides, adamantane and benzamide derivatives, compound 4g, chelerythrine and other benzophenenanthidine alkaloids, U73122 and U73343 (Table 1). Later, compounds such as cyanoguanidines and aminotetrazoles have been introduced [70]. The systemic administration of the P2X7R selective antagonists A-438079 and A-740003 showed a dose-dependent antinociceptive effect in models of neuropathic pain [71, 72]. In particular, A-740003 is more potent than A-438079 in reducing mechanical allodynia 2 weeks after spinal nerve ligation. The antinociceptive activity of P2X7R antagonists is comparable to their anti-inflammatory effect, and A-740003 was more effective in reducing nociception than paw edema in inflammatory pain models [71]. The antinociceptive action of A-438079 was due to the blockade of mechanical and thermal inputs to spinal neurons, and the spontaneous activity of all classes of spinal neurons was reduced in neuropathic, but not sham rats [72], which might have important clinical implications.

It has been reported that P2X4R and P2X7R knockout mice have a common pain phenotype, both showing reduced pain behavior through the inhibition of their receptor-specific signaling pathway. In P2X7R knockout mice, both chronic inflammatory and neuropathic pain were abolished, as well as IL-1β release [73], which has been demonstrated to play an upstream transductional role in the development of both types of pain [74]. Later, another study observed similar results and additionally suggested a role for microglial P2X7R activation in the p38 MAPK-dependent release of cathepsin, and hypothesized that this may represent an additional mechanism responsible for the efficacy of P2X7R antagonists in neuropathic pain [75]. Several other diffusible factors such as iNOS, PGE2, COX-2 and BDNF are also released by microglia upon P2X7R activation [76], and could account for the cross-talk between P2X7R- and P2X4R-dependent mechanisms that are associated with neuropathic pain (see also above). P2X7R-induced pore formation initiates several downstream effects, which may be involved in pain hypersensitivity. Thus, the specific inhibition of the pore formation without affecting the cation channel activity provides a mean to reduce chronic pain [77].

Recently, an increase in P2X7R mRNA and protein expression in spinal microglia has been observed in a neuropathic pain model induced by peripheral nerve injury [78]. Activation of P2X7Rs was also shown to induce the release of glutamate in the spinal cord, which contributes to mechanical

allodynia following partial nerve ligation of the sciatic nerve [79]. P2X7R turned out to be activated in a model of oxaliplatin-induced painful neuropathy [80], and the P2X7R antagonist A-438079 prevented the effects on allodynia induced by paclitaxel, another anticancer drug [81].

Peripheral P2X7R activation also induced mechanical hyperalgesia via inflammatory mediators, especially BK [82]. Genetic deletion and antagonists of P2X7R reduced pain in a mouse model of nitroglycerin-induced migraine [83]. Finally, a thalamic hemorrhagic rat model characterized by thermal and mechanical allodynia, which develops in the subacute to chronic phases upon central post-stroke pain onset, showed a significant increase in P2X7R expression in reactive microglia/macrophages in thalamic perilesion tissues at 5 weeks post-hemorrhage. Thalamic P2X7Rs were proved to be directly involved in pain transmission and hypersensitivity, and their systemic targeting rescued abnormal pain behaviors and neuronal activity in the thalamo-cingulate pathway [84].

One important issue in the research on P2X7Rs has been to demonstrate its possible interactions with membrane proteins, which could account for its ability to open a non-selective pore upon repeated stimulation. Recently, a research group suggested that Pannexin-1 (Panx1) hemichannel could represent a novel regulator of pain hypersensitivity, and showed its interaction with P2X7Rs in glial cells. Authors showed that ATP released from presynaptic neurons activates P2X7R on surrounding microglia, increasing intracellular calcium and activating Panx1, thus causing more ATP release. This ATP-induced ATP release potentiated purinergic signaling and enhanced the release of pro-inflammatory cytokines, thus participating to pain induction and chronicization [85]. Further supporting evidence for Panx1-P2X7R interaction in pain was also provided by another group suggesting that the increased P2X7R-dependent glutamate release through the recruitment of Panx1 is a downstream effector for the neuropathic syndrome generated by repeated administration of the anti-cancer agent oxaliplatin [86]. Moreover, a research group recently demonstrated that glial Panx1, and therefore its interaction with P2X7Rs, also contributes to tactile sensitivity in a mouse model of chronic orofacial pain [87].

A very recent study assessed the contribution of P2X7R in the antiallodynic and antihyperalgesic effects of the intraperitoneal daily administration of riluzole in a rat model of neuropathic pain induced by CCI, by demonstrating that riluzole downregulated P2X7R expression and inhibited microglial activation [88].

Interestingly, variations within the coding sequence of the *P2rx7* gene affect chronic pain sensitivity in both mice and humans. Using genome-wide linkage analyses, researchers discovered an association between nerve injury-induced mechanical allodynia and the P451L mutation of the mouse *P2rx7* gene: mice in which P2X7Rs have impaired pore formation, as a result of this mutation,

showed less allodynia than mice with the pore-forming *P2rx7* allele. Administration of a peptide corresponding to the P2X7R C-terminal domain, which blocked pore formation but not cation channel activity, selectively reduced nerve injury and inflammatory allodynia only in mice with the pore-forming *P2rx7* allele. Moreover, in two independent human chronic pain cohorts, authors observed a genetic association between lower pain intensity and the hypofunctional His270 allele of P2X7R. These findings suggest that selectively targeting P2X7R pore formation may be an innovative strategy to achieve a personalized treatment of chronic pain [77]. Subsequently, it has been shown that other P2X7R single nucleotide polymorphisms (SNPs) correlate with pain sensitivity [89]. Another research group conducted linkage disequilibrium analyses for 55 reported SNPs in the region within and around the P2X7R gene using genomic samples from 100 patients, and further analyzed associations between genotypes/haplotypes of these SNPs and clinical data, for a total of 355 samples. Authors demonstrated that cold pain sensitivity and the analgesic effects of fentanyl are related to the SNPs and haplotypes of the P2X7R gene [90], further underlining the involvement of P2X7R gene polymorphisms in pain sensitivity and their importance for personalized medicine.

2.1.2 P2Y G protein-coupled receptors and chronic pain

As mentioned, during inflammation and tissue injury, different cell types can release ATP and other nucleotides from their intracellular stores to the extracellular compartment (Figures 1 and 2) [91]. These nucleotides can bind to specific metabotropic receptors, the P2Y receptors (P2YRs) family, leading to the activation of different signaling pathways.

In particular, $P2Y_{1,12,13}$Rs are sensitive to ADP, $P2Y_{2,4}$Rs respond to both UTP and ATP, $P2Y_6$R is sensitive to UDP, $P2Y_{11}$R has ATP as endogenous ligand and finally $P2Y_{14}$R responds to the uridine sugars (such as UDP-glucose and UDP-galactose) [92]. Activation of each receptor subtype leads to the recruitment of various second messengers, which can vary also depending upon the tissue or cell expressing the receptor [92]. P2YRs are widely expressed throughout the body, and they are especially localized on endothelial cells ($P2Y_{1,2,4}$Rs), microglia ($P2Y_{6,12}$Rs), satellite glial cells ($P2Y_{1,2}$Rs) and other immune cells and leukocytes [93].

Unfortunately, selective available pharmacological entities targeting this receptor family are currently limited: $P2Y_{12}$R antagonists are used as antithrombotic agents [94], and recently a $P2Y_2$R agonist (i.e. Denufosol) reached phase III clinical trials for the treatment of cystic fibrosis, but has not been commercialized [95]. Conversely, another $P2Y_2$R agonist (i.e. Diquafosol) has been approved for topical treatment in dry eye syndrome [96]. Nonetheless, in the last years many published studies have shown the importance of P2YRs during chronic pain and inflammation, also thanks to the synthesis of new subtype-selective agonists and antagonists, thus increasing the interest

of the scientific community for the possible exploitation of these receptors as new pharmacological targets.

$P2Y_1Rs$. To date the role of this receptor subtype in chronic pain is controversial. It has been demonstrated that activation of $P2Y_1Rs$ has an antinociceptive action, since intraperitoneal administration of a selective $P2Y_1R$ agonist reduced allodynia in rats affected by neuropathic pain [97].

Conversely, other studies showed that $P2Y_1R$ antagonists reduced both thermal hyperalgesia [98] and bone cancer pain in rats [99]. It has also been found that $P2Y_1Rs$ are involved in formalin-induced pain at peripheral level. Activation of this receptor led to an increase in pain response, whereas inhibition decreased it [100]. Moreover, the same group found that $P2Y_1R$ expression was increased in the DRGs of different animal models of neuropathic pain, and this effect was exclusively observed in the early stages of the pathology, suggesting the involvement of this receptor in the development of neuropathic pain rather than in its maintenance [101]. Nontheless, the selective $P2Y_1R$ antagonist MRS2179 failed to reduce facial allodynia in a model of sub-chronic inflammatory trigeminal pain (i.e. CFA injection in the temporomandibular joint) [102].

