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REVIEW ARTICLE

Identification of A_3 adenosine receptor agonists as novel non-narcotic analgesics

Correspondence Daniela Salvemini, Department of Pharmacology and Physiology, Saint Louis University School of Medicine, 1402 South Grand Blvd, St. Louis, MO 63104, USA. E-mail: salvemd@slu.edu

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K Janes[1], AM Symons-Liguori[1], K A Jacobson[2] and D Salvemini[1]

[1]Department of Pharmacology and Physiology, Saint Louis University School of Medicine, St. Louis, MO, USA, and [2]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Chronic pain negatively impacts the quality of life in a variety of patient populations. The current therapeutic repertoire is inadequate in managing patient pain and warrants the development of new therapeutics. Adenosine and its four cognate receptors (A_1, A_{2A}, A_{2B} and A_3) have important roles in physiological and pathophysiological states, including chronic pain. Preclinical and clinical studies have revealed that while adenosine and agonists of the A_1 and A_{2A} receptors have antinociceptive properties, their therapeutic utility is limited by adverse cardiovascular side effects. In contrast, our understanding of the A_3 receptor is only in its infancy, but exciting preclinical observations of A_3 receptor antinociception, which have been bolstered by clinical trials of A_3 receptor agonists in other disease states, suggest pain relief without cardiovascular side effects and with sufficient tolerability. Our goal herein is to briefly discuss adenosine and its receptors in the context of pathological pain and to consider the current data regarding A_3 receptor-mediated antinociception. We will highlight recent findings regarding the impact of the A_3 receptor on pain pathways and examine the current state of selective A_3 receptor agonists used for these studies. The adenosine-to-A_3 receptor pathway represents an important endogenous system that can be targeted to provide safe, effective pain relief from chronic pain.

Abbreviations

ADA, adenosine deaminase; ADK, adenosine kinase; CCI, chronic constriction injury; CIPN, chemotherapy-induced peripheral neuropathy; CPP, conditioned place preference; ENT, equilibrative nucleoside transporter; PN, peroxynitrite; RVM, rostral ventromedial medulla; SO, superoxide; TLR4, toll-like receptor 4

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Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
A_1 receptor	ADA
A_{2A} receptor	Adenylyl cyclase (AC)
A_{2B} receptor	ADK
A_3 receptor	Ecto-5′-nucleotidase
Catalytic receptors[b]	ERK1
TLR4	ERK2
Transporters[d]	GAD65
CNTs	p38 MAPK
ENT1	PKA
ENT2	S-adenosylhomocysteine hydrolase
GAT1	TrkB
GLT1 (EAAT1)	
KCC2	

LIGANDS	
ABT-702	IL-1β
Adenosine	IL-10
ADP	Inosine
AMP	MRS1191
ATP	MRS1220
BDNF	MRS1523
cAMP	MRS5698
CCL2	Nitric oxide (NO)
GABA	NMDA
IB-MECA	TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d]Alexander *et al.*, 2015a,b,c,d).

Introduction

Chronic pain afflicts an estimated 10% of the world's adult population (Goldberg and McGee, 2011). The current therapeutic approaches for chronic pain include but are not limited to the use of nonsteroidal anti-inflammatory drugs (NSAIDs), antidepressants, anticonvulsants and opioid pain relievers; however, these strategies are frequently either inadequate or are associated with side effects that reduce quality of life or result in the discontinuation of therapy (Goldberg and McGee, 2011; Pizzo and Clark, 2012). The search for new therapeutic targets is therefore of great importance. Adenosine and two of its associated adenosine receptor subtypes, the A_1 and the A_{2A} receptor, have been investigated in the field of pain with varying degrees of success; however, these agents lack a useful therapeutic index due to cardiovascular side effects. In response, the focus of research has turned to the previously overlooked A_3 receptor, which displays both preclinical antinociceptive properties (Yoon *et al.*, 2004; Janes *et al.*, 2014b, 2015; Ford *et al.*, 2015; Little *et al.*, 2015) and, in trials for non-pain conditions including psoriasis, hepatitis, rheumatoid arthritis, and glaucoma, offers a therapeutic index and tolerability that would be suitable for the treatment of chronic pain (Fishman *et al.*, 2012). The aim of this review is to summarize the existing literature on adenosine and its receptors in the context of pain with a particular emphasis on the A_3 receptor and its prospect as a novel solution to the problem of chronic pain management.

Adenosine production and metabolism

Adenosine is an endogenous purine nucleoside that regulates a number of physiological processes and is an important neuromodulator in the CNS (Fredholm *et al.*, 2011). Concentrations of adenosine are generated by almost all cell types (Zimmermann, 2000), and accordingly, the physiological generation and neurobiology of adenosine has been thoroughly reviewed elsewhere (Hu *et al.*, 2014). Here, we will provide a brief, contextual overview of adenosine homeostasis in nervous tissues.

