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# Treatment of chronic neuropathic pain: purine receptor modulation

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## Abstract

Extracellular nucleosides and nucleotides have widespread functions in responding to physiological stress. The “purinome” encompasses four G protein-coupled receptors (GPCRs) for adenosine, eight GPCRs activated by nucleotides (P2YRs), seven adenosine 5’-triphosphate(ATP)-gated P2X ion channels, as well as the associated enzymes and transporters that regulate native agonist levels. Purinergic signaling modulators, such as receptor agonists and antagonists, have potential for treating chronic pain. Adenosine and its analogues potently suppress nociception in preclinical models by activating $A_1$ and/or $A_3$ adenosine receptors(ARs), but safely harnessing this pathway to clinically treat pain has not been achieved. Both $A_{2A}$AR agonists and antagonists are efficacious in pain models. Highly selective $A_3$AR agonists offer a novel approach to treat chronic pain. We have explored the structure activity relationship of nucleoside derivatives at this subtype using a computational structure-based approach. Novel $A_3$AR agonists for pain control containing a bicyclic ring system (bicyclo[3.1.0]hexane) in place of ribose were designed and screened using an *in vivo* phenotypic model, which reflected both pharmacokinetic and pharmacodynamic parameters. High specificity (>10,000-fold selective for $A_3$AR) was achieved with the aid of receptor homology models based on related GPCR structures. These $A_3$AR agonists are well tolerated *in vivo* and highly efficacious in models of chronic neuropathic pain. Furthermore, signaling molecules acting at P2X3, P2X4, P2X7 and $P2Y_{12}$Rs play critical roles in maladaptive pain neuroplasticity, and their antagonists reduce chronic or inflammatory pain, and, therefore, purine receptor modulation is a promising approach for future pain therapeutics. Structurally novel antagonists for these nucleotide receptors were discovered recently.

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Dr. Salvemini is founder of BioIntervene, Inc. a company developing $A_3$AR agonists as analgesics for chronic pain. All other authors declare no conflict of interest.

**Keywords**

adenosine receptor; P2Y receptor; P2X receptor; pain; agonist; antagonist

## Introduction

Chronic pain treatment remains one of the major unsolved medical needs and also accompanies many diseases and pharmacological interventions. Ion channels, G protein-coupled receptors (GPCRs) and kinases are common targets for analgesic drug discovery. However, modulators of purine receptors (either GPCRs or adenosine 5'-triphosphate (ATP)-gated channels) [28,151] are less often considered in pain research, compared to widely used treatments: sodium and calcium channel blockers, γ-aminobutyric acid (GABA) modulators, ligands of opioid and cannabinoid receptors and kinase inhibitors [32]. Nevertheless, the existing treatments are not generally effective in most patients, and the current treatments develop tolerance or have other serious side effect liabilities upon prolonged use. For example, opiates are more effective in acute pain than in chronic pain and can lead to addiction, desensitization and even hyperalgesia. Thus, novel treatment approaches for chronic neuropathic pain are needed.

Purine receptors belong to a ubiquitous signaling system in the body that has been termed the "purinome" (Figure 1) [80,185]. ATP and other adenine nucleotides are released during physiological stress, stemming from oxygen and nutrient deprivation, inflammation, cancer, tissue injury, etc., which are ultimately catabolized to adenosine. The immediate response to the stress is activation by ATP of P2X receptors to open ligand-gated cation channels. Each functional P2X ion channel consists of a homomer or heterotrimer of P2X subunits. A more diverse set of adenine and uracil nucleotides activate the eight G protein-coupled P2Y receptors (P2YRs), including nucleoside 5′-diphosphates, 5′-triphosphates and uridine diphosphate (UDP)-sugars. There are eight P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) and seven P2X receptor subunits (P2X1-7) that comprise an active trimeric channel. The P2X and P2Y receptors tend to boost the immune response in response to nucleotides, with their ligands acting as immediate danger signals [33]. Nucleotide-induced pain is included in this scheme as a beneficial function, as pain is a critical survival mechanism. Subsequent activation of adenosine receptors (ARs), also known as P1 receptors, of which there are four subtypes ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$), in general tends to put the brakes on the immune response, and their activation functions endogenously to suppress pain. This signaling system represents a temporal sequence of first activation of proinflammatory P2Rs by nucleotides, followed by the prolonged anti-inflammatory effect of adenosine, mainly formed gradually by enzymatic hydrolysis of released ATP, at the ARs [33]. Thus, in many cases, action of P2Rs increase, and ARs decrease, pain signaling [28,125]. Therefore, often, but not always, P2R antagonists and AR agonists are sought for controlling pain. RNA-seq (RNA-sequencing) analysis has shown that the expression of many of these genes in the purinome is enhanced in the dorsal root ganglia (DRG) and the spinal cord (SC) (Table 1) [146].

Many compounds are now available as selective modulators of the purinergic system, either as directly acting agonists and antagonists of P2Rs (Figure 2) or ARs (Figures 3 and 4) [80], or as inhibitors of associated enzymes and transporters that regulate native levels of adenosine (Figure 4) or nucleotides. Inhibitors of enzymes involved in processing of adenosine include the widely used adenosine kinase inhibitors ABT-702 **38**, a pyridopyrimidine, and the nucleoside A-134974 **39** (Figure 3) [87]. Inhibitors of these enzymes and transporters, in some cases, are approved drugs. For example, adenosine deaminase (ADA) inhibitor, pentostatin (dexycoformycin) **44**, is an anticancer drug. Dipyridamole **43**, a clinical vasodilator, inhibits equilibrative nucleoside transporters 1 and 2 (ENT1/2) and also acts as a phosphodiesterase (PDE) inhibitor.

## P2XR ligands and their use in relieving chronic pain

Various P2XR antagonists have shown efficacy in pain models [17]. For example, involvement of P2XRs has been explored in acupuncture analgesia and central pain syndrome [107,169]. In neuropathic pain, adrenomedullary chromaffin cells overexpress P2X3 and P2X7 [7]. Early studies of the mechanism of pain induction by ATP focused on the observation that the P2X3R and P2X2/3R are localized and function in neurotransmission at the DRG (Table 1), the main conduit for pain signaling by the peripheral nerve nociceptors [37,112]. ATP is produced by inflammatory cells, tumor cells, endothelial cells, sympathetic neurons, and Merkel cells in the skin and other cells. A local elevation of the extracellular levels of ATP can act to stimulate peripheral sensory nerves. It was demonstrated that clorodonate, a first-generation bisphosphonate was able to attenuate hyperalgesia in rodents models of carrageenan- and complete Freund's adjuvant (CFA)-evoked inflammatory pain via inhibition of vesicular ATP release, as well as in a partial sciatic nerve injury model of chronic neuropathic pain [98]. Furthermore, the acidification that accompanies both inflammation and the hypoxic tumor microenvironment can promote activation of the P2XRs by known phenomenon of pH modulation of the receptor protein [40].

### P2X3R antagonist antinociceptive effects:

Both pharma and academic labs have maintained a long-term effort to produce a P2X3R antagonist that might be effective in controlling pain [25,76,165,172]. However, there are currently no P2X3 antagonists in clinical trials for pain, but one is in trials for chronic cough [134]. Various P2X3 antagonists have been reported [65], including A-317491 **9**, which is potent and selective but not orally bioavailable. This first-reported selective P2X3R antagonist reduces neuropathic, inflammatory and chemogenic pain following intrathecal or intraplantar administration [129]. P2X3R antagonists have been shown to be efficacious in reducing chronic pain in animal models of cancer [55,94] and in several animal models of neuropathic pain such as chronic constriction injury (CCI) of sciatic nerve [152], spinal nerve injury [67], partial ligation of the unilateral infraorbital nerve [154], chronic pancreatitis [189], maternal separation [198] and inflammatory pain [88,91]. Recently, nucleotide derivatives with the ribose 2′,3′-hydroxyl groups in a chemically protected state were reported to be P2X3R antagonists, e.g. **10** [44]. Upregulation of various purine receptors in pain pathways is a consequence of the chronic pain state [123]. Peripheral

inflammation is associated with plasticity of purinergic signaling within sensory ganglia. The P2X3R in the DRG and trigeminal ganglia (TG, responsible for sensation in the face and cranium and motor functions in the mouth) are located on the neurons, at the pre- or post-synaptic terminals [27,135,139,157,186]. Neuronal plasticity leading to upregulation of the P2X3R on these ganglia has been observed in conditions of inflammation [49]. Novel P2X3R antagonists such as **11** were studied in models of chronic pain [93]. A methyl ester prodrug derivative appears to be cleaved *in vivo* to form the active carboxylic acid.