$P2Y_1Rs$ are also highly expressed in the rectosigmoid mucosa of diarrhea-predominant irritable bowel syndrome (IBS-D) patients [103], and further studies have shown that they are involved in the increased action potential firing of colonic sensory neurons that leads to visceral pain [104, 105].

Another study clearly indicates that $P2Y_1Rs$ play a role in chronic ischemia with severe acidosis. In particular, their activation led to the phosphorylation of TRPV1, a key receptor in nociceptive pathways, which in turn promoted thermal hyperalgesia [106]. This evidence seems to confirm a pro-nociceptive and pro-algogenic role of $P2Y_1Rs$, and thus the availability of selective antagonist could open up new strategies for innovative analgesic approaches.

$P2Y_2Rs$. A significant body of evidence is now depicting a clear role for this receptor subtype in several types of pain. Previous studies indicated that $P2Y_2Rs$ play an important role in thermal nociception, since mice lacking this receptor subtype failed to develop heat hyperalgesia in response to CFA-injection [107]. Recently, Molliver and colleagues confirmed these findings, by showing that lack of $P2Y_2Rs$ led to the reduction of the responsiveness of unmyelinated polymodal afferents to heat. Moreover, $P2Y_2R$ deletion altered the mechanical properties of these cutaneous afferents, which translated into the acquisition of mechanical responsiveness by a subset of TRPV1-expressing afferents [108].

Our group has recently demonstrated that $P2Y_2Rs$ expressed by satellite glial cells in the TG are upregulated by pro-algogenic stimuli, and that their inhibition by a selective antagonist led to a total recovery from allodynia in a sub-chronic inflammatory trigeminal pain model (i.e., CFA injection in the temporomandibular joint) [102]. This is in agreement with the results obtained in a previous study that showed the same antinociceptive effect of $P2Y_2Rs$ antagonist in a rat model of trigeminal neuropathic pain [109].

Finally, further evidence validates the involvement of $P2Y_2Rs$ in IBS-D and it has been suggested that its increased expression in the rectosigmoid mucosa of IBS-D patients correlates with the presence of abdominal pain [103].

$P2Y_4Rs$. This receptor subtype is known to be expressed by satellite glial cells in the TG [110, 111], and could therefore affect sensory neurons during pain development. Nonetheless, to date its role in pain transmission has been only speculated but not demonstrated.

$P2Y_6Rs$. Together with $P2Y_{12}Rs$, $P2Y_6Rs$ are crucially involved in controlling microglial cell functions. In fact, their activation led to increased production of chemokines [112], and promoted microglial phagocytic activity [113]. In accordance to this, expression of $P2Y_6Rs$, together with $P2Y_{13}Rs$ and $P2Y_{14}Rs$, has been found upregulated in spinal microglial cells after peripheral nerve injury [114]. This increased expression lasted at least 2 weeks, and the pharmacological inhibition of this receptor subtype attenuated mechanical pain hypersensitivity [114]. Similar results on the pronociceptive role of $P2Y_6Rs$ were confirmed by another research group, which showed that the expression of $P2Y_6Rs$ and $P2Y_{11}Rs$ is increased in a model of neuropathic pain (spinal nerve ligation, SNL), and that the pharmacological inhibition of these receptors improved tactile allodynia [115]. Moreover, the activation of $P2Y_6Rs$ (and also $P2Y_1Rs$ and $P2Y_{11}Rs$) increased the flinch behavior due to formalin-induced pain, and pre-treatment with receptor antagonists relieved pain [100].

Conversely, opposite results have been obtained in other studies. In a model of SNI, the pharmacological inhibition of $P2Y_6Rs$ failed to reduce allodynia [116]. In sharp contrast with the pronociceptive role of $P2Y_6Rs$ observed in other studies, Bernier et al. showed that $P2Y_6R$ activation led to a decreased P2X4R activity, suggesting that $P2Y_6Rs$ might have an antinociceptive role [117].

$P2Y_{11}Rs$. It has been shown that the expression of this receptor subtype is increased in different animal models of pain, and that its inhibition decreased tactile allodynia in both neuropathic [115] and formalin-induced pain models [100]. It is, however, worth mentioning that the existence of a rodent ortholog of human $P2Y_{11}R$ is still a matter of debate, since no cloned receptor but only

functional data are currently available. This claims for caution when postulating the involvement of this receptor in preclinical models of pathology.

$P2Y_{12}$Rs. This receptor subtype is mainly expressed by microglial cells both in the brain and spinal cord [118-120], where it crucially modulates microglial chemotactic activity [118]. Its expression in these cells increases following partial nerve injury (PNI) in the spinal cord [121, 122], and its genetic deletion improved tactile allodynia [123]. These results strongly suggest the involvement of microglial $P2Y_{12}$Rs in the pathogenesis of neuropathic pain.

Moreover, $P2Y_{12}$Rs also carry out an important role in controlling the engulfment of myelinated axon by activated microglia in the spinal dorsal horn, and also this pathway could be critical for the development of neuropathic pain [124].

Recently, it has been demonstrated the involvement of microglial $P2Y_{12}$Rs in a model of spinal nerve transection, since its genetic deletion ameliorated pain hypersensitivity and reduced the morphological and electrophysiological changes observed in microglial cells upon nerve transection [125].

$P2Y_{13}$Rs. Recent studies clearly show the involvement of $P2Y_{13}$Rs in neuropathic pain. In particular, it has been demonstrated that the expression of these receptors increased during neuropathic pain in spinal microglia. Therefore, authors proposed $P2Y_{13}$R as a key receptor in the induction and maintenance of neuropathic pain through ROCK, a protein suggested to be involved in morphological changes that occur during microglia activation [114, 126].

2.2. Adenosine modulation of chronic pain conditions

As shown in Figure 1, adenosine (Ado) is extracellularly generated as the final product of the hydrolysis of purine nucleotides, i.e. its peak of concentration follows that of ATP and ADP. Also thanks to this relationship, Ado-mediated effects are often opposite to that of nucleotides, thus providing an efficient system to fine-tune and/or turn off nucleotide-mediated cellular responses. These modulatory properties of Ado on neuronal functions and on glial cell activation have important outcomes in pain transmission. Ado itself has been demonstrated to exert important analgesic actions in various types of both inflammatory and neuropathic pain models [127], and even in clinical settings [128]. Nevertheless, significant cardiovascular side effects have been detected, due to the wide distribution of G protein-coupled adenosine receptors (ARs, collectively referred to as P1 receptors, encompassing the $A_{1, 2A, 2B, 3}$ AR subtypes) and to the lack of selectivity of the endogenous nucleoside towards the various receptor subtypes. Research has, therefore, mostly focused on the identification

of the actual AR subtypes involved in Ado-mediated analgesia, in order to elaborate a more selective therapeutic strategy devoid of major side effects.

A_1ARs. The antinociceptive activity of Ado has been initially only attributed to the activation of A_1ARs, mostly due to their neuronal localization and their known inhibitory activity on cell firing, thanks to the modulation of specific K^+ channels. Marked analgesic effects of selective A_1AR agonists have been demonstrated in a wide variety of preclinical models of pain, spanning from neuropathic pain due to nerve injury, diabetic neuropathy, peri-operative pain, and central pain following spinal cord injury [reviewed in [129]]. Multiple sites of action have been identified, due to the widespread expression of A_1ARs along the pain signaling pathways. In fact, they are expressed on peripheral nerve endings, in the superficial layers of dorsal horn spinal cord, and in specific supraspinal sites crucially involved in pain integration and perception[129]. Unfortunately, A_1ARs are also the AR subtype mostly involved in the modulation of cardiovascular functions, thus reducing the possible clinical exploitation of selective agonists due to dose limitation and significant side effects[129]. It is worth mentioning that various series of new potent and selective A_1AR agonists have been synthesized in recent years, which could separate their antinociceptive activity from the modulation of cardiovascular functions [130]. It has been in fact hypothesized that limitations with earlier agonists could be due to both pharmacokinetic (e.g., low central nervous system permeability) and pharmacodynamic (e.g., limited intrinsic activity) issues. Additional strategies to overcome potentially harmful side effects are represented by: i) the use of partial agonists, which could show better receptor selectivity due to receptor plasticity under painful conditions (e.g., increased receptor density and/or coupling to second messengers in the presence of nerve injury) [131], and ii) the use of allosteric modulators, acting on distinct binding site with respect to agonists, that have already proven effective in various models of acute and chronic pain [132]. In fact, one of the main issue which has long limited the clinical exploitation of Ado receptor ligands is their widespread distribution, which could account for serious side effects. An innovative strategy to overcome this problem is represented by the synthesis of allosteric modulators that can selectively enhance P1 receptor activity only at sites where Ado concentrations are increased, due to tissue damage and/or inflammatory processes [133]. The first interesting example of this strategy is represented by T62, which showed promising results in the management of mechanical allodynia associated with nerve injury, and in inflammatory pain [134]. Unfortunately, despite a concluded Phase II clinical trial aimed at evaluating its efficacy in postherpetic neuralgia ClinicalTrials.gov Identifier: NCT00809679), no further data on the clinical application of this compound have been provided.