In the context of pain, the most notable function of adenosine in the CNS is its role as a neuromodulator for neurotransmitter systems including glutamate, GABA, ACh and dopamine. In these systems, adenosine limits the extent of neuroexcitability and also regulates neuroplasticity (Sebastiao and Ribeiro, 1996). However, CNS adenosine is not restricted to neuronal synapses nor is it a directional signalling molecule., Adenosine plays a regulatory role in glial activation state and function, such that adenosine can be said to impact the entire synaptic unit (Cunha, 2008; Dias *et al.*, 2013).

Concentrations of adenosine in CNS tissue are reported to persist at a basal level of 25–250 nM (Dunwiddie and Masino, 2001). Adenosine is generated as a metabolic intermediate in both the intracellular and extracellular spaces and passively exchanged along its concentration gradient via the ubiquitously expressed equilibrative nucleoside transporters (ENTs), ENT1 and ENT2 (Brundege and Dunwiddie, 1998; Peng *et al.*, 2005) or through concentrative nucleoside transporters (CNTs) along the concentration gradient of sodium (Bonan, 2012; Choi and Berdis, 2012). The extracellular *function* of adenosine is regulated by (i) changes in the ratio of intracellular: extracellular adenosine generation and consequently its driving gradient across passive transport systems and (ii) the local expression profile of adenosine receptors mediating its response (Deussen *et al.*, 1999; Zimmermann, 2000). Together, these two elements are modified in the pathological cellular environment in order to facilitate the action of adenosine as an anti-inflammatory, inhibitory neuromodulator.

Intracellular adenosine generation in the CNS is predominantly a resultant from the dephosphorylation of AMP by

soluble 5′-nucleotidases (Latini and Pedata, 2001). Unlike other physiological tissues, adenosine generated from S-adenosylhomocysteine hydrolysis does not significantly contribute to adenosine concentrations in the CNS. In the extracellular space, ATP is released as a co-transmitter or in response to cellular insult (e.g. inflammation, cellular stress or excitotoxicity) (Ballarin *et al.*, 1991; Engler, 1991; Latini and Pedata, 2001) and can be dephosphorylated by ectonucleoside triphosphate diphosphohydrolases (CD39 family) and either ecto-5′-nucleotidase (CD73) (Robson *et al.*, 2006; Bonan, 2012) or by tissue-nonspecific alkaline phosphatase (Sebastian-Serrano *et al.*, 2015) to form extracellular adenosine (Figure 1). Adenosine generation is therefore tightly coupled to the availability of ATP and ADP in the intracellular and extracellular spaces.

A second factor limiting the ratio of intracellular: extracellular adenosine is the metabolic inactivation of adenosine through adenosine kinase (ADK) phosphorylation (Spychala *et al.*, 1996) or, to a lesser extent, the activity of adenosine deaminase (ADA) (Blackburn and Kellems, 1996). The function of these enzymes limits the physiological half-life of adenosine to <1 s (Moser *et al.*, 1989). Inhibition of ADK is the most effective strategy for increasing the extracellular concentration of adenosine and occurs by potentiating intracellular concentrations of adenosine and supporting an outward driving gradient through passive ENTs (Keil and DeLander, 1992; Zhang *et al.*, 1993). Pharmacological blockade of ADK activity in the CNS results in adenosine-mediated inhibition of spinal nociceptive transmission via the ENT-dependent release of adenosine (Otsuguro *et al.*, 2015), and inhibitors of ADK are efficacious in rodent experimental neuropathic pain models (Kowaluk *et al.*, 2000; McGaraughty *et al.*, 2005). It is important to note that ADK expression shifts from neurons to astrocytes during postnatal development, and accordingly, astrocytes are central to this aspect of adenosine homeostasis (Studer *et al.*, 2006).

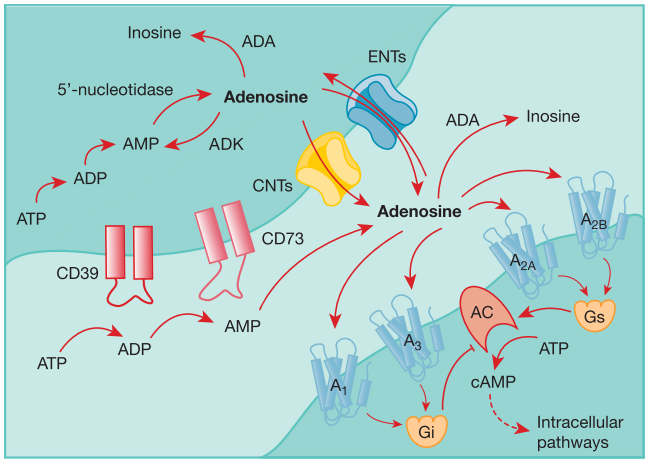

Figure 1

Adenosine synthesis and metabolism. ATP can be released from various cell types in response to cell excitation or insult. ATP can be deophosphorylated in sequence to form ADP, AMP and finally adenosine. In the extracellular space, ectonucleotidases (CD39 and CD73) facilitate formation of adenosine. Adenosine can act on its cognate receptors (A_1, A_{2A}, A_{2B} and A_3) or be removed from the extracellular space by metabolic enzymes (adenosine deaminase, ADA) or by transport back into the cell via equilibrative nucleoside transporters (ENTs) or concentrative nucleoside transporters (CNTs). In the intracellular space, adenosine can be converted to AMP (by adenosine kinase, ADK) which in turn is catalysed to ADP and then ATP. Intracellular adenosine can also be generated from AMP by 5′-nucleotidase.