### P2X4R antagonist antinociceptive effects:

The P2X4R, P2X7R and $P2Y_{12}R$ are upregulated in chronic pain states leading to hypersensitivity of the microglia and associated neurons [64]. This is a function of peripheral nerve injury (PNI), but not peripheral tissue inflammation. P2X4R activation has an additional consequence of the release of brain-derived neurotrophic factor (BDNF) from microglial cells, which acts upon neighboring neurons to interfere with the GABAergic anti-nociceptive system. Fibronectin acts upon pain-activated microglia to upregulate the expression of P2X4 [14,128]. BDNF binds to neuronal tropomyosin receptor kinase B (TrkB) in spinal lamina I to interfere with $K^+$-$Cl^-$co-transporter (KCC2), which normally maintains a low level of intracellular chloride in the SC neurons [43,111]. Consistent with a lack of microglial role in the development and maintenance of neuropathic pain in females, this mechanism was found to be essential only in male mice [156] since the *P2rx4* gene was upregulated only in dorsal horn of male, but not female mice, after PNI [47,95,147,156]. Despite qualitative sex differences in neuroimmune signaling after nerve injury, neurons in males and females ultimately experience a similar reduction in KCC2 function, leading to comparable levels of pain hypersensitivity [127]. The rise in intracellular chloride ions as a result of macrophage P2X4R activation diminishes the ability of drugs that directly or indirectly activate $GABA_A$ ion channels, to reduce chronic pain. Therefore, if a clinical P2X4R antagonist were to be made available, it might make the existing pain medicines more effective as well as having its own antinociceptive activity. Furthermore, a peripheral action of P2X4R antagonists on macrophages reduces the release of prostaglandin E2 (PGE-2) by a p38-mitogen-activated protein kinase (MAPK)-dependent mechanism, which acts as a pro-nociceptive signal [179]. Therefore, both spinal and peripheral sites of action of such antagonists could contribute to an analgesic effect [28].

Thus, P2X4R and P2X7R antagonists have been considered for pain control through their action on spinal microglial and other cells. Pharmaceutical companies and academic labs have developed numerous compounds to antagonize these receptors that are drug-like molecules and well tolerated in the body (Figure 2) [35,45,138,168]. PSB-12062 **12** is a P2X4R antagonist of μM affinity (human 1.38 μM, rat 0.928 μM, mouse 1.76 μM) [161], but it has low aqueous solubility. P2X4R knockout (KO) mice displayed normal acute pain responses and tissue damaged-induced pain, but reduced chronic pain, especially tactile allodynia [181]. P2X4R antagonists have been shown to be efficacious in reducing chronic pain in diabetes-induced neuropathic mechanical hyperalgesia [170] and in a model of trigeminal allodynia [117]. IgG#191 is a potent and selective antibody binding the head domain of P2X4 and able to reverse mechanical hyperalgesia in a mouse model of neuropathic pain induced by the Seltzer partial sciatic nerve ligation. IgG#191 does not cross

the blood-brain barrier (BBB) but a chimeric construct containing a domain which can be transported across the BBB into the central nervous system (CNS) showed dose-dependent reversal of mechanical hyperalgesia, indicating that with appropriate molecular modification, IgG#191 could access the SC from the periphery [190]. The selective P2X4R antagonist NP-1815-PX **13** displayed anti-allodynic effects in traumatic nerve damage and herpetic mouse models without affecting acute pain, however it does not readily cross the BBB [128]. NP-1815-PX was discovered as a result of a screen of chemical libraries and displays high aqueous solubility. NC-2600 (structure not disclosed) was developed in a collaboration between Prof. K. Inoue at Kyushu Univ. and academic and pharma collaborators (Nippon Chemipharma) and entered a Phase 1 clinical trial for chronic neuropathic pain in Japan, in June 2016.

### P2X7R antagonist antinociceptive effects:

Various P2X7R antagonists have been reported to be active against pain and inflammation [31]. P2X7R antagonists A-438079 **14** and A-740003 **15** have been shown to be efficacious in reducing chronic neuropathic and inflammatory pain [71,137]. Furthermore, the more potent antagonist A-740003 is highly P2X7R-selective in both human and rodent species. Microglial P2X7R activation contributes to maintaining advanced bone cancer pain by releasing proinflammatory cytokine interleukin-18 (IL-18) [197]. However, there have been clinical trials for P2X7R antagonists in inflammatory diseases, cancer, and depression. JNJ 47965567 **16** is a brain penetrant P2X7R (human, mouse and rat) antagonist of nearly nM affinity. Several positron-emission tomography (PET) ligands (**17**, **18**) for brain imaging of the P2X7R have been reported. P2X7R antagonist GSK1482160 **19** developed to target peripheral pain and has been in a clinical trial for inflammatory pain (Table 2). It was also radiolabeled with $^{11}$C for PET imaging [57,191]. AZ 11645373 **20** is a highly selective human P2X7R antagonist but does not bind to rodent P2X7Rs.

Pannexin-1 (Panx1) hemichannels are permeable to small molecules (<1 KD) and are the source of much extracellular ATP in the SC subsequent to morphine treatment, which can activate P2XRs [26]. ATP originating in spinal microglia in the dorsal horn contributes to long-term synaptic facilitation that is characteristic of opioid withdrawal. Pharmacological inhibition of ATP breakdown exacerbates opioid withdrawal, suggesting its detrimental action occurs through P2Rs, although a potential beneficial effect of adenosine produced from ATP hydrolysis was not addressed.

## P2YR ligands and their use in relieving chronic pain

Certain P2Rs present on the DRG and on the associated SGCs (satellite glial cells) are upregulated in pain states [123]. The receptors that are upregulated or activated in sensory neurons, such as the DRG and sciatic nerve include: neuronal and glial $P2Y_1R$, $P2Y_2R$, $P2Y_6R$, $P2Y_{11}R$ (all Gq-coupled) as well as $P2Y_{12}R$ and $P2Y_{14}R$ ($G_i$-coupled) [11,133,207]. Adenosine-5'-diphosphate (ADP) is the principal endogenous agonist of $P2Y_1R$, $P2Y_{12}R$ and $P2Y_{13}R$. UDP is the principal endogenous agonist of $P2Y_6R$. UDP-glucose is the principal endogenous agonist of $P2Y_{14}R$.

### $P2Y_1R$ antagonist antinociceptive effects:

$P2Y_1R$ antagonists have been shown to be efficacious in reducing chronic and acute pain in several animal models such as neuropathic pain model of spinal nerve ligation (SNL), CCI, spared nerve injury (SNI) [12], formalin and carrageenan-induced inflammatory pain [11,109], visceral hypersensitivity in rats with experimental irritable bowel syndrome [192], cancer-induced bone pain (CIBP) [38] and migraine [124]. The different cell types involved include: dorsal root, trigeminal, nodose sensory neurons, SGCs [145] and in the SC, astrocyte [59] and some of the dorsal horn neurons [102]. Potent and selective $P2Y_1R$ antagonist MRS2500 **2** reduces peripheral interleukin-1β (IL-1β)-mediated thermal hypersensitivity [109]. $P2Y_1R$ activation stimulates visceral nociceptors, an effect blocked by MRS2500 [68]. The upregulation of neuronal purine receptors in pain states can be interrupted by receptor antagonists. For example, MRS2500 reduced upregulation of $P2Y_1R$ in DRG induced by nerve injury [33]. Also, the $P2Y_1R$ intensifies the thermal hyperalgesia mediated by transient receptor potential cation channel subfamily V member 1 (TRPV1) in peripheral nerves, through its protein kinase C-dependent phosphorylation, leading to a pro-nociceptive TRPV1 as shown in an ischemic model [110]. This process was blocked by $P2Y_1R$ antagonist MRS2179 **1**.

### $P2Y_{12}R$ antagonist antinociceptive effects:

The Gi-coupled P2Y subfamily consists of three subtypes: $P2Y_{12}R$, $P2Y_{13}R$ and $P2Y_{14}R$. $P2Y_{12}R$ already has antagonists that are used clinically for the prevention of thrombosis. This receptor is considered a primary sensor of ATP and ADP *in vivo*, is present on neurons of peripheral sensory ganglia, and its presence in SGCs is induced in pain states [97]. A neuronal $P2Y_{12}R$ is also involved in excitability. 3 days of successive administration of uncharged $P2Y_{12}R$ antagonist MRS2395 **8** reversed glial cell activation in rat SGCs of the TG and alleviated mechanical and heat sensitivity of the tongue [125]. Also, PSB-0739 **5**, a competitive, polyanionic antagonist of $P2Y_{12}R$ has proven useful in studies of pain [72]. The microglial cells are the resident macrophage-like immune cells in the brain and SC, accounting for roughly 10-15% of the total cell numbers. Naturally, purine receptors have prominent roles in the function of microglial cells. In the SC, the microglial cells tend to amplify the pain signals by producing proinflammatory cytokines and other nociceptive modulators. Potential mechanisms have been described for purine receptors on activated microglial cells to induce hyperexcitability in the spinal dorsal horn, to increase neuropathic pain [146,180].