New insights on the role of A_1ARs in pain transmission have also emerged from the demonstration of their expression and function in spinal cord microglia. Exposure to ATP, as it happens following tissue damage, led to the upregulation of A_1ARs on spinal cord microglia, and their activation with selective agonists inhibited both the morphological changes linked to microglia activation and its ability to promote neuronal sensitization [135]. More interestingly, the chronic administration of a new, potent and selective A_1AR agonist, named 5'-chloro-5'-deoxy-(±)-ENBA (Table 1), alleviated neuropathic pain in the mouse model of the SNI of the sciatic nerve, in parallel with a reduction of microglia activation and with no effect on motor coordination and blood pressure [136]. Taken together, these data support the growing notion that targeting the purinergic system on glial cells represents a promising therapeutic strategy against various forms of pain that are currently poorly controlled.

A_{2A}ARs. In the case of A_{2A}ARs, the connections between inflammation and pain is stronger than with all the other Ado receptors (see also 4.2). In fact, A_{2A}ARs are highly expressed on cells of the immune system where they mostly exert anti-inflammatory effects (see 4.2), whereas in the CNS their localization is both neuronal and glial. A_{2A}ARs expressed by post-synaptic neurons in the striatum are key modulators of movements, and act as functional antagonists of dopamine D2 receptors [137]. Their expression in other brain areas directly involved in pain integration and modulation is more controversial. It is known that traumatic and ischemic conditions, or chronic stress and ageing can induce A_{2A}AR expression in the CNS, and that increased concentrations of inflammatory cytokines exert similar effects on microglia [138]. Moreover, as conflicting results on the pro- or anti-nociceptive action of A_{2A}ARs have been provided, it is foreseen that the overall outcome of a pharmacological approach targeting this receptor subtype is not easily predictable [139].

Initial data seem to overall point to a pro-nociceptive role of A_{2A}AR activation. However, it was lately demonstrated that the spinal administration of low doses of a selective A_{2A}AR agonist reverted neuropathic pain in rats, and that the i.c.v. injection of an antibody with A_{2A}AR agonist-like activity was antinociceptive in naïve mice [reviewed in [139]]. Discrepancies can be due to the different sites of action (e.g., peripheral versus central; neuronal versus inflammatory cells), but also to the low selectivity and bioavailability of older molecules. According to their fundamental role in inflammatory conditions, activation of A_{2A}ARs with the new selective agonist LASSBio-1359 (Table 1) led to a significant reduction of thermal and mechanical hyperalgesia in several models of monoarthritis in rodents [140]. These effects were accompanied by normalization of TNF-α and iNOS expression levels, thus suggesting a direct action on inflammatory cells.

One A_{2A}AR agonist named BVT-115959 has reached phase II clinical trial for diabetic neuropathy. The study has been concluded (ClinicalTrials.gov identifier: NCT00452777) but no results are available, and the development of the drug has been discontinued.

To consider possible side effects, it must be remembered that A_{2A}AR activation mediates vasodilation of the local microvessel circuits and of coronary arteries, as well as inhibits platelet aggregation, through increased cAMP levels [141, 142]. These actions might be responsible for undesired and potentially life threatening events.

A_{2B}ARs. This is the last P1 receptor subtype in order of characterization, and therefore its role in many physiological and pathological conditions is still elusive. Its prominent expression on inflammatory cells suggests a role in inflammatory pain (see 4.2), while in the CNS low levels have been described with a specific localization on astrocytes [139]. Interestingly, as for the A_3AR subtype, this receptor is activated by high Ado concentrations, which suggests its crucial involvement in pathological conditions when extracellular concentrations of nucleosides and nucleotides increase several folds. Overall, the few available reports on a possible role of A_{2B}ARs in nociception indicate pro-nociceptive effects, also in the periphery [143]. Conversely, the acute intratechal administration of the selective A_{2B}AR agonist BAY 60-6583 (Table 1) showed a potent antiallodynic activity in a CCI model of neuropathic pain [144]. Thus, as observed for the A_{2A}ARs which is coupled to similar intracellular signaling pathways (Figure 1), it seems that opposite outcomes on nociception can be obtained depending on the peripheral or central site of action. This must be taken into careful account when planning either the synthesis of new selective chemical entities or their protocol of administration.

A_3ARs. The A_3AR subtype has more recently emerged as a previously unexpected player in the development of chronic pain, and currently represents the most promising target for innovative purinergic-based analgesic therapies, due to the different mechanisms at the basis of its action and to the availability of selective agents which have been already tested in humans (see below). This is the only AR subtype that has been identified by cloning (and not based on pharmacological evidence) in early ‘90s [145], and later pharmacological studies have demonstrated its low (in the micromolar range) affinity for the endogenous ligand Ado, similar to the A_{2B}AR, thus leading to the hypothesis of its key involvement in pathological conditions. Selective synthetic agonists (i.e., IB-MECA and Cl-IB-MECA; Table 1) and antagonists [146] were almost immediately made available by Prof. Jacobson’s group at the NIH. These tools have greatly help identifying the involvement of a specific AR subtype in various physiological and pathological conditions, with the initial demonstration of its

involvement in the modulation of reactive astrogliosis, mast cell degranulation, recovery after stroke [147] and later in cancer and autoimmune pathologies, like rheumatoid arthritis and dry eye disease [148].

In early years 2000s, some papers with contradictory results have suggested an involvement of A_3ARs in inflammatory chronic pain, with the initial demonstration of its analgesic role [reviewed in [149]]. Interestingly, all these papers pointed to the ineffectiveness of A_3AR agonist to alter "physiological" protective painful responses and basal threshold nociception. This suggests that targeting this Ado receptor subtype could represent an interesting approach to pathological painful conditions. It has been only starting from 2012 that robust evidence has unveiled a clear analgesic role played by A_3ARs. First of all, the availability of novel, selective A_3AR agonists (like MRS1898 and MRS5698; Table 1), more potent than IB-MECA and Cl-IB-MECA, has significantly fostered research in this field [149]. Thanks to these new tools, multisite analgesic actions of A_3AR agonists have been demonstrated in neuropathic pain, with antinociceptive effects exerted both in the periphery, and at the spinal and supraspinal levels along the ascending nociceptive pathway. Moreover, receptors expressed by neurons, glial cells and immune system cells are involved in the overall analgesic outcome of A_3AR agonist administration, further demonstrating the complexity of the signaling pathways involved in nociception and modulated by this receptor subtype [reviewed in [149]]. The analgesic properties of A_3ARs have been also demonstrated in various cancer-related painful conditions, thus suggesting the modulation of common pathways involved in different forms of chronic painful states. Interestingly, although A_3ARs are likely undergoing rapid receptor down-regulation, no tolerance has developed following chronic administration of agonists. Additionally, no involvement of both the endocannabinoids and opioids systems has been observed [150], thus suggesting a total lack of risk of abuse for A_3AR-based analgesics, and further strengthening the necessity to foster the clinical application of these preclinical observations. The fact that the oldest A_3AR agonists IB-MECA and Cl-IB-MECA (now known as CF-101 and CF-102, respectively, thanks to their acquisition by CanFite Therapeutics) have been already tested in clinical trials for psoriasis, rheumatoid arthritis, dry eye syndrome and as anti-cancer agents with excellent results and, equally important, with no significant side effects [151], unveils the feasibility of this pharmacological approach also for different pain states in the near future (see also 4.2).

Figure 3 provides a simplified schematic summary of the sites of activity of the purinergic system in pain pathways from the periphery to the spinal cord and above.