Adenosine receptors

As mentioned previously, the abundance of adenosine receptor subtypes is the second critical factor mediating the effects of extracellular adenosine, and much remains to be understood regarding the dynamics of adenosine receptor expression during cellular insult and pathological nociception. The extracellular actions of adenosine are mediated by its four cognate GPCRs: the A_1, A_{2A}, A_{2B} and A_3 receptor.

In the CNS, the A_1 receptor is highly expressed both pre-synaptically and post-synaptically on neurons as well as on glia in the brain (cortex, cerebellum and hippocampus) and specifically in the superficial laminae of the spinal cord dorsal horn (Gessi *et al.*, 2011). It is known that glial expression of the A_1 receptor can be depressed in multiple sclerosis patients (Johnston *et al.*, 2001), and expression of the A_1 receptor in the lumbar dorsal horn is decreased in a model of post-operative pain. However, expression of the A_1 receptor is increased following sciatic nerve constriction (Yamaoka *et al.*, 2013). These data suggest that the A_1 receptor is responsive to neuroinflammatory and nociceptive pathology, and that A_1 receptor antinociception may involve differential expression of the receptor post-injury. However, in the context of off-target effects, the presence of the A_1 receptor in cardiovascular tissue—particularly the atrioventricular node—is responsible for high-grade atrioventricular block mediated by A_1 receptor agonists (Kiesman *et al.*, 2009) and represents an unfortunate hurdle to the therapeutic exploitation of the A_1 receptor

Expression of the A_{2A} receptor in the brain is most notable in the striatum on post-synaptic neurons, to a lesser extent pre-synaptically in areas of the hippocampus and cerebral cortex and on glia (Svenningsson *et al.*, 1997; Rebola *et al.*, 2005). A_{2A} receptor expression is known to increase in response to insults such as hypoxia, spinal cord injury, streptozotocin-induced diabetes and in other circumstances such as chronic behavioural stress and ageing (Janes *et al.*, 2014b). Of relevance to chronic pain, pro-inflammatory mediators including IL-1β and TNF-α are also known to enhance expression of the A_{2A} receptor in monocytes (Morello *et al.*, 2006) such as microglia. Lastly, vasodilator A_{2A} receptors expressed on the epithelium of coronary blood vessels is of note with respect to the cardiovascular side effects of A_{2A}-specific agents (Jacobson and Gao, 2006; Gao and Jacobson, 2007; Fredholm *et al.*, 2011). The lower-affinity A_{2B} receptor is also expressed in the CNS—particularly on immune-related cells—and in the cardiovascular system, but because of the low-expression profile and micromolar affinity of adenosine for the A_{2B} receptor, we will not extensively discuss the A_{2B} receptor in this review (Fredholm *et al.*, 2000; Aherne *et al.*, 2011).

For many years, the A_3 receptor was largely overlooked in CNS tissue due to a reported low profile of expression; however, it is now known that the A_3 receptor can be found in high levels on many immune cell types, including glial cells (Abbracchio *et al.*, 1997; Poulsen and Quinn, 1998; Ochaion