$P2Y_{12}R$ antagonists have been shown to be efficacious in reducing chronic and acute pain in the CFA-induced inflammatory pain model, in some models of neuropathic pain induced by partial sciatic nerve ligation (PSNL) [72], SNI [103] and spinal nerve transection (SNT) [64], in CIBP [118] and in some orofacial pain models (tongue cancer pain [167] and neuropathic tongue pain [97,163]). This likely involves action at multiple cell types: neurons, microglia and satellite cells of the dorsal and TG [163,177,178,188]. $P2Y_{12}R$ serves as a find-me signal for microglial cells, i.e. it induces motility [70]. Because $P2Y_{12}R$ antagonists are already in widespread use in the clinic, there are opportunities for testing purine-based hypotheses in humans that are not yet feasible for most other purine receptors. However, a liability of $P2Y_{12}R$ antagonists would be the potential for increased bleeding.

Currently there is no basis to separate the two-mechanism based actions of $P2Y_{12}R$ antagonists, i.e. analgesic and antithrombotic effects. Numerous $P2Y_{12}R$ antagonists and prodrug inhibitors have been developed as antithrombotics. Among the clinically accepted antagonists of the $P2Y_{12}R$, cangrelor (ARC-69931MX **6**) is a competitive $P2Y_{12}R$ antagonist of sub-nM affinity. However, as a nucleotide analogue it is not orally bioavailable, but it is active upon injection *in vivo*. Ticagrelor **7** is also a competitive $P2Y_{12}R$ antagonist, but it additionally inhibits ENT1, thus raising the levels of endogenous adenosine. Other $P2Y_{12}R$ antagonists, e.g. thienopyridines clopidogrel and prasugrel (structures not shown), used widely in the clinic act non-competitively by binding to the receptor after preactivation in the body; thus, they are prodrugs. MRS2395 **8** is a weak, uncharged $P2Y_{12}R$ antagonist that has been used in pain studies [97,118,167].

### $P2Y_6R$ antagonist antinociceptive effects:

Other microglial P2YRs that have a role in pain are $P2Y_4R$, $P2Y_6R$ and $P2Y_{13}R$ [123]. $P2Y_6R$ serves to induce phagocytosis in microglial cells [105]. SGCs express $P2Y_{14}R$, which is associated with inflammatory cytokine release [114]. There are few known antagonists of the $P2Y_6R$, but agonist structure-activity relationships (SAR) has been extensively explored. MRS2578 **3** is a potent, but hydrophobic and likely noncompetitive $P2Y_6R$ antagonist, and the more recently reported TIM-38 **4** is a weak antagonist [78]. $P2Y_6R$ activation suppresses the P2X4R-induced current amplitude, activation and channel permeability in microglia, suggesting that a $P2Y_6R$ agonist might provide therapeutic benefit [16]. However, a recent paper reports the opposite effect based on a protective effect elicited by $P2Y_6$ antagonist MRS2578 but not $P2Y_6$ agonist UDP in a model of neuropathic pain. In particular, intraperitoneal administration of MRS2578 alleviated CCI-induced hyperalgesia. Conversely, treatment of UDP on CCI rats increased pain intensity [18].

### $P2Y_{14}R$ antagonist antinociceptive effects:

$P2Y_{14}R$ is widely expressed throughout the body [34], and it is found in multiple parts of the nervous system [133]. Its mRNA is expressed in various immune cells as well as glia and neuronal cells [34,133], therefore $P2Y_{14}R$ is speculated to play a role in neuroimmune responses. Evidence supporting this was observed after acute challenge of rats with lipopolysaccharide (LPS) where an increase in $P2Y_{14}R$ mRNA expression across various regions of the brain occurred [36,133]. In addition, $P2Y_{14}R$ seem to be involved in chronic pain. One study observed increase in $P2Y_{14}R$ mRNA levels in the dorsal horn of SC from days 3 to 14 after a spared nerve injury [103]. Moreover, the *in-situ* hybridization of dorsal horn sections showed that expression of $P2Y_{14}R$ mRNA was elevated selectively on the ipsilateral side and that it co-localized with Iba1, which indicates that the upregulation happened in microglia [103]. The same study found that the administration of antisense-locked nucleic acid (AS-LNA) to inhibit $P2Y_{14}R$ for 7 days reversed SNI-induced mechanical allodynia on days 5 and 7 [103]. The $P2Y_{14}R$ is expressed on both neurons and SGCs in the rat TG [114]. Its upregulation in TG was observed in an inflammatory pain model (CFA-induced pain) and that was associated with increased release of anti-inflammatory cytokines and the activation of MAP kinases [115]. In addition, using PPTN, which is a selective $P2Y_{14}R$ antagonist, attenuated mechanical hyperalgesia in this inflammatory pain model [115].

Functional modal shifts of purine receptors occur upon microglial activation: P2X4R and $A_{2A}$AR are upregulated, and $P2Y_{12}$R, $A_1$AR and $A_3$AR are downregulated [104]. Thus, when considering the application of purinergic modulation to chronic neuropathic pain it is important to consider the relationships in the pathological state, as distinct from the normal state of a tissue.

## AR ligands and their use in relieving chronic pain

The ARs are activated by endogenous adenosine, the ubiquitous agonist produced by all cells. AR activity can also be modulated by exogenous agonists and antagonists or by inhibition of proteins that control adenosine transport, formation and degradation [5,33]. AR agonists and antagonists can be competitive with adenosine by binding to the same common site conserved among the four ARs (the orthosteric binding site), or they can interact non-competitively by binding to a separate, allosteric site (Figure 3). Each of the four ARs has numerous selective orthosteric agonists and antagonists, and their ligand binding SAR have been reviewed elsewhere [89]. Extensive molecular modeling of the ARs has also been performed based on X-ray structures of the $A_{2A}$AR and more recently of the $A_1$AR [61]. This modeling is informative in interpreting the SAR at any given subtype and guiding the discovery of new AR ligands [89]. Allosteric enhancers, i.e. positive allosteric modulators (PAMs), for the $A_1$AR and the $A_3$AR have been well characterized pharmacologically [82].

### $A_1$AR antinociceptive effects:

A number of clinical trials of potential analgesic agents acting as agonists at the Gi/o-coupled $A_1$AR (e.g. GW493838 **24**) and the Gs-coupled $A_{2A}$AR (e.g. BVT.115959 **27** and sonedenoson **28**) have been performed in recent years (Table 2, Figure 3) [208]. However, all of the trials were discontinued due to lack of efficacy or the presence of side effects. The reason for the lack of efficacy is unclear, because the same compound tested clinically, i.e. $A_1$AR PAM T-62 **40**, produced significant benefit in extensive preclinical tests of efficacy in pain and related conditions. The side effects, in general, may or may not be AR mechanism-related. Therefore, it may not be appropriate to rule out testing any new potential analgesic agents acting at the $A_1$AR or the $A_{2A}$AR, simply based on the unsuccessful trials so far. However, it must be noted that $A_1$AR or $A_{2A}$AR agonists would likely display cardiovascular side effects that would limit the dose range and utility of these agents in the general population. Only two AR agonists have been approved for clinical use, but not for pain: adenosine itself and regadenoson **31**, but both lack oral bioavailability. Several non-selective AR antagonists are used clinically, but the only selective AR antagonist currently approved for human use is istradefylline **51** [21]. Nonselective AR antagonist caffeine **47** is sometimes present in over-the-counter pain medications.