3. ROLE OF PURINES IN NON-CONVENTIONAL THERAPEUTIC APPROACHES TO PAIN

An intriguing new field of research on the role of the purinergic system in nociception has open after the publication of Prof. Burnstock's hypothesis on the involvement of purines in acupuncture in 2009 [152]. The evidence at its basis was simple and incontrovertible: i) purinergic receptors, especially neuronal P2X3Rs, are key modulators of chronic and inflammatory pain; ii) acupoints (i.e., the specific sites for insertion of needles) generally overlie major neuronal bundles [153]; iii) massive amounts of ATP are released following traumatic injuries or mechanical deformation of tissues; iv) needle insertion and manipulation (i.e., twisting) in acupuncture is likely to provoke micro-traumatic injuries or mechanical deformation of the tissue. A scenario was therefore depicted where increased extracellular ATP concentrations at the site of needle insertion (or due to the application of an electrical current in electroacupuncture, EA) can locally act on neuronal P2X3Rs, leading to their desensitization. This, in turn, would reduce neuronal firing and sensitization in the spinal cord, with the final result of a reduction of the painful sensation [152]. Additionally, activation of specific purinergic receptors on nerve endings could engage specific central inhibitory neuronal pathways in the spinal cord, brainstem and cerebral cortex, which could promote analgesia and, in parallel, account for the modulation of various physiological functions by acupuncture [152].

Since then, several studies have experimentally confirmed this hypothesis by demonstrating the involvement of P2X3Rs in the analgesic effects exerted by EA [reviewed in [154]]. Moreover, it has also become increasingly clear that the whole purinergic system is possibly involved in acupuncture-mediated analgesia. In fact, the rapid degradation of ATP to ADP and, more importantly, to Ado has prompted researchers to verify whether these mediators are also involved. A seminal paper has indeed demonstrated that A_1ARs are necessary for the analgesic actions of acupuncture against inflammatory and neuropathic pain, since full analgesia and reduction of hypersensitivity was obtained by injecting the A_1AR agonist CCPA into the so-called "Zusanli" acupoint (or St 36, located close to the deep peroneal nerve), whereas a complete loss of effect of acupuncture was observed in A_1AR knockout animals [155]. Moreover, the transient effect of acupuncture could be prolonged by pharmacologically inhibiting adenosine deaminase by deoxycoformicin, thus confirming that Ado generation is a fundamental step for the development of the observed beneficial effects [155]. This has also suggested that enzymes known to increase Ado concentrations (like, for example, nucleotidases; see paragraph 5) could be locally injected into acupoints to sustain and prolong the analgesic effects of acupuncture [139]. Interestingly, increased Ado levels have been later observed in humans subjected to acupuncture practice [156], thus suggesting that the above-mentioned hypothesis can be translated to patients as well.

Although few data are currently available, it is conceivable that other ARs apart from A_1ARs are also involved, based on the above-mentioned evidence of their key role in specific types of pain and inflammatory conditions. One single report shows that A_{2A}ARs mediate the analgesic effects of EA on synovitis in collagen-induce arthritis (i.e., a type of inflammatory pain) [157], while the recruitment of A_3ARs in the treatment of neuropathic pain can at the moment only be hypothesized, based on the role exerted by this receptor subtype in neuropathic pain (see 2.2).

Not only Ado receptors, but also P2 nucleotide receptors have been implicated in the analgesic actions of acupuncture. A recent study in rats subjected to CCI of the sciatic nerve showed that EA depresses the currents created by ATP and the upregulation of the P2X3Rs. Additionally, EA turned out to be more potent in reducing mechanical allodynia and thermal hyperalgesia when combined with the intrathecal administration of the P2X3R antagonist A-317491, indicating that EA and A-317491 might have an additive effect in inhibiting the transmission of pain mediated by P2X3Rs [158]. Moreover, another study on CCI rats reported that EA ameliorated tactile allodynia by down-regulating excessive expression of IFN-γ in the spinal cord and subsequently reducing the expression of P2X4Rs [159].

Overall, the above-mentioned evidence clearly demonstrates that the purinergic system is as involved in mediating acupuncture-based analgesia as other known signaling systems, like opioids [160]. Based on the temporal relationship existing between the increases in extracellular nucleotide and nucleoside concentrations, with the latter derived from the hydrolysis of the former, the logical question is: are P1Rs or P2Rs mostly responsible for the observed effects? [154]. At the light of available evidence, and due to the extremely rapid (and virtually immediate) degradation of ATP to Ado, it can be hypothesized that the main analgesic activity is to be ascribed to P1Rs, with a subsequent normalization of P2Rs taking place consequently. Additional studies possibly employing more selective receptor subtype antagonists are needed to definitively clarify this issue and, eventually, to exploit these results in clinics.

Interestingly, the involvement of the purinergic system has been also proposed in other branches of traditional medicine, like Chinese herbal medicine where herbal preparations have been administered for centuries based on the long-lasting experience of elderly and on the empiric demonstrations of their therapeutic effects. To favor the spreading of traditional medicine to Western countries and to overcome the diffidence of the scientific community against these types of treatment, in the last decades Chinese researchers are putting enormous efforts to unveil the biochemical and molecular bases for the efficacy of traditional herbal products. In this respect, it has been shown that several chemical components of traditional analgesic herbal products, like sodium ferulate, tetramethylpyrazine and puerarin, act by inhibiting P2X3Rs [161-163]. Lappaconitine, a Chinese

herbal medicine used as analgesic for centuries, reduced neuropathic pain acting on P2X3Rs on rat DRG [164]. Finally, the active compound anthraquinone emodin, constituent of rhubarb extract, reduced neuropathic pain mediated by P2X3Rs in primary sensory nerves [165]. Recent literature data reported that the flavonoid luteolin reduced the severity of CFA-induced arthritic scores also via the suppression of P2X4R activity, thus becoming a potential preventive or therapeutic candidate for the treatment of inflammation and arthritis [166]. Additionally, Aconitum species are known to suppress purinergic receptor expression. *In vitro* studies demonstrated that the Aconitum suppresses ATP-induced P2X7R-mediated inflammatory responses in microglial cell lines [167]. Additionally, a recent paper investigated the effect of intrathecal administration of thermally processed *Aconitum jaluense* on pain behavior, showing an anti-allodynic effect of the compound on spared nerve ligation (SNL)-induced neuropathic pain, possibly by suppressing P2X7R expression as well as reducing microglial activation in the spinal cord [168].

Moreover, norisoboldine extracted from *Radix Linderae* has proved efficacious against inflammatory pain through the activation of the A_1AR subtype [169], and Uliginosin B, a dimeric acyphloroglucinol occurring in *Hypericum* species native to South America, showed analgesic effects in the hot plate test in mice through activation of the A_1ARs and A_{2A}ARs [170].

4. ROLE OF PURINES IN INFLAMMATION

4.1 Involvement of nucleotides in inflammatory processes

4.1.1 P2X receptors and inflammation

Extensive literature data clearly indicate that inflammatory diseases are associated with increased extracellular release of nucleotides. Moreover, experimental evidence shows changes in P2XRs expression in inflammatory conditions in various *in vitro* and *in vivo* models. However, for several years the majority of these studies was lacking demonstration for a causative relationship between P2XR activation and inflammation. Nowadays, besides neuronal P2X3Rs, which are mainly involved in chronic pain mechanisms, most literature is addressed toward a pivotal role for microglial P2X4Rs and P2X7Rs in the development and maintenance of inflammatory pain.

P2X3Rs. It is now clearly evident that ATP plays a broad range of activities in inflammatory pathways, acting on many different cell types and receptors that have been implicated in chronic inflammatory diseases [171]. As mentioned above, P2X3Rs are almost exclusively expressed by neurons in sensory and sympathetic ganglia, and their expression in inflammatory cells has not been

reported. Previous reports have described possible functions of P2X3Rs in the activation of inflammatory responses in chondrocytes and synovial fibroblasts in joints [172, 173] but without any *in vivo* correlate. However, studies on sensory responsiveness of tissues in models of chronic inflammation showed a clear potential for P2X3R antagonism in inflammatory pain conditions. In fact, following activation of primary afferent neurons, sensory axon reflexes give rise to the release of factors, including prostanoids, substance P, CGRP and ATP itself, that could contribute to or modulate neurogenic inflammatory responses [174].

Increased P2X3R expression and function have been reported in several rodent joint inflammation models, obtained by application of noxious irritants or by mechanical damage to paw, knee, vertebral, or temporomandibular sites. Acute administration of CFA, formalin, carrageenan, and mono-iodoacetate (mIOA) in rodents induced the development of inflammatory pain-related behavior, such as reduced tolerance threshold to mechanical and thermal stimulation. In these models, P2X3R mRNA and protein expression were increased in small- and medium-sized neurons in DRG or cranial ganglia, as well as in the peripheral terminal fields in the joint and/or in central terminals in the dorsal horn of spinal or brainstem projections [29]. Knockout mice with deletions of P2X3R, P2X2R or both receptor subunits showed reduced response to formalin injection into the paw [33], while rats intrathecally injected with antisense oligonucleotides and siRNA probes showed a significant reduction of mechanical and thermal hyperalgesia in paw and knee joint irritation models [34, 35]. ATP was also reported to be increased in inflamed and damaged tissues and joints, thus activating arthritic knee-joint afferent fibers. Literature data showed that, in patients with arthritic knee joints, synovial ATP release was proportionally related to symptom severity and decreased during symptom ameliorating therapy with intraarticular administration of hyaluronic acid [175]. P2X3R and P2X2/3R antagonists also reduced spontaneous firing and evoked responses of spinal nociceptive neurons in inflamed rats [176].