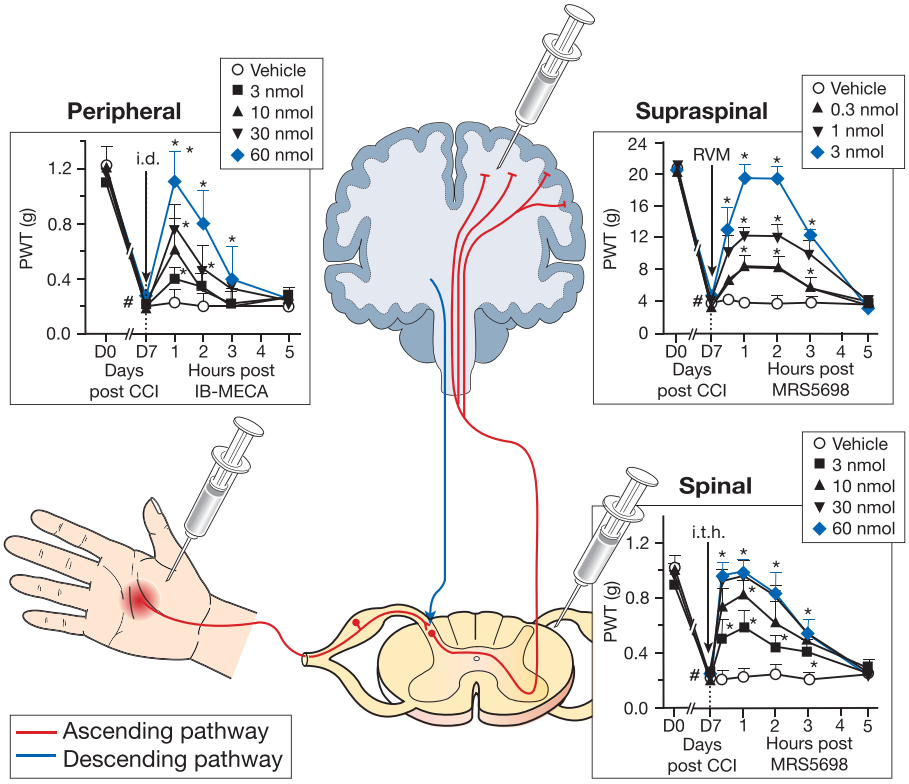

Figure 2

A_3 receptor's multiple sites of action. Studies employing selective A_3 receptor agonists have uncovered the efficacy of A_3 receptor activation at several sites important for pain processing. In the ascending pathway (red), a stimulus is conducted from the periphery to the spinal cord where it is then transported to the brain to be interpreted. The brain is able to modulate these events via the descending pathway (green). The i.d. administration of IB-MECA (3–60 nmol; Salvemini unpublished results) dose-dependently attenuated the reduction in paw withdrawal thresholds (PWTs) associated with chronic constriction injury (CCI). These results were extended by MRS5698 administration via surgically implanted catheters placed into the intrathecal (i.t.; 3–60 nmol) space or rostral ventromedial medulla (RVM; 0.3–3 nmol). Data are mean ± SD for $n = 5$ animals per group and analysed by two-way ANOVA with Bonferroni comparisons. #$P < 0.05$ vs. D0; *$P < 0.05$ vs. D7. The i.t. data are reprinted with permission from J Neuro (Ford *et al.*, 2015) . Intra-RVM data are reprinted with permission from Brain (Little *et al.*, 2015).

et al., 2009), as well as on both peripheral (Ru *et al.*, 2011) and central neurons (Jacobson *et al.*, 1993; Lopes *et al.*, 2003; Giannaccini *et al.*, 2008; Zhang *et al.*, 2010) in the brain and spinal cord (Borea *et al.*, 2015; Haeusler *et al.*, 2015). Both mRNA and protein for the A_3 receptor have been documented in the lumbar spinal cord and in supraspinal areas including the rostral ventromedial medulla (RVM): indeed, A_3 receptors at the level of the peripheral afferent, the spinal cord and the RVM are functionally relevant in pain as selective A_3 receptor agonists administered via i.d., intrathecal (i.t.) or intra-RVM routes dose-dependently attenuate neuropathic pain behaviours (Little *et al.*, 2015). It can therefore be concluded that the A_3 receptor is functionally expressed at multiple levels of pain processing (Figure 2).

The A_3 receptor is unique among the adenosine receptors in that species disparities exist for both the receptor structure and distribution; the highest expression of the A_3 receptor in rats exists in testis and mast cells, whereas the highest levels in human are found in the liver and lung (Borea *et al.*, 2015). Importantly, while no direct evidence has been found for the existence of the A_3 receptor in cardiomyocytes, several studies have demonstrated A_3 receptor-mediated cardioprotection from ischaemic injury (Tracey *et al.*, 1997; Thourani *et al.*, 1999a, 1999b; Cross *et al.*, 2002; Harrison *et al.*, 2002; Headrick and Peart, 2005) and doxorubicin-induced cardiotoxicity (Shneyvays *et al.*, 1998; Shneyvays *et al.*, 2001). Additionally, high A_3 receptor expression has been documented in the human coronary and carotid arteries (Hinze *et al.*, 2012; Grandoch *et al.*, 2013).

Given the distribution of adenosine receptors, it is also important to note the coupling mechanisms of these receptors. The A_{2A} and A_{2B} subtypes are G_s-coupled GPCRs which stimulate AC and produce elevations in intracellular cAMP and associated signalling cascades (Fredholm *et al.*, 2001). A_1 and A_3 receptors differ both in their coupling to the opposing G_i cascade which inhibits AC and in the ability to respond to inosine as a partial agonist (Boison *et al.*, 2010; Fredholm *et al.*, 2011). It is important to note that while these receptors have, at face value, opposing intracellular effects, the expression of these receptors on differing cell types can be responsible for commonalities in their activation in the CNS.

Adenosine and pain

Adenosine plays an integral role in CNS processing of pain through its regulation of excitatory neurotransmission,

persistent neuronal signalling and regulation of glial activation and proliferation. Accordingly, the ability of adenosine and its analogues to inhibit pain behaviour has been documented in models of various aetiologies, including neuropathic pain associated with spinal cord injury, spinal nerve ligation and in the mustard oil, formalin and carrageenan pain models (Dickenson *et al.*, 2000). Indeed, in the clinical setting, i.t. adenosine administration has been shown to provide sustained relief of chronic neuropathic pain lasting for several hours up to months in some patients (Hayashida *et al.*, 2005). Adenosine therapy has also been employed for the prevention of post-operative pain with mixed results: prophylactic i.v. administration of adenosine prior to surgical procedures conferred persistent pain relief in several studies (Hayashida *et al.*, 2005; Gan and Habib, 2007), while another similar clinical trial did not observe a prophylactic effect (Habib *et al.*, 2008). Unfortunately, the i.v. administration of adenosine is associated with undesirable cardiac side effects (Zylka, 2011) that limit its utility and route of administration (e.g. i.t.) in patients. Thus, isolating the antinociceptive qualities of adenosine from its cardiovascular side effects by evaluating receptor involvement has become an important focus for the development of adenosine-based therapeutics in pain.