$A_1$AR KO mice ($A_1AR^{-/-}$) were used to evaluate the role of the $A_1$AR in nociception. Under normal conditions, as well as during inflammatory or neuropathic pain, $A_1AR^{-/-}$ animals showed hypersensitivity to heat in comparison to the wild-type (WT) mice. No significant differences were found in terms of mechanical withdrawal threshold and cold response. $A_1AR^{-/-}$ mice also showed reduced antinociceptive effect of morphine given intrathecally, but not systemically [193]. The basis for the analgesic actions of $A_1$AR

activation are multifold, as reviewed by Sawynok [151]. $A_1$AR is highly expressed in the nervous system, including on the central terminals of primary afferent neurons of the SC and cell bodies in the dorsal horn. $A_1$AR activation produces presynaptic inhibition of primary afferent neurotransmission onto dorsal horn neurons by decreasing release of glutamate, substance P and other transmitters from primary afferents. $A_1$AR also hyperpolarizes dorsal horn neurons by increasing $K^+$ conductance and potentiates the inhibitory postsynaptic transmission mediated by glycine receptors [9]. $A_1$AR activation on nociceptive neurons by adenosine generated from locally-released ATP has also been suggested as a mechanism of action of acupuncture [62]. Consistently, it is reported that caffeine, a non-selective AR antagonist, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX **49**), a selective $A_1$AR antagonist, reduce the antinociceptive effect of acupuncture [56,201]. Central or peripheral administration of $A_1$AR agonist CPA **21** in the mouse improved mechanical allodynia resulting from diabetic neuropathy [99]. Data on $A_1$AR antagonists or $A_1$AR KO mice in diabetic neuropathy models has not been reported. CPA showed the ability to attenuate the mechanical hyperalgesia induced by $PGE_2$, but when injected repeatedly it causes tolerance, dependence and changes in nociceptor function producing mechanical hyperalgesia similar to the effect of μ-opioid receptor agonists [6]. Downregulation of $A_1$AR contributes to neuropathic pain in a mouse model of neuropathy induced by resiniferatoxin, a capsaicin analogue that acts on TRPV1 [96]. Luongo et al. [121] demonstrated the analgesic properties of an $A_1$AR agonist in a mouse model of neuropathic pain (SNI) that were antagonized by the $A_1$AR-selective antagonist DPCPX. The agonist used was Cl-(±)-ENBA **3**, which is particularly potent and selective (>3000-fold selective in binding to the mouse $A_1$AR) [30]. However, Cl-(±)-ENBA is a mixture of two diastereoisomers, each of which can have distinct pharmacokinetic and pharmacodynamic properties. An $A_1$AR agonist of similar affinity, selectivity and *in vivo* activity is MRS7469 **26**, which is a pure isomer [176]. A caveat in the use of $A_1$AR agonists *in vivo* is that even compounds that have only 100s of fold selectivity in binding to the $A_1$AR compared to $A_{2A}$AR and $A_3$AR can display nonselective activity at the latter subtypes. For example, Reitman and coworkers found that $A_1$AR agonist CPA **1** activates mast cell $A_3$AR to produce hypothermia in mice at the same modest doses (e.g. 0.3 mg/kg, intraperitoneal (i.p.)) at which a central $A_1$AR activation was seen [30]. Thus, it is essential to use antagonists to probe the receptor subtype selectivity of an assumed selective agonist in a given model. The use of AR subtype KO mice also can strengthen the conclusion that a particular AR subtype is involved.

Paeoniflorin **45**, a natural phytochemical and a component of Chinese traditional medicine, protected against mechanical and thermal pain in the PSNL mouse model at high doses. This protection was blocked by moderately selective $A_1$AR antagonist CPT **48** and in $A_1$AR KO mice [199]. However, the mechanism of $A_1$AR activation by paeoniflorin was not established. Neuropeptide S injected intracerebroventricularly significantly reduced formalin-induced nociception during both phases of the formalin test by activating both $A_1$AR and $A_{2A}$AR during phase 1, but only the $A_{2A}$AR during phase 2 [69]. UNC32A **25** is an $A_1$AR agonist structurally related to adenosine 5′-monophosphate (5′-AMP), a nucleotide able to activate $A_1$AR. UNC32A did not show cardiovascular side effects and caused dose-dependent antinociceptive effects in WT mice when administered orally [106].

Allosteric modulation of the ARs is one means of potentially avoiding their cardiovascular side effects [77,184]. $A_1$AR PAM TRR469 **42**, an aminothiophene, was anti-allodynic in a chronic neuropathic pain model without locomotor or cataleptic side effects. It attenuated nociceptive responses in the formalin and writhing tests, similarly to morphine's effects. Coadministration of TRR469 and an $A_1$AR agonist CCPA **2** produced additive protection in a pain model. The effects of coadministered CCPA and TRR469, or each agent alone were antagonized by PSB36 **50**, a highly $A_1$AR-selective antagonist. The anti-nociceptive effect of TRR469 was additive with those of morphine. VCP171 **41**, an $A_1$AR PAM of the same chemotype as **40** and **42**, reduced the evoked excitatory postsynaptic current (eEPSC) amplitude in the SC dorsal horn to a larger degree in the nerve injured state than in control rats, suggesting that an elevated adenosinergic tone in that tissue would enable the use of $A_1$AR PAMs in pain therapy [77].

The role of the $A_{2A}$AR in pain is somewhat controversial in that pro- and anti-nociceptive have been documented.

### $A_{2A}$AR antinociceptive effects:

$A_{2A}$AR activation as well as $A_1$AR activation can reduce the release of excitatory neurotransmitters such as glutamate [63]. Watkins and colleagues showed that selective $A_{2A}$AR agonist ATL-313 **30** infused in the spinal column decreased chronic pain in a manner that was long lasting, and repeated administrations in four-week intervals remained efficacious [120]. They also demonstrated that a single intrathecal injection of $A_{2A}$R agonists CGS21680 **29** or ATL313 administered between 1 and 7 weeks after SC injury reversed spinal neuropathic avulsion pain (SNAP) for up to 6 weeks [108]. A single i.p. injection of CGS21680 was also able to significantly decrease the formalin-induced licking behavior in both the early (0-15 min) and late (15-60 min) phase in a mouse model of formalin-induced inflammatory pain [136]. Furthermore, a single peripheral injection of the potent but nonselective $A_{2A}$-agonists, 5'-(*N*-ethyl)-carboxamido-adenosine and 2-phenylaminoadenosine induced a decrease in mechanical nociceptive threshold in the hindpaw of the rat that was antagonized by an $A_{2A}$AR antagonist, PD 081360-0002 (HTQZ, **52**) [166]. Intracerebroventricular injection of adonis, an agonist-like monoclonal antibody with high specificity for the $A_{2A}$AR, led to a significant dose-dependent increase in hot-plate and tail-flick latencies in mice. This effect was prevented by caffeine and ZM241385 **53**, a specific $A_{2A}$AR antagonist [29]. In another study, LASSBio-1359 **32**, an $A_{2A}$ AR agonist of atypical structure, inhibits the hyperalgesic response caused by acute and chronic inflammation induced by formalin, carrageenan or CFA injection [131].

### $A_{2A}$AR pronociceptive effects:

$A_{2A}$AR activation might enhance the release and stimulatory effects of excitatory neurotransmitters such as glutamate [153]. It has been demonstrated that KO mice lacking the $A_{2A}$AR are less sensitive to nociceptive stimuli; this is due to a large reduction in the density of NMDA glutamate receptors [74] as well as a reduced neuronal activity in the SC [75]. In $A_{2A}$AR KO mice, carrageenan-induced hyperalgesia was significantly reduced compared to WT controls; furthermore, a selective $A_{2A}$AR antagonist ZM241385, when injected into the hindpaw, reduced the mechanical hyperalgesia in female mice, but not in

males [113]. $A_{2A}$AR KO mice also showed a significant decrease of mechanical allodynia in a model of neuropathic pain induced by sciatic nerve injury when compared to the WT group [24].

In acetic acid-induced writhing and tail immersion tests in the mouse, a series of novel $A_{2A}$AR-selective antagonists administered i.p. decreased acute pain [183]. These antagonists were described as promising for the treatment of chronic pain, as well. In another study, the $A_{2A}$AR agonist CGS21680 induced mechanical sensitization of esophageal C fibers that was abolished by pretreatment with selective $A_{2A}$ antagonist SCH58261 **54** [23]. Moreover, thermal and chemical stimuli, induced respectively by hot plate and acetic acid showed that an i.p. injection of the agonist CGS21680 produces pro-nociceptive effects [13]. Therefore, although most studies showed analgesic effects, it seems that $A_{2A}$AR activation might in some cases intensify response to nociceptive stimuli. The role of $A_{2A}$AR in pain transmission needs to be explored further.