Moreover, a recent study investigated the involvement of P2XRs in TG sensitization caused by LPS-induced dental pulp inflammation. The expression of P2X2Rs, P2X3Rs, and P2X5Rs was found increased in the V1-V2 division of the TG, primarily in small- and medium-sized neurons. Markers of glutamatergic afferents and GABAergic afferents were induced by LPS and co-expressed with P2X3Rs in small-sized TG neurons [177].

It has been recently shown that cAMP-dependent guanine nucleotide exchange factor 1 (Epac1) is upregulated after inflammatory injury in rat DRGs, where it plays a critical role in P2X3R sensitization by activating protein kinase C epsilon (PKCε), the major PKC isoform mediating CFA-induced inflammatory hyperalgesic responses [178].

Another study showed that P2X3Rs in masseter muscle afferent neurons and in the TG are involved in muscle pain induced by inflammation caused by chemical agents or eccentric muscle contraction. Authors showed an upregulated expression of P2X3Rs in the TG subnucleus caudalis and periaqueductal gray (PAG), which may contribute to the development of inflammatory orofacial pain [179].

Recent studies using P2X3R knockout mice or antagonists suggest that P2X3Rs also contribute to the development of visceral inflammation. A recent paper investigated the effect of the selective P2X3R antagonist A-317491 (Table 1) on visceral sensitivity under physiological conditions, during acute and post-inflammatory phases of colitis. Rats with acute colitis and in the post-inflammatory phase displayed significant visceral hypersensitivity, which was dose-dependently reversed by A-317491. Moreover, A-317491 did not modify visceral sensitivity in control animals, indicating that P2X3Rs are not involved in sensory signaling under physiological conditions, whereas they modulate visceral hypersensitivity during the course of the pathology, thus validating P2X3Rs as potential new targets in the treatment of abdominal inflammatory pain syndromes [180].

P2X7Rs. Among the seven P2XR subtypes, the strongest body of evidence for an involvement in mediating inflammation so far exists for P2X7Rs. It is currently accepted that P2X7Rs are widely distributed throughout the mammalian body in a wide variety of cells involved in inflammatory processes [67]. After its discovery, this receptor subtype was thought to be restricted to cells of the hematopoietic lineages, i.e. macrophages, mast cells, monocytes, lymphocytes, erythrocytes and eosinophils. However, it is now accepted that P2X7Rs are expressed by other cell lineages, including osteoblasts, fibroblasts, endothelial, and epithelial cells. Furthermore, P2X7Rs are present on cells in both the central and peripheral nervous system, including microglia, astrocytes, oligodendrocytes and Schwann cells. In addition, P2X7Rs expression has been reported on some populations of neurons in the spinal cord, cerebellum, hypothalamus, and substantia nigra [67].

Gene-linking and epidemiological studies have implicated P2X7Rs in different inflammatory CNS diseases [89]. *In vivo* studies have been a fundamental tool to demonstrate the involvement of the P2X7Rs in activating the inflammasome in a broad variety of rodent CNS disease models, including cerebral ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, depression and anxiety and multiple sclerosis [69].

Different features of the P2X7R make it an optimal mediator of cellular responses to inflammation. Indeed, the low affinity of the receptor for ATP and its slow desensitization mean that it is unresponsive to micromolar variations in extracellular ATP concentrations. This allows ATP signaling to exert its actions in different ways at different concentrations, with phasic micromolar

ATP signaling operating via other P2XRs to modulate a number of physiological pathways, while millimolar concentrations, released into the extracellular milieu in response to injury, can act via P2X7Rs, in turn initiating the inflammatory cascade [181]. Moreover, the pore-forming functionality of P2X7R facilitates the release of large hydrophilic molecules, and seems to be necessary for its role in activating the inflammasome [182].

The molecular and cellular mechanisms through which P2X7R exerts its pro-inflammatory role still represent a matter of debate. The inflammasome is a protein complex that regulates the interaction with caspase molecules, which cleave precursor protein substrates into immunomodulatory molecules. Six or possibly eight inflammasome subtypes have been currently identified, with the main but not exclusive function to catalyze conversion of pro-IL-1β and pro-IL-18 into their respective mature forms [183]. Inflammasome activated by damage-associated molecular pattern molecules (DAMPs) promotes a massive K^+ efflux, which in turn leads to the processing of procaspase-1 into caspase-1, followed by the cleavage of pro-IL-1β. It has been demonstrated that ATP acts as a DAMP, via P2X7Rs, activating the inflammasome and caspase-1 and that it is also involved in cytokine release and in the production of superoxide products and TNF-α, all of which have roles in generation and/or maintenance of inflammatory pain [184]. P2X7R activation leads to an increase in K^+ permeability either directly through the P2X7R pore, or through the opening of Panx1 hemichannels. While some reports indicate that Panx1 channel opening is a mandatory step of inflammasome activation [185], other groups have described a P2X7R-induced IL-1β release independent from Panx1 [186]. A further study hypothesized that Panx1 is responsible for the ATP release from dying cells, upstream of P2X7R in the signaling cascade [187]. Alternatively, Panx1 hemichannels may open in response to an increase in extracellular K^+ as a result of P2X7R pore opening, thus amplifying K^+ efflux [188].

Considering the overall distribution of P2X7Rs in pro-inflammatory cells and their functional properties, it is not surprising that several studies have been carried out to determine the role of this receptor subtype in inflammation. As mentioned, in the CNS P2X7Rs are expressed in highest concentrations on microglial cells, but also on astrocytes, oligodendrocytes and neurons, particularly at presynaptic terminals [189]. P2X7R expression on different cell types, upregulated in response to CNS insult, combines to mediate a neuroinflammatory response. In particular, ATP-activated microglial cells are main actors of the neuroinflammatory response, releasing IL-1β in response to a pro-inflammatory stimulus, acquiring an activated morphology, proliferating and migrating towards the site of the damage to form an inflammatory focus [185].

Relief from inflammation-induced mechanical hyperalgesia in rats treated with the P2X7R antagonist oxidized ATP has been reported [190], and the systemic administration of bacterial LPS

markedly increased the expression of P2X7Rs in the CNS, offering a mechanism for changes in CNS function in response to systemic infection [191]. Moreover, it has been reported that P2X7R stimulation on enteric neurons elicited a direct release of ATP, which stimulated glial cells through Panx1 [192]. Through this paracrine signaling, P2X7Rs can function as gatekeepers between glial cells and neurons to regulate inflammatory cascades [193].

Also astrocytic P2X7Rs contribute to the inflammatory response, with their activation leading to a neurotoxic phenotype in a model of Amyotrophic Lateral Sclerosis (ALS) [194]. Previous studies also showed that, following trauma, astroglial P2X7R activation led to upregulation of monocyte chemoattractant protein-1 (MCP-1) and, subsequently, to an increased infiltration of systemic immune cells at the site of damage [195].

The *in vivo* investigation of the role of P2X7Rs in inflammation has been greatly supported by the development of P2X7R knockout mice, which gave a further confirmation of the role of this receptor subtype in promoting inflammation. A first study examined the response of P2X7R knockout mice in a monoclonal antibody-induced arthritis model, showing that arthritis severity was significantly attenuated in P2X7R knockout mice compared to wild-type [196]. In a more detailed study, another research group showed that P2X7R-deficient animals did not develop measurable pain symptoms following induction of inflammatory status [73]. More recently, in an acute inflammatory pain model, microglial P2X7Rs were demonstrated to play a major role in the development of sensitization of nociceptive neurons in rat medullary dorsal horn *in vivo* [197].

Surprisingly, a recent study analyzed the P2X7R secretome in wild-type and P2X7R-deficient macrophages polarized either to M1 or M2, providing evidence that proteins released after P2X7R stimulation goes beyond the caspase-1 secretome. Authors found that P2X7R stimulation in macrophages is able to release potent anti-inflammatory proteins independently from their polarization state, suggesting for first time a potential role for P2X7R during resolution of the inflammation [198].

In light of the role of P2X7Rs in mediating inflammatory pain, a search for selective antagonists has been carried on by different pharmaceutical companies. Systematic compound screening led to the discovery of several selective P2X7R antagonists. Among these, AZ-11645373 (Table 1), a highly potent antagonist at human P2X7Rs, proved to be effective in inhibiting ATP- and Bz-ATP-elicited currents. In parallel, systemic administration of the selective P2X7R antagonists A-438079 and A-740003 was able to reduce thermal hyperalgesia in both CFA- and carrageenan-induced inflammatory pain [70].