A_1 and A_{2A} receptors and pain

It is not our intent to discuss in detail the A_1 and A_{2A} receptors, because these targets have been the topic of many excellent reviews (Sawynok, 1998; Fredholm *et al.*, 2011; Zylka, 2011; Chen *et al.*, 2013), but these receptors have played an important role in the understanding of adenosine antinociception. The antinociceptive properties of adenosine were long attributed to the activation of the A_1 and A_{2A} receptor subtypes (Zylka, 2011; Sawynok, 2013). Genetic deletion of the A_1 receptor produces thermal hypersensitivity and exacerbates neuropathic behavioural responses to cold and heat (Fedorova *et al.*, 2003; Wu *et al.*, 2005), and it is well documented in the literature that A_1 receptor activation leads to antinociception in a range of pain models. A_1 receptor activation produces beneficial outcomes in preclinical models of acute and chronic pain including nerve injury-induced pain (Cui *et al.*, 1997; Gong *et al.*, 2010), peri-operative pain (Gan and Habib, 2007), inflammatory pain (Sowa *et al.*, 2010), central pain following spinal cord injury (Sjolund *et al.*, 1998) and painful diabetic neuropathy (Vincenzi *et al.*, 2014; Katz *et al.*, 2015). Additionally, a spinally administered A_1 receptor agonist reduces non-evoked spontaneous pain behaviours resulting from a surgical model of pain (Zahn *et al.*, 2007). The preclinical robustness of A_1 receptor pain relief resulted in clinical trials for multiple A_1 receptor agonists and an A_1 receptor allosteric enhancer; however, these drug trials were discontinued due to limited efficacy, presumably driven by a low therapeutic index and dose limitation (Romagnoli *et al.*, 2010; Gessi *et al.*, 2011).

The findings regarding the A_{2A} receptor have been controversial. Mice lacking the A_{2A} receptor demonstrate depressed responses to acute pain stimuli as measured by the hot-plate and tail-flick tests (Ledent *et al.*, 1997). Peripheral administration of an A_{2A} receptor agonist is associated with nociceptive behaviours (Taiwo and Levine, 1990). However, very low doses of A_{2A} receptor agonists administered spinally have been shown to promote the sustained reversal of nerve injury-induced pain in rats for weeks after a single i.t. injection (Loram *et al.*, 2009). In models of post-surgical pain (Zahn *et al.*, 2007) and inflammatory pain (Poon and Sawynok, 1998), i.t. administration of A_{2A} receptor agonists had only transient antinociceptive effects that were minimal-to-negligible. However, an i.c.v. injection of an A_{2A}-targeted antibody with agonist-like activity produces antinociceptive effects in naïve mice (By *et al.*, 2011). At the clinical level, a phase II trial of an A_{2A} receptor agonist BVT-115959 in the treatment of diabetic neuropathy was completed in 2008, but the trial has been since abandoned (Swedish Orphan Biovitrum, 2014). The differing observations of A_{2A} receptor agonists in pain highlight an apparent dichotomy of peripheral versus central A_{2A} receptors in pain processing.

For the past decade, a narrow therapeutic focus on the A_1 and A_{2A} receptor has failed to harness adenosine antinociception effectively and without cardiovascular side effects (Zylka, 2011; Boison, 2013). In response, we anticipate that a greater emphasis on the A_3 receptor is a better utilization of adenosine antinociception to provide safe, effective pain relief at the clinical level.

A_3 receptors and pain

Our understanding of A_3 receptor signalling in pain has evolved greatly since the human A_3 receptor was first cloned in 1993 (Salvatore *et al.*, 1993). Early investigations reached conclusions that were not necessarily correct due to the use of A_3 receptor-targeted compounds with poor specificity such as N^6-benzyl–NECA (Sawynok *et al.*, 1997; 1999) and the A_3 receptor$^{-/-}$ mouse (Wu *et al.*, 2002). A 1997 publication examining the contribution of the A_3 receptor in pain reported that s.c. administration of N^6-benzyl–NECA into the rodent hindpaw produced dose-dependent flinching behaviour that was blocked by administration of a histamine H_1 receptor antagonist or a 5-HT_2 receptor antagonist, but not by antagonists of A_1 or A_2 receptors (Sawynok *et al.*, 1997). These studies lead to the incorrect hypothesis that the A_3 receptor was likely to mediate the pro-nociceptive, pro-inflammatory effect of N^6-benzyl–NECA via the induction of mast cell degranulation. A subsequent study clarified that N^6-benzyl–NECA nociception was not influenced by an A_3 receptor antagonist (MRS1191) but was in fact abolished by blockade of the A_{2B} receptor, a subtype previously implicated in inflammation (Feoktistov and Biaggioni, 2011).