A few studies have examined the involvement of the $A_{2B}$AR in pain; however it is known that activation of the $A_{2B}$AR has both pro-inflammatory and anti-inflammatory effects [164]. The lack of studies in pain is partly a result of the fact that selective agonists for this subtype are not as plentiful or well-characterized as agonists of the other ARs. Nevertheless, a study of ADA KO mice showed that prolonged increase in plasma adenosine activates $A_{2B}$AR on myeloid cells that results in increased sensitivity and chronic pain [73]. Furthermore, the $A_{2B}$AR-selective adenosine receptor antagonists PSB-1115 **56**, PSB-50, PSB-53, PSB-55 and enprofylline (structures not shown) displayed a dose-dependent analgesic effect in the hot-plate test in mice, an acute animal pain model [4,73]. The selective $A_{2B}$AR antagonist PSB-603 **57** has also proven useful as a pharmacological probe of this AR subtype. Although limited, such data suggests a pronociceptive role of this receptor.

### $A_3$AR antinociceptive effects:

Until recently, the dogma was that the pain relieving effects of adenosine were mediated predominantly by actions at the $A_1$AR and perhaps at the $A_{2A}$AR [208]. These conclusions were made without examining the contributions of the Gi-coupled $A_3$AR [85]. A focus on the $A_1$AR and $A_{2A}$AR failed to harness the potent analgesic effects of adenosine [20,208]. Thus, despite demonstrated preclinical efficacy in several pain models, agonists of $A_1$AR and $A_{2A}$AR have not been the focus of clinical trials due to significant cardiovascular side effects [208]. $A_3$AR mRNA is predominantly expressed in human and rodent testis, lung, kidney, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon, eye, DRG and SC (Table 1) [22,146]. Early in the study of the $A_3$AR, soon after its cloning in 1992-3 [150,206], it was thought that the $A_3$AR was absent in the brain [148]. However, subsequent studies established its neuronal expression in the thalamus, hypothalamus, cortex, cerebellum and other brain regions in rodents [195]. Based on exonal RNA sequencing, in human cadaver-derived tissue, $A_3$AR mRNA is highly expressed in testes, SC, substantia nigra, adrenal gland, spleen, small intestine, amygdala, hypothalamus, tibial nerve, tibial nerve, hippocampus, bladder, lung, adipose tissue, whole blood, transverse colon and coronary arteries [79]. When compared to $A_1$R and $A_{2A}$AR, $A_3$AR is expressed at

much lower levels in the CNS [21]. However, $A_3$AR has higher expression on many immune cell types, including glial cells [3,140,144]. In rodents, it is expressed in astrocytes, neurons, oligodendrocyte precursor cells, newly formed oligodendrocyte, myelinating oligodendrocyte, microglia/macrophage and endothelial cells [204]. In humans, it was found in fetal and mature astrocytes, oligodendrocyte, microglia/macrophage and endothelial cells [205].

$A_3$AR can be found on both peripheral [149] and central neurons [60,81,119,202] of the brain and SC [22,66]. In pain processing centers in rodents, $A_3$AR transcript and protein have been identified in the lumbar SC and rostral ventromedial medulla (RVM) [116]. The $A_3$AR is significantly upregulated in settings of inflammation and cancer in numerous cell types and both in rodents and humans [22,53,79,140]; noteworthy activation of phosphoinositide 3-kinases (PI3K), cAMP response element-binding protein (CREB) and nuclear factor-κB (NFκB) plays critical roles in $A_3$AR transcription [41]. The intracellular signaling pathways associated with the $A_3$AR include the inhibition of adenylate cyclase through the $G_i$ protein and also activation or deactivation of a wide range of kinases, such as MAPK, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38), jun N-terminal kinases (JNK), PI3K, protein kinase B (AKT), and glycogen synthase kinase 3β (GSK3β) [79]. These kinases and the transcription factors that they modulate are relevant to cancer, cell survival and proliferation, as well as pain [90,126,143,155,194,203]. In general, $A_3$AR agonists tend to restore a balance in signaling that is subject to pathological deviation in disease states, such as cancer and autoimmune inflammatory diseases [79]. This generality applies to chronic pain states, as well [86].

The development of highly selective $A_3$AR agonists such as MRS5698 **35** and MRS5980 **37** [173,174] provide exquisite tools to now probe the mechanistic roles of the $A_3$AR axis in pain; their selectivity for $A_3$AR is >1000-fold when compared to $A_1$AR and $A_{2A}$AR [173,174]. The high affinity and selectivity of MRS5698 was observed for several species, with Ki values of 3 nM at both human and mouse $A_3$ARs [175]. MRS5841 **16**, another member of the 2-arylethynyl series [141], was selective at both human and mouse $A_3$ARs and was excluded from diffusing across biological membranes. Thus, MRS5841 was a useful tool in separating effects of $A_3$AR activation in the peripheral and the CNS because of its permanently charged sulfonate group. Another means of distinguishing classes of GPCR agonists is according to their preferred signaling pathway, which is potentially a means of reducing side effects. Several studies have addressed the question of biased $A_3$AR agonists. Although a high degree of bias has not yet been found, some agonists tend to favor the Gi-dependent pathway over the β-arrestin pathway [58,162]. These ligand tools, some of which are now commercially available, are anticipated to further our understanding of the role of the $A_3$AR axis in pain states.

Between 2004 and 2006, the early literature examining the role of $A_3$AR in pain was confounded by results from three contradictory papers gathered from $A_3$AR-targeted compounds with poor specificity, e.g. $N^6$-benzyl–NECA (structure not shown) or from a single study performed in $A_3AR^{-/-}$ mice [85]. In 2007, we revisited the $A_3$AR hypothesis and demonstrated in a series of studies using pharmacological tools and genetic $A_3$AR KO mice ($A_3AR^{-/-}$) that activation of $A_3$AR exerts potent antinociceptive effects in models of

traumatic nerve-injury induced neuropathic and chemotherapy-induced neuropathic pain and bone cancer pain [39,54,116,160,175,187] validating the observations in models of non-neuropathic pain states [200]. Pharmacological probes used included moderately selective agonists IB-MECA **33**, Cl-IB-MECA **34** and highly selective $A_3$AR agonists such as MRS5698 and MRS5980, as well as an antagonist for the mouse $A_3$AR, MRS1523 **60**. It is to be emphasized that human $A_3$AR antagonist MRS1220 **59** is not selective when applied in rodent species. Noteworthy, $A_3$AR agonists do not interfere with anti-tumor effects of widely used chemotherapeutics but instead are of themselves antitumor agents [8,41,50,51,53,182]. Cl-IB-MECA is currently in phase II clinical trials for hepatocellular carcinoma as an anti-cancer agent [1,2,159]. Therefore, the use of $A_3$AR agonists may provide dual benefits in the treatment of a variety of cancer-related pain states.

In addition to traumatic-nerve injury and chemotherapy-induced neuropathic pain [39,54,116,160,171,175,187], $A_3$AR agonists are effective in formalin-induced inflammatory pain [142], breast cancer bone metastasis [182] and diabetic neuropathy [196].

The beneficial effects of $A_3$AR agonists are exerted in the periphery and in the central nervous system (SC and RVM); we have proposed that their high degree of potency may be the outcome of their potential synergy at multiple pain transmission sites [85], just like opioids [19]. We are currently examining this possibility. Moreover, $A_3$AR agonists exert their effects through multiple molecular mechanisms; we postulate that such polypharmacology may also account for their potency and efficacy. In neuropathic pain models where nerve damage is induced by CCI or chemotherapy treatment (oxaliplatin), $A_3$AR agonists exert their beneficial effects by suppressing spinal microglial and astrocytic activation, respectively [86,171,187]. In animal models of chemotherapy-induced neuropathic pain, $A_3$AR appear to be disease-modifying, i.e. achieving not only symptomatic relief, but also reversing some of the pathological processes in pain signaling pathways that contribute to vicious cycles [39,50,86,116,187]. $A_3$AR signaling inhibits neuropathic pain by attenuating the production of proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-1β, and increasing formation of the anti-inflammatory interleukin-10 (IL-10) and interleukin-4 (IL-4) in the SC [86,187]. The functional role of IL-10 in the beneficial effects exerted by $A_3$AR has been established [10,187]. $A_3$AR agonists can also inhibit activation of MAPKs (p38 kinase) and of NFκB in SC in response to nerve injury [84,100,171]. Noteworthy, $A_3$AR agonists have been reported to inhibit MAPKs, NFκB activation and release of inflammatory cytokines in models of inflammation (i.e. rheumatoid arthritis) and in cells and tissues harvested from patients with rheumatoid arthritis, psoriasis and Crohn's disease [41].