The PET ligand ^{11}C-GSK1482160, recently reported by GSK, was derived from medicinal chemistry efforts directed toward the generation of pyroglutamate and imidazolidine carboxamide-

based P2X7R antagonists. These antagonists are highly potent at the human P2X7R and showed excellent exposure in the CNS; they also have modest potency at the rat receptor allowing for their use in preclinical pain models. Recently, a phase I clinical trial has been conducted by GSK to evaluate the pharmacokinetic, pharmacodynamics, safety and tolerability of GSK1482160 compound. In preclinical studies, GSK1482160 was shown to be efficacious in the chronic joint pain model of inflammatory pain and in the CCI model of neuropathic pain. A reduction of ATP-mediated release of IL-1β following LPS stimulation was observed in patients' blood samples. Later, authors postulated that >90% inhibition of IL-1β release was necessary throughout the dosing interval to test the P2X7R hypothesis, and that GSK1482160 did not have the safety margins to achieve such sustained inhibition. Therefore, the further development of GSK1482160 was terminated [70].

In addition to the discovery of P2X7R PET ligands, a number of novel CNS-penetrant P2X7R antagonists is now appearing in the literature, proving increased interest in centrally penetrating compounds for the treatment of pain and inflammation [70].

Finally, an essential step towards personalized medicine has been recently made, with literature showing that P2X7R SNPs (see 2.1.1) could be exploited as diagnostic biomarkers for the development of tailored therapies [199].

Interestingly, a recent study shed new light on the functional role of P2X7R in the regulation of microglial effector functions during substance abuse. Authors suggested that P2X7Rs play an important role in methamphetamine-induced microglial activation responses, and thus selective antagonists may represent a novel therapeutic approach to neuroinflammatory conditions in stimulant abuse by regulating pathologically activated glial cells [200].

P2X4Rs. As previously described, plenty of evidence demonstrate changes in P2XR expression during inflammation, but no clear demonstration that other P2XRs than P2X7Rs mediate the process. Evidence is accumulating, however, that P2X4Rs may also play a role.

It has been recently demonstrated that, similarly to P2X7Rs, also P2X4Rs form a large conductance pore on the cell membrane, facilitating ion efflux and subsequently inflammasome activation [181], but this process seems to be Panx1-independent [188]. Since P2X4Rs have higher affinity than P2X7Rs to extracellular ATP, sequential inflammasome activation by distinct P2XRs might represent responses to insults of different intensity, i.e. while P2X4Rs may act as an initial trigger, P2X7Rs in concert with Panx1 may later amplify the signal [201].

Concerning CNS inflammation, i both systemic and i.c.v. injection of LPS resulted in thermal hyperalgesia and tactile allodynia, and LPS has been shown to enhance the responses to low concentrations of ATP through P2X4R activation [202]. Moreover, evidence for the involvement of

microglial P2X4Rs in post-ischemic inflammation in the brain ischemic injury has been recently provided [203].

Again, strong evidence on the pro-inflammatory role of P2X4Rs derived from *in vivo* studies using knockout mice. P2X4R knockouts showed less microglial activation and loss of the prostaglandin E2-mediated inflammatory pathway [204]; a more recent study showed that P2X4R knockout mice displayed impaired inflammasome activation, resulting in a decrease in extracellular IL-1β and reduced infiltration of neutrophils and monocyte-derived M1 macrophages following spinal cord injury [205].

Finally, in the high-throughput screening of a compound library, a research group recently identified the phenylurea BX430 (Table 1) with antagonist properties on human P2X4R-mediated Ca^{2+} uptake. BX430 proved to be highly selective, having virtually no functional impact on all other P2XR subtypes at the surface of human macrophages. Therefore, this ligand provides a novel molecular probe to assess the specific role of P2X4Rs in inflammatory and neuropathic pain conditions, where ATP signaling has been shown to be dysfunctional [206].

4.1.2 P2Y receptors and inflammation

At variance from P2XRs and P1Rs, the role of P2YRs in inflammatory pathways is still elusive, mostly due to the long-lasting lack of subtype-selective agonists and antagonists (see also 2.1.2). Recent data are, however, clearly demonstrating that also this receptor family is participating to inflammatory processes, thus adding further complexity to the role of purinergic system in controlling these events.

$P2Y_1$Rs. This receptor subtype is involved in vascular inflammation and in the recruitment of leukocytes, which could be inhibited by its deletion or inhibition, thus suggesting a possible therapeutic approach for inflammatory vascular diseases such as atherosclerosis. In particular, authors reported that this effect is mediated through the regulation of the p38 MAPK pathway and they addressed the pharmacological inhibition of the $P2Y_1$Rs as new tool for the treatment of p38 MAPK-mediated vascular inflammation [207].

The activation of $P2Y_1$Rs expressed by platelets induces shape changes as well as weak and transient aggregation [208], and its deletion leads to impaired platelet aggregation [209]. As for $P2Y_{12}$Rs (see below), these observations suggest caution in planning a therapeutic approach based on its pharmacological modulation, due to possible life-threatening side effects (i.e., thrombosis or bleeding). Nonetheless, platelet $P2Y_1$Rs activation, plays a fundamental role in the recruitment of leukocytes in the lungs of allergic mice, and this effect is mediated by RhoA signaling [210]. $P2Y_1$Rs

are also involved in macrophage phagocytosis and migration [211]. It has been recently discovered that inorganic polyphosphate interacts with this receptor. This compound is known to have a pro-inflammatory effect, and its interaction with endothelial $P2Y_1Rs$ promoted the expression of cell adhesion molecules and induced barrier-disruptive effects, thus fostering leukocyte infiltration and recruitment [212].

Moreover, it has been shown that astrocytic $P2Y_1Rs$ are upregulated in the brain after ischemia [213]. A more recent study indicated the presence of $P2Y_1R$-dependent neuroinflammation in the hippocampus after focal cerebral stroke, and the inhibition of this receptor ameliorated the associated cognitive deficits [214].

$P2Y_2Rs$. This receptor is involved in the resolution of inflammation by mediating the recruitment of leukocytes and promoting the clearance of apoptotic cells by macrophages [215] and neutrophils [216, 217]. Nonetheless, its contribution to lung inflammatory diseases like asthma or contact hypersensitivity has been also demonstrated [218, 219]. Moreover, $P2Y_2Rs$ also contribute to the release of pro-inflammatory chemokines and cytokines from neutrophils, monocytes and macrophages [220], further highlighting its pro-inflammatory actions.

In the last decade, the pharmacological interest around $P2Y_2R$ agonists has raised, due to the capacity of this receptor to induce chloride secretion by epithelial cells, which is drastically impaired in cystic fibrosis [221]. For this reason, Denufosol, a selective agonist at $P2Y_2Rs$, has been developed as potential pharmacological agent that would be able to compensate the effect caused by non-functional chloride CFTR channel. This compound was tested in phase III for the treatment of cystic fibrosis, but long term follow-up of patients did not show beneficial effects in reducing the symptoms of the disease [95], probably due to the concomitant pro-inflammatory role of $P2Y_2Rs$. Accordingly, more studies are needed to better evaluate the role of $P2Y_2Rs$ in inflammation. Nevertheless, another $P2Y_2R$ agonist, Diquafosol, has been recently approved in Japan for the topical treatment of the dry eye syndrome [96, 222, 223], a pathology in which immunoinflammatory processes play a major role [224].

Furthermore, it has been demonstrated that peripheral inflammation leads to upregulation of $P2Y_2Rs$ in cutaneous sensory neurons [225], thus further suggesting a possible role for this receptor in inflammatory pain.

$P2Y_4Rs$. Recent studies have shown the involvement of this receptor in inflammation. For example, $P2Y_4Rs$ on endothelial cells act as key regulators of the inflammatory response after cardiac

ischemia [226]. Moreover, this receptor subtype is expressed by eosinophils, but its action on chemotaxis still has to be demonstrated [227].

$P2Y_6$Rs. The activation of this receptor subtype in monocytes, eosinophils, dendritic and endothelial cells leads to the release of chemokines, which in turn increase the recruitment of inflammatory cells at the site of inflammation or bacterial infection [228-230]. Indeed, $P2Y_6$Rs are important in the development of inflammation, suggesting that their inhibition could help improving the inflammatory states [231]. Moreover, it has been shown that neurons release UTP/UDP after injury, thus causing the upregulation of $P2Y_6$Rs on microglial cells and increasing their phagocytic activity [113]. To support these findings, another study showed that blocking $P2Y_6$Rs helps preventing neuronal loss *in vitro* [232].