The first definitive characterizations of the A_3 receptor as antinociceptive resulted from the use of a more specific A_3 receptor agonist, IB-MECA (N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide): IB-MECA is 50-fold selective for the A_3 receptor over the A_1 or A_{2A} receptor, as compared with the 14-fold selectivity of N^6-benzyl–NECA (Gallo-Rodriguez *et al.*, 1994; Jacobson, 1998). In 2005, a study demonstrated that systemically administered IB-MECA exerts a significant antinociceptive effect during the second phase of the formalin test without altering protective nociceptive responses (i.e. response to noxious thermal or mechanical stimuli) (Yoon *et al.*, 2005). i.t. administration of an A_3 receptor antagonist (MRS1220) prevented the antinociceptive actions of adenosine in the second phase of the formalin test, supporting a role for spinal A_3 receptors in the effect of adenosine (Yoon *et al.*, 2006).

Genetic work in the A_3 receptor knockout mice $(A_3AR)^{-/-}$ mouse has followed a similar pattern of evolution. In 2002, a study of carrageenan-induced peripheral inflammatory pain in the $A_3AR^{-/-}$ mouse noted a mild increase in the development of thermal hyperalgesia as compared with wild-type controls (Wu *et al.*, 2002). There was no alteration in the protective (i.e. non-pathological) nociceptive responses of $A_3AR^{-/-}$ animals to noxious heat and mechanical stimuli, implicating the A_3 receptor in pathological rather than protective pain states. This report was partially contradicted by a later study that observed a decrease in the protective hotplate but not tail-flick responses of $A_3AR^{-/-}$ mice (Fedorova *et al.*, 2003). Consequently, it was unclear whether the A_3 receptor affected normal protective nociception or was solely implicated in pathological pain states.

There were no studies published between 2006 and 2012 that examined the contribution of A_3 receptors in pain. In 2012, our laboratory revisited the A_3 receptor hypothesis using the agents IB-MECA and Cl-IB-MECA in neuropathic pain (Chen *et al.*, 2012; Little *et al.*, 2015). Importantly, we have observed no impact of these agents and others on baseline nociceptive thresholds, and we have therefore concluded that A_3 receptor agents do not alter normal protective nociception. In neuropathic pain, both IB-MECA and Cl-IB-MECA blocked the development of mechano-allodynia following chronic constriction injury (CCI) in a manner prevented by an antagonist of the A_3 receptor but not antagonists of the A_1 or A_{2A} receptor (Chen *et al.*, 2012). Low doses of IB-MECA given in combination with morphine, gabapentin or amitriptyline increased the potency of these agents as analgesics (Chen *et al.*, 2012). The antinociceptive effects of IB-MECA and Cl-IB-MECA have since been corroborated by better-selective A_3 agonists including MRS1898 [>100-fold over A_1 or A_{2A} receptors (Gao *et al.*, 2009)] and more recently MRS5698 [>10 000-fold over A_1 or A_{2A} receptors (Tosh *et al.*, 2012)] in rodent CCI, spared nerve injury and spinal nerve ligation models (Chen *et al.*, 2012; Ford *et al.*, 2015; Little *et al.*, 2015). The specificity of a newer generation A_3 receptor agonists has been corroborated by the attenuation of MRS5698 antinociception in the $A_3AR^{-/-}$ mouse, and alternatively in the presence of a specific A_3 receptor antagonist. (Little *et al.*, 2015). In CCI, A_3 receptor agonists administered via i.d. (ipsilateral paw to nerve injury) injection (IB-MECA, 3–60 nmol), i.t. cannula (MRS5698, 3–60 nmol) or RVM cannula (MRS5698, 0.3–3 nmol) dose-dependently attenuate mechanical allodynia (Little *et al.*, 2015). Antinociception conferred via systemic administration of the CNS-permeant MRS5698 is attenuated with i.t. or intra-RVM delivery of an A_3 receptor antagonist (Little *et al.*, 2015). However, systemic administration of a peripherally restricted A_3 receptor agonist also reverses CCI-induced peak mechanical allodynia, and the effect is not reversed by administration of an i.t. A_3 receptor antagonist (Paoletta *et al.*, 2013). It can therefore be concluded that the A_3 receptor produces antinociceptive input at central and peripheral levels, which is an important characteristic of successful analgesic agents (e.g. opioids). Further studies are warranted to explore the relationship between peripheral and central A_3 receptors in pain.