$A_3$AR agonists modulate redox-dependent events in the SC, reducing the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and avoid the posttranslational modification and inactivation of glutamate transporter glutamate transporter-1 (GLT-1) and glutamine synthetase (GS) which regulate synaptic glutamate homeostasis [84]. Also, inhibitory neurotransmission can be triggered by $A_3$AR agonists. In a model of CCI both IB-MECA and MRS5698 were able to reverse neuropathic pain via a spinal mechanism of action that modulates GABA activity directly or through KCC2 function [54]. An *in vivo* study conducted on sensory DRG neurons isolated from rats

revealed $A_3$AR expression in DRG neurons and showed that $A_3$AR agonists Cl-IB-MECA, and the highly selective MRS5980 are able to inhibit $Ca^{2+}$ currents evoked by a voltage-ramp protocol [42]. The pain-relieving effects observed following $A_3$AR activation in the rat could be mediated by DRG neurons, which underscores the potential of $A_3$AR agonists as effective therapies to relieve pain in different pathologies.

$A_3$AR agonists are also mitoprotective as they preserve the activity of the major mitochondrial enzyme, manganese superoxide dismutase, preventing the sustained formation of oxygen and nitrogen-derived free radicals (superoxide and peroxynitrite); here mitoprection in peripheral sensory afferents may contribute to their ability to attenuate increased abnormal spontaneous discharge observed in models of chemotherapy-induced neuropathic pain [15,83,84]. Some of the mechanisms of action depicted so far for $A_3$AR are captured in Figure 5. The development of highly selective $A_3$AR agonists such as MRS5698 and MRS5980 [173,174] provide exquisite tools to now probe the mechanistic roles of the $A_3$AR axis in pain; their selectivity for $A_3$AR is >1000-fold when compared to $A_1$AR and $A_{2A}$AR [173,174]. These tools which are now commercially available are anticipated to further our understanding of the role of the $A_3$AR axis in pain states.

The antinociceptive effects of $A_3$AR agonists are not dependent upon endogenous opioid or endocannabinoid pathways [116,205]. Moreover, $A_3$AR agonists produce conditioned place preference (CPP) only in nerve injured rodents, with no effects in sham animals, suggesting that $A_3$AR agonists provide relief of spontaneous pain [116]. Therefore, $A_3$AR agonists have the potential to selectively modify pathological but not protective pain, while avoiding the tolerance and abuse potential associated with opioid therapy [85]. To this end, antinociceptive effects of $A_3$AR agonists persists even with prolonged treatment (at least up to 2 weeks of treatment) [116,160,187] suggesting that tolerance does not develop to $A_3$AR agonism. Similar findings have been observed in animal models of autoimmune disorders and cancer, where chronic administration of $A_3$AR agonists maintains anti-inflammatory/anti-cancer effects even during $A_3$AR down-regulation [122]. Fishman postulated that the functionality of $A_3$AR agonists in inflammation/tumor-growth may be dependent on the down-regulation of $A_3$AR to inhibit downstream regulatory proteins [52].

In addition to their potential use as stand-alone analgesics, $A_3$AR agonists may be able to be given in conjunction with currently used drugs as these can increase the analgesic potency of morphine, gabapentin, and amitriptyline [39]. Recently, Kim and co-workers demonstrated that the anti-allodynic effects of amitriptyline and its inhibitory effects on the activation of ERK and CREB and inflammatory cytokines in the SC in a rat model of spinal nerve ligation induced neuropathic pain was abrogated by an $A_3$AR antagonist MRS1191 **58** [100]. This suggests that part of the action mediated by amitriptyline is mediated via activation of the $A_3$AR [100]. The link between amitriptyline and the adenosine/$A_3$AR axis remains to be elucidated.

## Conclusions

Modulation of purinergic signaling can be used to reduce pain in various models, generally through AR activation or P2YR or P2XR inhibition. Among AR ligands, both $A_1$AR

agonists and an $A_1$AR PAM have been tested in the clinic without success so far, but optimism remains based on mechanistic considerations. The efficacy of $A_{2A}$AR ligands in pain depends on the route of administration: antagonists in the periphery and agonists in the spinal column. $A_3$AR agonists would be relatively free of serious cardiovascular side effects and are also efficacious in diverse rodent models of chronic pain. Benefit in pain models has been observed with various P2XR antagonists: P2X3R, P2X4R and P2X7R, although P2X4R is the more promising target because of its functional presence in microglial cells. There is conflicting data concerning the role of various P2YRs, but $P2Y_{1,}$ $P2Y_{12}$R and $P2Y_{14}$R antagonists appear to be consistently antinociceptive. Thus, the prospects are encouraging for the eventual use of purine receptor modulators as non-addictive treatment of chronic neuropathic pain. $A_3$AR agonists (IB-MECA and Cl-IB-MECA) are currently in clinical trials for chronic inflammatory diseases, liver diseases and cancer with a good safety profile [1,2,46,158,159]. Significant effects are across rodent models with pharmacological tool compounds, such as $A_3$AR agonists, which provide the impetus to discover new drugs acting through purinergic pathways. Highly selective $A_3$AR agonists are anticipated to provide an advantage by providing a wide therapeutic index for chronic administrationThe $A_3$AR has been identified as an exciting target for AR agonists for pain management, and highly selective $A_3$AR agonists are moving through preclinical and clinical development for the treatment of neuropathic pain states.

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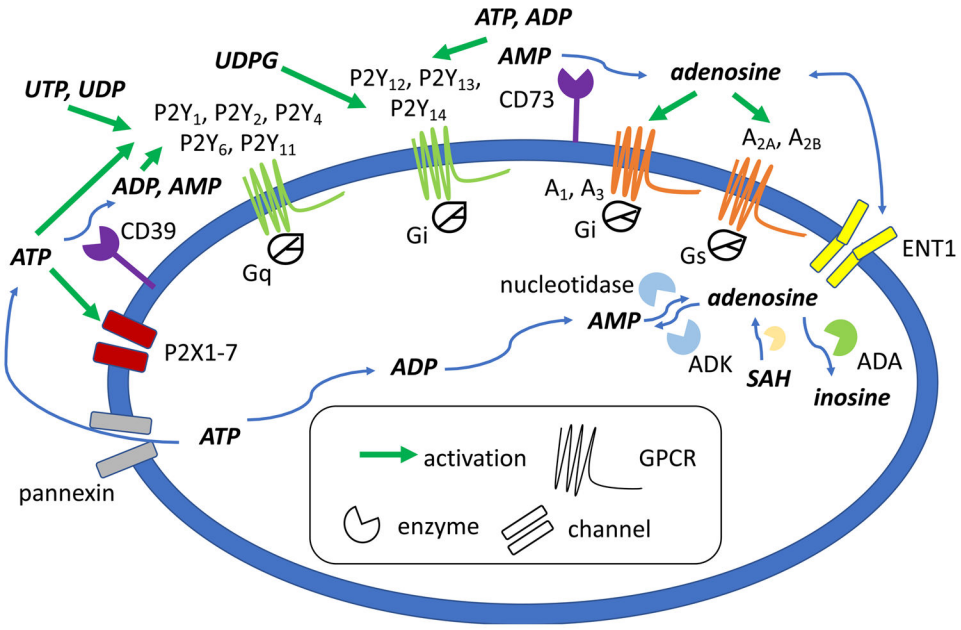


**Figure 1.**

Purinergic signaling pathways for purine nucleosides and nucleotides, and pyrimidine nucleotides. Extracellular ATP and other nucleotides originate from intracellular sources through cell damage, cotransmission, pannexin hemichannels, and other mechanisms. These nucleotides act on P2Y (GPCRs, activated by triphosphates, diphosphates and UDP-sugars) and P2X (ion channels, mainly by ATP) receptors. Ectonucleotidases (CD39, CD73) are largely responsible for the formation, from ATP, of adenosine that activates its four receptors. In general, adenosine receptor agonists and P2X/P2Y receptor antagonists induce pain relief in various models.