In IBS-D, the inhibition of $P2Y_6$Rs improves the disease outcome in mice [233, 234]. Moreover, $P2Y_6$Rs activation induces atherosclerotic disease in murine models [235], whereas their inhibition or deletion contribute to a favorable the outcome of the pathology by developing smaller plaque [236].

$P2Y_{11}$Rs. A recent *in vitro* study showed that $P2Y_{11}$Rs are involved in the autocrine regulation of macrophages, and thus their antagonism could help in inflammatory diseases [237].

$P2Y_{12}$Rs. The $P2Y_{12}$R is the most studied subtype of purinergic metabotropic receptors, due to its peculiar expression on platelets where it controls aggregation, and to the use of selective antagonists (i.e., clopidogrel, ticlopidine, and ticagrelor) as effective antithrombotics [94]. It is worth notice that the circulating levels of inflammatory mediators are decreased in patients treated with clopidogrel [238], thus suggesting a role for this receptor also in the modulation of inflammatory processes. Consistently, the genetic deletion of $P2Y_{12}$Rs in mice exerted a protective role against the development of atherosclerosis [239-241]. In contrast, the inflammatory response to LPS is more potent in $P2Y_{12}$R null than in wild type mice [242].

Furthermore, it has been shown that leukotriene E4 exerts its pro-asthmatic role through $P2Y_{12}$Rs expressed by platelets, and this effect can be deduced from the lack of inflammation after platelet depletion, treatment with clopidogrel or following the genetic deletion of the receptor [243]. Nonetheless, a more recent study points out that $P2Y_{12}$Rs expressed by platelets were not involved in inflammatory response in the lungs of allergic mice [210]. Moreover, $P2Y_{12}$R SNPs in asthmatic children were associated with altered lung function [244]. Thus, the role of $P2Y_{12}$Rs in inflammation is still elusive and needs to be fully understood and clarified.

$P2Y_{13}$Rs. A recent a study showed an increase in the incidence of atherosclerosis in double-knockout mice for apoE and $P2Y_{13}$Rs, compared to single apoE knockout mice [245]. This finding has unveiled a novel role for this receptor subtype in inflammatory vascular diseases.

$P2Y_{14}$Rs. This receptor subtype is expressed by leukocytes, thus pointing out a possible effect in inflammation [246]. It has been shown that the activation of $P2Y_{14}$Rs promoted the chemotaxis of neutrophils [247, 248], specifically in the lungs [249].

4.2. Adenosine and inflammation: a well-known connection with possible future clinical exploitations

Hypoxic/anoxic conditions foster the local generation of Ado, since available ATP is rapidly degraded to produce energy in the absence of oxidative phosphorylation. Therefore, high micromolar concentrations of Ado are detected at any site of inflammation. In this condition, it has been initially demonstrated that Ado acts as "STOP" signal on inflammatory cells mostly through the activation of the A_{2A}ARs, thus turning down the acute beneficial inflammatory response of the tissue to avoid the development of a sustained, chronic, and potentially harmful reaction of the damaged tissues [250]. Research in recent years has significantly increased our knowledge on the complex role played by Ado on the modulation of inflammatory and immune cell functions, through the activation of its different receptor subtypes. For a detailed overview on this issue, we recommend to refer to excellent reviews that have been recently published [133, 251]. Here, we shall highlight the most important actions exerted by the different Ado receptor subtypes in the course of inflammation, to identify the most promising targets to be possibly exploited in clinics.

A_1ARs. This receptor subtype is mainly expressed by activated neutrophils where it mediates stimulatory activities on various cell functions, and by dendritic cells where it promotes chemotaxis and maturation [133]. Overall, activation of A_1ARs has been linked to pro-inflammatory activities, with a specific role in asthma also in response to exposure to allergens. It is worth mentioning that the methylxanthine theophylline has been used for decades as anti-asthmatic drug, with its main mechanisms of action linked to its ability of inhibiting phosphodiesterases but also to its antagonistic activity at Ado receptors (see also Conclusions) [252]. A crucial role for A_1ARs in asthma has been provided by the demonstration that administration of selective antisense oligonucleotides reduced bronchoconstriction in rodents [253], although to date data on humans have not led to their clinical exploitation [254].

A_{2A}ARs. As already mentioned, A_{2A}ARs have long been recognized as crucially involved in the fine-tuning of inflammatory processes, by acting at different levels with an overall anti-inflammatory action. A_{2A}ARs are expressed by virtually all immune cells, and their expression is upregulated in inflammatory conditions, like asthma, inflammatory kidney diseases and arthritis [133]. Concerning the latter, it has been recently demonstrated that receptor upregulation is paralleled by increased expression of CD73, the nucleotide-metabolizing enzyme whose activity increases Ado concentrations (Figure 2) [255]. This has led to the development of a pro-drug of a selective A_{2A}AR agonist that is converted in its active form by CD73, i.e. at the site of the pathology where increased density of A_{2A}ARs can be also found [255], thus avoiding unwanted cardiovascular side effects. In fact, currently available drugs acting selectively on this receptor subtype (like Regadenoson) are employed as coronary dilatory agents in diagnostic procedures [256], thus highlighting the importance of this receptor subtype in controlling vascular and cardiac functions (see 2.2).

By activating A_{2A}ARs on T cells, Ado mediates immunosuppression in the hypoxic tumor microenvironment. As already mentioned, high Ado concentrations are generated at any hypoxic sites due to rapid ATP breakdown [251]. A significant contribution to this pathway is provided by the transcription factor Hypoxia-Inducible Factor 1-alpha (HIF-1α), which upregulates the nucleotide metabolizing enzymes CD39 and CD73, thus fostering Ado production [257]. This Ado-enriched tumor microenvironment is now considered a fundamental barrier that needs to be overcome to allow anti-tumor T lymphocytes and natural killer cells to infiltrate and eliminate tumor cells. One possible therapeutic strategy is represented by the use of selective A_{2A}AR antagonists to weaken Ado-mediated immunosuppression and to facilitate immunotherapies in cancer [257].

Other pathological settings were the modulation of A_{2A}ARs could prove beneficial are intestinal inflammation, enteritis, sepsis induced by various pathogens, as well as autoimmune diseases like multiple sclerosis, where pharmacological stimulation of these receptors on lynmphocytes from patients led to a reduced release of several pro-inflammatory mediators [133].

A_{2B}ARs. Contradictory data on the role of this receptor in inflammation have been published so long, thus pointing to the need of additional research on the molecular pathways activated by this receptor subtype under different physiological and pathological conditions. For example, a clear anti-inflammatory effect of A_{2B}AR antagonists has been foreseen following the demonstration of increased pro-inflammatory cytokines by receptor activation. In line with this, a selective A_{2B}AR antagonist, CVT 6883 (Table 1), has proven effective in reducing airway hyperreactivity in several models of inflammatory lung disorders. The compound showed no side effects and good tolerability

in healthy subjects, but no further clinical exploitation of these data is currently available [258]. The same receptor antagonist was able to ameliorate the course of experimental autoimmune encephalomyelitis, the rodent model of human multiple sclerosis, thanks to a reduction in the production of pro-inflammatory mediators by immune cells [259]. As already mentioned in nociception (see 2.2), opposite results have been obtained in other experimental settings. In fact, the selective A_{2B}AR agonist BAY 60-6583 (Table 1) was able to potentiate the anti-inflammatory activity of dexamethasone on human airway epithelial cells *in vitro* [260]. A pro-inflammatory role of this receptor subtype in intestinal inflammation has been also suggested, although once again conflicting results have been provided [133].

A_3ARs. Since its cloning in early 1990's, this receptor subtype has been linked to immune cell functions, with a role in degranulation, chemotaxis, and cytokine production. As mentioned in 2.2, the immediate availability of selective agonists, like IB-MECA and its chloro derivative Cl-IB-MECA has allowed their testing in various pre-clinical models of inflammatory conditions, like arthritis, and uveitis [for review see [133]]. These data have prompted CanFite Therapeutics to undertake clinical trials in patients affected by rheumathoid arthritis, uveitis, psoriasis and dry eye syndrome, with excellent encouraging results [261], which suggest that these molecular entities will soon reach the market for several clinical inflammatory conditions.

5. MODULATION OF PURINE METABOLIZING ENZYMES AND TRANSPORTERS

Not only P1 and P2 receptors, but also other molecular components of the purinergic system have already been (or could be) targeted pharmacologically to modulate the balance between the extracellular concentrations of Ado and ATP, especially when increased Ado concentrations are needed. For example, the nucleoside uptake inhibitor, dipyridamole, has long been utilized as anti-thrombotic agent thanks to its ability to inhibit membrane transporters, thus leading to increased extracellular Ado concentrations which in turn activate platelet A_{2A}ARs [262]. The same overall effect can be obtained by inhibiting adenosine deaminase (ADA)-mediated Ado metabolism or its reconversion to AMP mediated by adenosine kinase and/or by increasing its production through the administration of CD39 or CD73 ectonucleotidase enzymes (Figures 1 and 2). The issue of the site of action of these agents is not trivial, since increased Ado concentrations are needed only in the target organ or tissue to avoid potentially life-threatening side effects. Moreover, due to the opposite effects on pain and inflammation exerted by some receptor subtypes (see above) the development of selective agents acting only at peripheral or central sites would be desirable.