A_3 receptor agonists have also been validated in a number of cancer-related pain states. In models of chemotherapy-induced peripheral neuropathy (CIPN), IB-MECA (Chen *et al.*, 2012; Janes *et al.*, 2014b) and MRS5698 (Janes *et al.*, 2015; Little *et al.*, 2015) blocked the development of neuropathic pain without interfering with the antitumour effects (Chen *et al.*, 2012). In a rat model of breast cancer bone metastasis, treatment with Cl-IB-MECA reduced tumour growth and the related bone pain (Varani *et al.*, 2013), and MRS5698 had similar antinociceptive effects in a mouse model of breast cancer bone metastasis (Little *et al.*, 2015). High expression of the receptor is detected on many malignant cell types, and A_3 receptor agonists have been shown to produce direct anticancer effects on their own and have been documented to enhance the actions of several widely used chemotherapeutics and attenuate the associated myelosuppression (Fishman *et al.*, 2002; Fishman *et al.*, 2009; 2012). Indeed, a successful phase I/II clinical trial of Cl-IB-MECA as an anticancer agent in hepatocellular carcinoma was recently completed by Can-Fite BioPharma. Therefore, the use of A_3 receptor agonists may provide dual benefits in the treatment of a variety of cancer-related pain states.

Finally, it is important to note that because the antinociceptive effects of IB-MECA and other selective A_3 receptor agonists are not dependent upon endogenous opioid or endocannabinoid pathways (Ford *et al.*, 2015; Little *et al.*, 2015), studies have evaluated whether A_3-specific antinociception lacks classical tolerance and inherent reward properties. In preclinical studies, A_3 receptor agonists are not subject to analgesic tolerance: in rats and mice, the effect of A_3 receptor agonists persisted following six repeated daily injections (compared with morphine, which demonstrated tolerance following repeated injections for 6 days) or when administered as a continuous infusion for 7 days (Little *et al.*, 2015). These findings are interesting as repeated or continuous exposure to adenosine receptor agonists usually results in diminished responses and receptor down-regulation known as 'desensitization phenomenon,' a response that is characteristic for all adenosine receptor subtypes (Klaasse *et al.*, 2008). However, similar findings have been reported in animal models of autoimmune disorders and cancer, wherein chronically administered A_3 receptor agonists do not lose their anti-inflammatory/anticancer effects in spite of A_3 receptor protein down-regulation (Madi *et al.*, 2003). It was demonstrated that down-regulation of the receptor is associated with downstream inhibition of key regulatory proteins involved in inflammation/tumour growth, such that receptor down-regulation in fact represents receptor functionality in these cases (Fishman *et al.*, 2006). It is possible that a similar mechanism exists for the action of IB-MECA and other A_3 receptor agonists in pain, but this hypothesis requires further investigation.

In order to evaluate the inherent reward and therefore abuse potential of A_3 receptor agonists, the conditioned place preference (CPP) method was combined with the induction of nerve injury pain in rats to evaluate spontaneous pain behaviours. Results from these studies indicate that A_3 receptor agonists such as MRS5698 produce CPP in nerve-injured but not sham rats (unlike opioids and other drugs of abuse, which can elicit CPP from both naïve and injured animals), suggesting that A_3 receptor activation attenuates spontaneous pain behaviours without inherent reward (Little *et al.*, 2015). Taken together with the observation that A_3 receptor agonists

selectively modify pathological but not protective pain, it can be hypothesized that A_3 receptor agonists may circumvent the classical complications of tolerance and abuse potential associated with opioid therapy.

Mechanisms of A_3 receptor-induced antinociception

Due to the recent emergence of the A_3 receptor as a valid target for pain relief, much remains to be explored regarding the specific mechanisms of action downstream of receptor activation. To date, it is known that in the CCI model of neuropathic pain, the effects of A_3 receptor agonists are mediated independently of the opioidergic and cannabinoid systems, but do act supraspinally to recruit the activation of 5-hydroxytryptaminergic and noradrenergic bulbospinal circuits, and reduce the excitability of wide dynamic range spinal neurons (Little *et al.*, 2015). Here, we will discuss the mechanisms by which A_3 receptor activation in other disease states alters processes involved in the development of central sensitization and pain, including protein kinase activity, glutamatergic neurotransmission, ion conductance and neuroinflammation. A summary of this discussion can be found in Figure 3.

The GABAergic system is an important component regulating correct nociceptive transmission. Following the release of GABA from interneurons within the CNS, the neurotransmitter GABA can act upon its receptors to dampen neuronal excitability and reduce nociceptive signalling. During pathological pain, dysfunction of the GABAergic interneuron

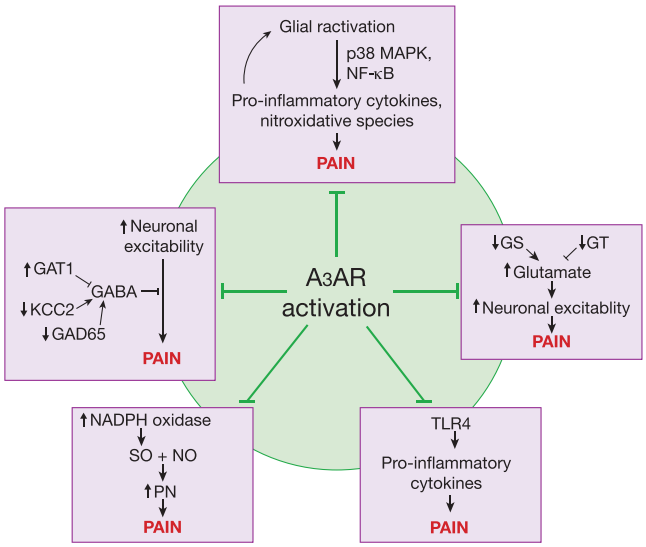

Figure 3

Potential mechanisms of A_3 receptor (A_3AR)-mediated antinociception. Several pathways are known to be important in the establishment of pain states including impairment of GABAergic neurotransmission, enhanced neuroinflammation characterized by increased glial hyperactivation and TLR4 signalling, increased glutamatergic signalling and heightened production of nitroxidative species. The A_3 receptor has been shown to modulate these pathways through the use of selective agonists, potentially explaining A_3 receptor's antinociceptive actions.