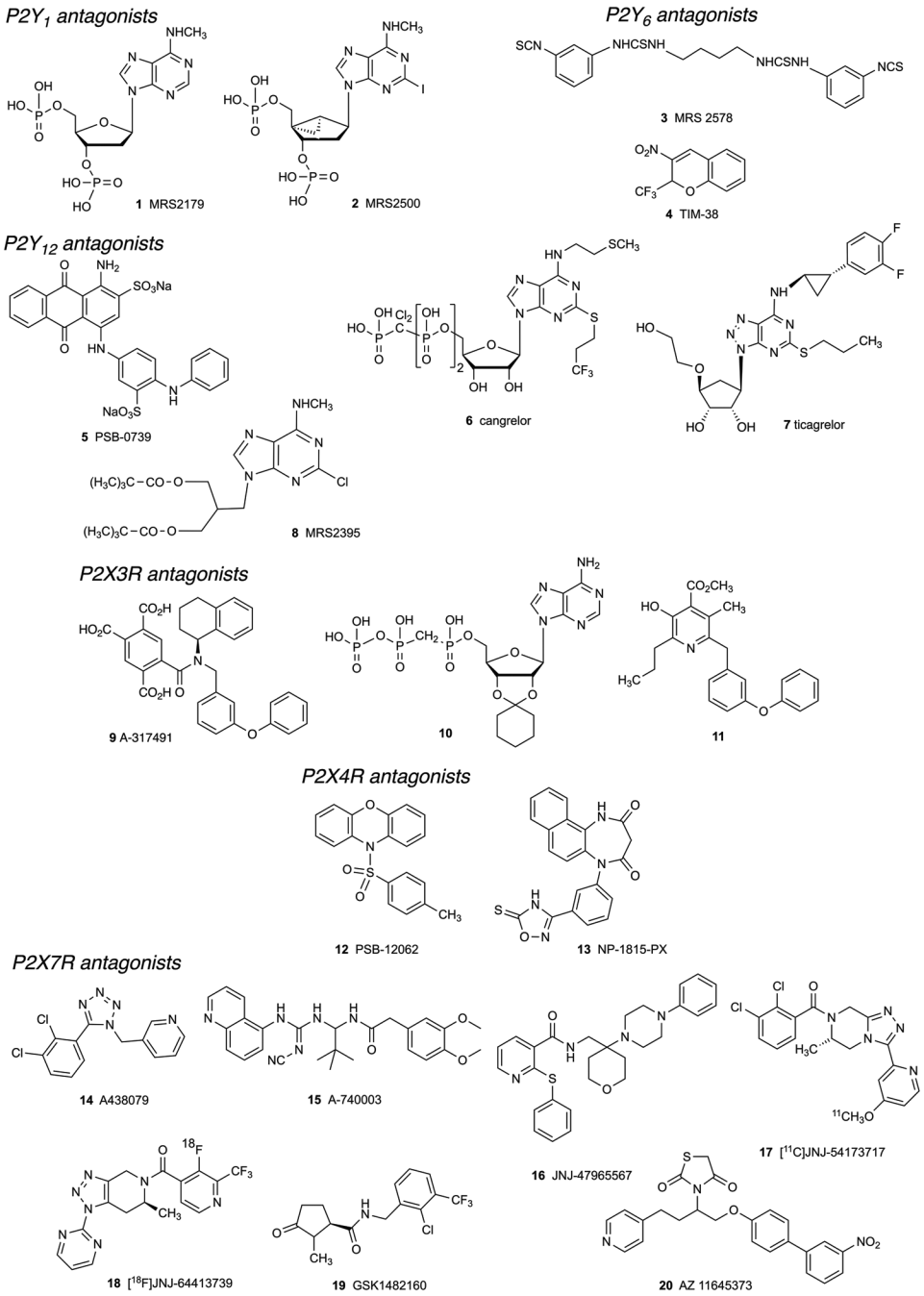


**Figure 2.**

Structures of representative P2YR and P2XR antagonists that have been used in pain studies.

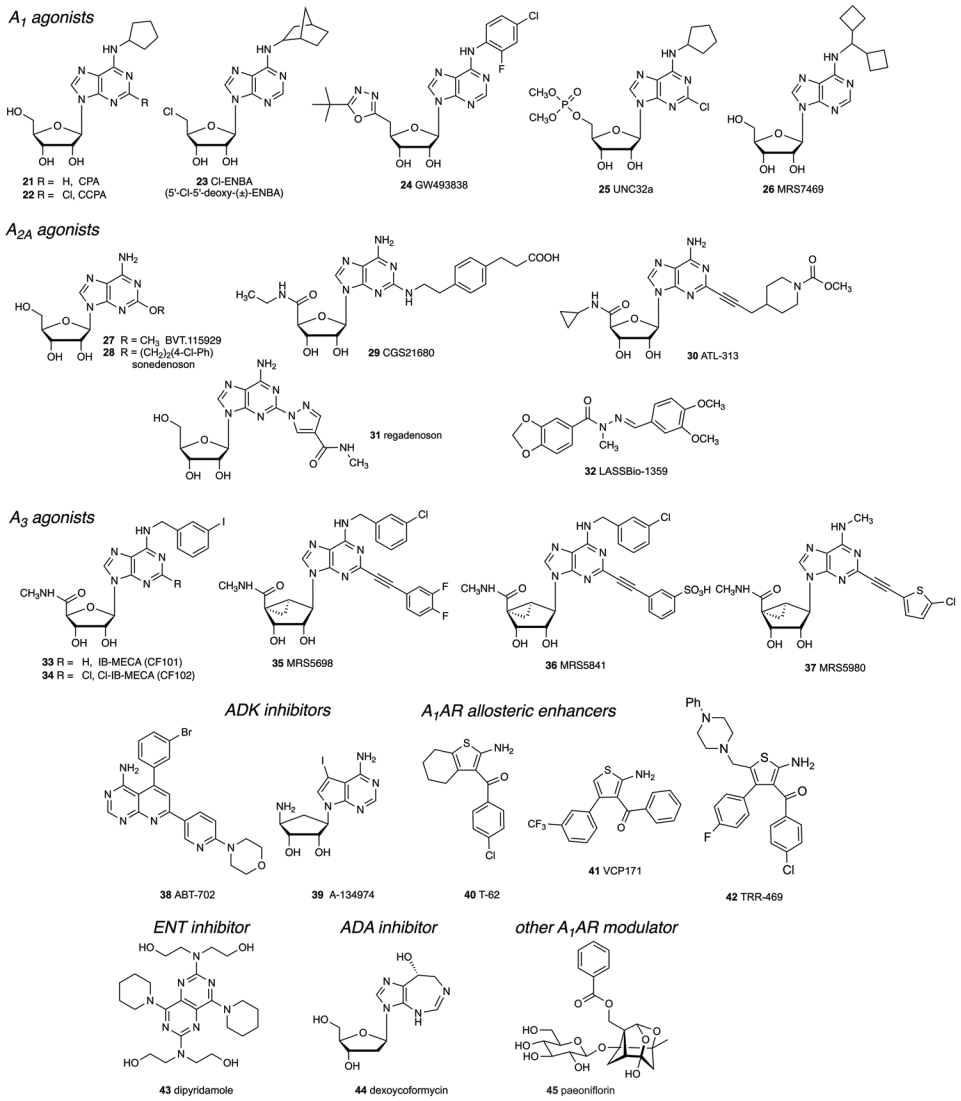


**Figure 3.**

Structures of representative AR agonists that have been used in pain studies.

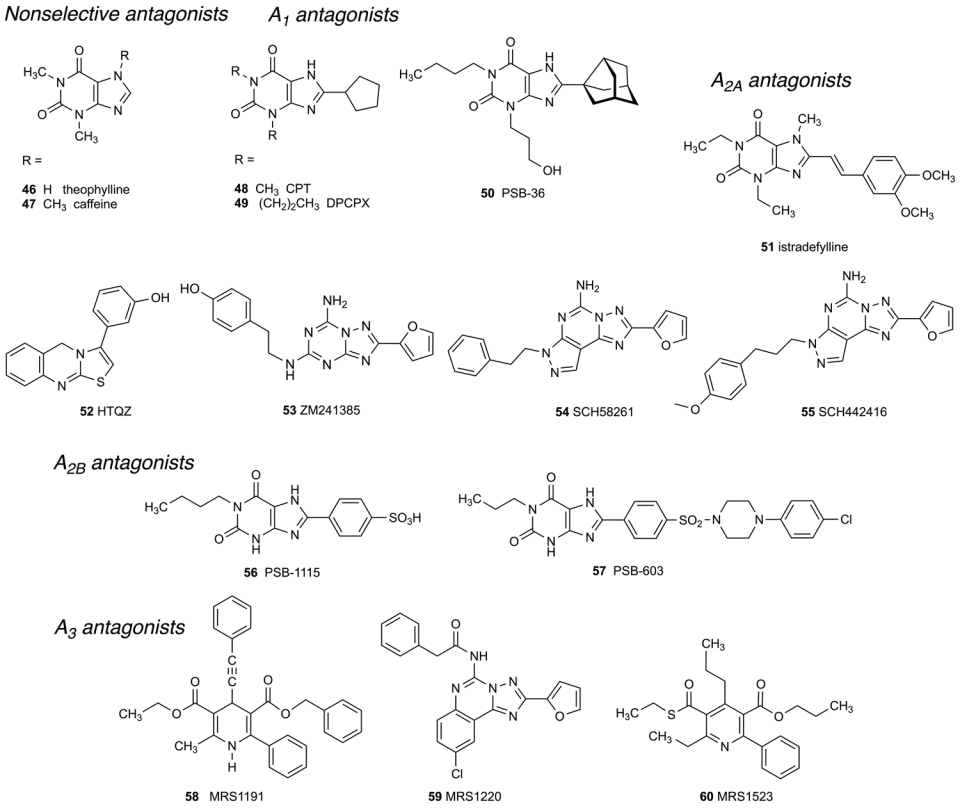


**Figure 4.**

Structures of representative AR antagonists that have been used in pain studies.