Nevertheless, similar pharmacological approaches aimed at modulating endogenous Ado

concentrations has been already applied to pain and inflammatory conditions. For example, inhibitors of adenosine kinase have proved beneficial in inflammatory and neuropathic pain models, although severe side effects have led to discontinue their further development [139]. Also dipyridamole and other uptake inhibitors have shown significant analgesic properties in guinea pigs [263], accompanied by hypothermia and alterations of motor activity, due to central actions. A single intrathecal administration of the recombinant AMP-metabolizing enzyme CD73 (also known as 5'-ecto-nucleotidase) showed long-lasting anti-nociceptive activity in wild type mice which was lost in A_1ARs knockout animals [264, 265].

Concerning inflammatory conditions, administration of the ADA blockers EHNA (Table 1) and pentostatin has significantly improved the outcome of experimental colitis and sepsis [for review see [133]]. Similar beneficial effects have been observed by adenosine kinase inhibitors in colitis, diabetic retinopathy, and carrageenan-induced arthritis [133]. Finally, dypiridamole has provided significant protection against lung injury induced by LPS inhalation in rodents [266]. Results have driven a randomized, double-blind, placebo-controlled study to evaluate its use in human experimental endotoxemia, with the demonstration of increased circulating concentrations of Ado and a steep reduction in the production of pro-inflammatory cytokines in patients [267].

6. CONCLUSIONS

Available literature pre-clinical data together with the results of some clinical trials clearly highlight the purinergic system as a new target for the development of new analgesic and anti-inflammatory agents. Unfortunately, to date, no molecule has reached the market, and many promising clinical trials have failed (Table 2). Since no results are available for many of these studies, only speculations on the possible reasons for failure can be made. As already mentioned, the intrinsic complexity and widespread distribution of the purinergic signaling system is likely to contribute to the difficulties in translating pre-clinical results to humans, where the development of previously unforeseen side effects can represent an important issue. . Moreover, for many purinergic receptors (e.g., the A_3ARs, and several P2YRs) [92, 148] significant differences in pharmacology and in agonists/antagonists affinity and selectivity between rodents and humans have been observed. For example, in the case of some P2X7R antagonists with very poor affinity at rodent receptors, a study on patients with rheumatoid arthritis has been directly performed following in vitro proof-of-principles experiments, leading to no results [70]. Additionally, many chemicals that are utilized as pharmacological tools in preclinical studies are not sufficiently centrally permeant when administered to patients through classical routes. This could significantly limit their efficacy and their clinical development. The availability of modified molecules with enhanced CNS permeability could help

overcome this important issued [70]. It is worth mentioning that, to date, the most promising candidates for a purine-based therapy against inflammatory pain and rheumatoid arthritis are represented by A_3AR selective agonists (Table 2). This is possibly due to their multiple sites of actions, both in the CNS and in the periphery [149], which overall contribute to the final positive outcome of the treatment. Additionally, several promising agents and new pharmacological approaches (like allosteric modulators) have been studied to overcome these issues [133], and we are confident that some new purine-based drugs will soon reach the market for pain and inflammation.

When thinking about innovative pharmacological strategies targeting the purinergic system, it should be remembered that it is the main target of the most widely diffused substance of abuse worldwide, i.e. caffeine. Its estimate average intake in Western countries spans from about 227 mg/die to 70 mg/kg/die, deriving mostly from coffee but also from food, cold drinks, and tea. Concerns are raised by the increasing assumption of energy drinks, which contain twice as much caffeine as cold drinks, especially by adolescents [268, 269]. From a pharmacological point of view, methylxanthines in general, and caffeine in particular, are known to act as antagonists at Ado receptor subtypes apart from being inhibitors of phosphodiesterases. Most of the stimulating effects of caffeine are due to its antagonistic activity versus Ado receptors, especially the A_1ARs and A_{2A}ARs; based on studies on rodents, it was long generally believed that the A_3ARs are virtually insensitive to methylxanthines. However, at variance from rodents, caffeine shows antagonistic activity versus *all* four human ARs, thus including the A_3AR subtype [270]. Thus, it is conceivable that caffeine-containing beverages and foods could exert a significant inhibitory action on ARs-based analgesics and anti-inflammatory drugs in patients, whatever the receptor subtype targeted by the drug. This issue must be taken into careful account, and specific advices to avoid or limit the assumption of caffeine in the course of therapy with Ado receptor agonists must be given to patients. Conversely, inhibition of pro-nociceptive A_{2A}ARs could prove beneficial. On this basis, low doses of caffeine are utilized as adjuvant component in combination with antidepressant, acetaminophen and non-steroidal anti- inflammatory drugs in many over the counter (OTC) analgesics [139, 271].

In line with this, it has been reported that a moderate caffeine intake at doses relevant to average daily human consumption can hide acupuncture-mediated analgesia in mice [269]. Apart from further confirming the role of caffeine-sensitive Ado receptors in acupuncture, this observation opens to the evaluation of coffee intake in patients as a predictive factor for possible beneficial effects before starting an acupuncture treatment for painful conditions. Once again, the issue of caffeine assumption is not trivial for patients subjected to this alternative therapy, and to any future patient that will be exposed to new purine-based pharmacological approaches.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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FIGURE CAPTIONS

Figure 1. Purine metabolism and Adenosine receptors. Schematic picture showing the pathways of ATP release under pathological conditions, its sequential degradation to AMP and Adenosine, through the activity of CD39 and CD73 surface enzymes. Extracellular adenosine can be then reuptaken by surrounding cells or bind to four different G-protein-coupled receptors that either stimulate (A_{2A}ARs and A_{2B}ARs) or inhibit (A_1ARs and A_3ARs) adenylyl cyclase activity. Moreover, all adenosine receptors couple to mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase 1 (ERK1), ERK2 and p38 MAPK. In the extracellular space, adenosine concentrations are controlled by adenosine deaminase (ADA; which catalyses the conversion of adenosine into inosine) and by the activity of nucleoside transporters (NTs). cAMP, cyclic AMP; JNK, JUN N-terminal kinase. Much less is known about the release and catabolism of uracil and uracil-sugar nucleotides, which nevertheless contribute to the modulation of inflammatory and painful conditions. Reproduced with permission (licence #3976430230660) from [1].

Figure 2. ATP release pathways, receptors and degrading enzymes involved in purinergic signaling. Schematic representation of the contribution of P2 receptors to purinergic signaling. ATP is released into the extracellular space via secretory vesicles (exocytosis), plasma membrane-derived microvesicles, transporters (for example, ABC cassettes), channels (for example, pannexin-1 or connexins) or through the ion channel P2X7R itself. Once in the extracellular milieu, ATP acts at P2X and some P2Y receptors (like the $P2Y_2$Rs and $P2Y_4$Rs). It is also hydrolyzed to ADP which activates $P2Y_{12}$Rs but also $P2Y_1$Rs and is further degraded to adenosine (ADO) which activates its specific membrane receptors. Uracil nucleotides which activate other P2Y receptor subtypes are not included in this scheme. Reproduced with permission (licence #3977540979535) from [2].

Figure 3. Involvement of purinergic receptors in chronic pain pathways. P2XR ion channels have been identified as the main players at sensory nociceptive terminals in the periphery, together with G protein-coupled P2YRs. Although less potent than ATP, adenosine (ADO) also exerts an action on sensory terminals through P1 receptors, and can potentiate P2XR activation. Painful stimuli are integrated within peripheral sensory ganglia (i.e. DRG and TG), where ATP induces the sensitization of nociceptive neurons, thus increasing the sensitivity to ATP of surrounding satellite glial cells, which in turn can modulate neuronal firing. In the sensory synapsis in the spinal cord, ATP acts postsynaptically through P2XRs, and after being degraded to ADO, it acts on P1 receptors expressed

on the presynaptic primary afferent terminal, which play a modulatory role on neurotransmitter release. In the spinal cord, both P1 and P2 receptors on activated microglia have been implicated in chronic pain generation and maintenance. A role for purinergic receptors expressed by activated astrocytes has been also hypothesized, although no direct proofs are available yet. Due to the paucity of data, uracil nucleotides have not been reported in this scheme, although it is foreseen that they significantly contribute to the modulation of the whole process at different levels. See text for details.