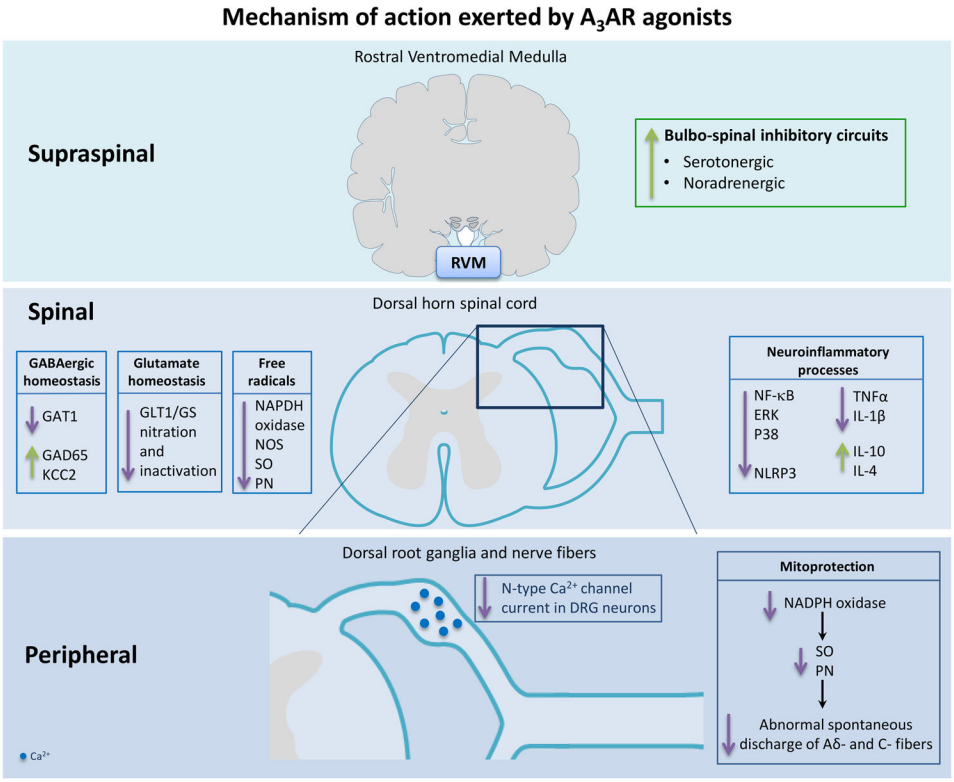


**Figure 5.**

Schematic representation of the molecular signaling pathways of $A_3$AR agonists. $Ca^{2+}$, calcium ions; ERK, extracellular signal-regulated kinase; GABA, gamma-aminobutyric Acid; GAD65, glutamic acid decarboxylase 65; GAT1, GABA transporter type 1; GLT-1, glutamate transporter 1; GS, glutamine synthetase; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-10, interleukin-10; KCC2, potassium-chloride cotransporter protein; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NF-kB, nuclear factor-κB; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; NOS, nitric oxide synthase; p38, p38 mitogen-activated protein kinase; PN, peroxynitrite; RVM, rostral ventromedial medulla; TNFα, tumor necrosis factor alpha; SO, superoxide.

**Table 1.**

Message for purinergic receptors and selected associated enzymes and a transporter in DRG and SC, expressed as transcripts per million (TPM) using RNA-seq data [146]. Other names for enzymes listed below are: ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1, CD39), NT5E (ecto-5'-nucleotidase, CD73), ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), SLC29A1 (equilibrative nucleoside transporter 1, ENT1).

| **A.** | | |
|---|---|---|
| **Mouse GENE** | **DRG** | **SC** |
| ADORA1 | 96.424 | 143.604 |
| ADORA2A | 6.218 | 1.342 |
| ADORA2B | 0.969 | 6.501 |
| ADORA3 | 0.01 | 1.411 |
| P2RY1 | 25.792 | 10.238 |
| P2RY2 | 29.923 | 0.521 |
| P2RY4 | 0.01 | 0.01 |
| P2RY6 | 2.968 | 4.65 |
| P2RY12 | 3.069 | 11.464 |
| P2RY13 | 0.343 | 4.303 |
| P2RY14 | 1.383 | 1.862 |
| P2RX1 | 0.091 | 0.01 |
| P2RX2 | 2.332 | 0.37 |
| P2RX3 | 120.479 | 1.932 |
| P2RX4 | 48.646 | 27.775 |
| P2RX5 | 26.276 | 13.083 |
| P2RX6 | 40.086 | 15.987 |
| P2RX7 | 23.197 | 5.529 |
| ENTPD1 | 9.469 | 96.8 |
| NT5E | 7.621 | 2.776 |
| ENPP1 | 6.814 | 8.653 |
| ADK | 422.075 | 531.732 |
| SLC29A1 | 93.294 | 57.227 |
| **B.** | | |
| **Human GENE** | **DRG** | **SC** |
| ADORA1 | 16.475 | 48.6 |
| ADORA2A | 2.237 | 8.659 |
| ADORA2B | 0.847 | 1.49 |
| ADORA3 | 65.881 | 33.548 |
| P2RY1 | 1.275 | 0.621 |
| P2RY2 | 1.484 | 0.838 |
| P2RY4 | 0.01 | 0.124 |

**A.**

| Mouse GENE | DRG | SC |
|---|---|---|
| P2RY6 | 3.889 | 1.924 |
| P2RY11 | 15.252 | 15.424 |
| P2RY12 | 23.365 | 10.179 |
| P2RY13 | 4.851 | 3.693 |
| P2RY14 | 6.429 | 2.638 |
| P2RX1 | 0.575 | 2.017 |
| P2RX2 | 0.115 | 0.093 |
| P2RX3 | 53.911 | 0.01 |
| P2RX4 | 21.389 | 24.89 |
| P2RX5 | 36.934 | 1.334 |
| P2RX6 | 22.026 | 11.886 |
| P2RX7 | 1.464 | 18.807 |
| ENTPD1 | 7.077 | 23.276 |
| NT5E | 6.377 | 5.71 |
| ENPP1 | 1.307 | 0.683 |
| ADK | 61.783 | 207.371 |
| SLC29A1 | 29.553 | 23.028 |

**Table 2.**

Representative recent clinical trials of AR agonists, an $A_1$AR PAM and P2R antagonists in pain conditions (data from clinicaltrials.gov, accessed 5-8-2019). The only ongoing clinical development is P2X4R antagonist NC-2600 (unpublished).

| Receptor | Condition | Compound (route) | Years | Phase, NCT# | References |
|---|---|---|---|---|---|
| $A_1$ | Neuropathic pain | adenosine **1** (i.t.) | 2014-2018 | **2**, 00349921 | [208] |
| | Perioperative pain | adenosine **1** (i.v.) | 2006 | **2**, 00298636 | [92] |
| | Neuropathic pain | GW493838 **5** | 2002-2003 | **2**, 00376454 | [48] |
| | Postherpetic neuralgia | T-62 **18** | 2008-2012 | **2**, 00809679 | [130] |
| $A_{2A}$ | Diabetic nerve pain | BVT.115929 **7a** | 2007-2014 | **2**, 00452777 | [101] |
| | Diabetic foot ulcers | Sonedenoson **7b** | 2006-2012 | **2**, 00318214 | [132] |
| $A_{2B}$ | none | - | - | | [164] |
| $A_3$ | none | - | - | | [85] |
| P2X4 | neuropathic pain | NC-2600 | 2016-2017 | **1** | www.chemiphar.co.jp |
| P2X7 | inflammatory pain | GSK1482160 **50** | 2009-2017 | **1**, 00849134 | [57] |