system liberates excitatory neuronal signalling and ultimately results in a state of system hyperexcitability (Zeilhofer *et al.*, 2012). An abnormality in GABA signalling can occur through a variety of mechanisms, including reduced GABA synthesis by the enzyme GAD65 (Stiller *et al.*, 1996; Eaton *et al.*, 1998), enhanced expression of the GABA reuptake transporter GAT-1 (Eaton *et al.*, 1998; Moore *et al.*, 2002) and impairment of the K^+-Cl^- cotransporter KCC2, which maintains the anion gradient required for the inhibitory action of Cl^- through $GABA_A$ channels (Coull *et al.*, 2003; Price *et al.*, 2005). It was recently demonstrated that the A_3 receptor agonist MRS5698 reverses CCI-induced pain via a GABA-mediated mechanism, wherein the CCI-related dephosphorylation of GAD65 and GAT-1 and the phosphorylation of KCC2 are ameliorated by treatment with an A_3 receptor agonist (Ford *et al.*, 2015). These results indicate that restoration of the GABAergic inhibitory system contributes to the reversal of neuropathic pain following A_3 receptor activation. In addition to modifying phosphorylation of these enzymes, the A_3 receptor may also exert its effects on GABA-mediated inhibition indirectly through the attenuation of brain-derived neurotrophic factor (BDNF) signalling. Glial-derived BDNF–TrkB signalling has been shown to reduce GABAergic signalling in the CCI model of neuropathic pain (Biggs *et al.*, 2010; Ferrini and De, 2013; Smith, 2014). A_3 receptor activation is associated with the attenuation of astrocyte reactivity, neuroinflammatory response (Janes *et al.*, 2015) and reactive microglial chemotaxis (Choi *et al.*, 2011), such that A_3 receptor agonists may reduce BDNF associated with glial hyperactivation and free the GABAergic system to function properly.

A_3 receptor agonists have demonstrated neuroprotection in animal models that is potentially mediated through the induction of pro-survival RhoA-PLD signalling pathways (Jacobson, 1998; Fredholm *et al.*, 2011). For example, A_3 receptor agonists prevent the decrease in PLD activity that occurs in response to prolonged reactive oxygen species exposure during apoptosis in cardiomyoctytes (Lee *et al.*, 2001; Asemu *et al.*, 2005). Accordingly, the protection of proper PLD function can then go on to increase the production of choline, which leads to activation of α7 nicotinic ACh receptors (Lee *et al.*, 1993). This effect is known to be both neuroprotective and antinociceptive in chronic neuropathic pain (Feuerbach *et al.*, 2009).

Many studies implicate spinal glia in the development and maintenance of chronic pain, and a variety of pain states can be prevented or attenuated with agents that disrupt the stimulation of glial cells (Milligan and Watkins, 2009; Gwak *et al.*, 2012; Old *et al.*, 2015). During an enhanced response state, glial cells can release pro-inflammatory cytokines and nitroxidative species, which can then sensitize the neurons in the dorsal horn leading to pain (Cao and Zhang, 2008; Milligan and Watkins, 2009). These glial-derived pro-inflammatory mediators act not only on neurons but also on glial cells leading to an amplification loop that is potentially responsible for the long-lasting hypersensitivity underpinning some chronic pain states (Bradesi *et al.*, 2001). A_3 receptor agonists have been shown to impart their beneficial actions at least partly through modulation of spinal neuroinflammatory processes, as IB-MECA treatment results in a reduction of hyperactive astrocytes and reduced

production of pro-inflammatory/neuroexcitatory cytokines in models of CIPN (Janes *et al.*, 2014a; Janes *et al.*, 2015). Interestingly, A_3 receptor activation also enhances formation of the anti-inflammatory cytokine IL-10 (Hasko *et al.*, 1996; Janes *et al.*, 2014a; Janes *et al.*, 2015) and the production of glial-derived neuroprotective substances including CCL2 (Wittendorp *et al.*, 2004). Both *in vitro* and *in vivo* studies have revealed that A_3 receptors produce these effects by inhibiting the p38 MAPK and NF-κB signalling pathways (Madi *et al.*, 2007; Varani *et al.*, 2010; Varani *et al.*, 2011; Janes *et al.*, 2014a). A better understanding of whether this mechanism occurs in A_3 receptor-mediated antineuroinflammatory pain relief is critical to the development of an A_3 therapeutic strategy in pain.

Increased formation of nitroxidative species including superoxide, NO and their highly pro-nociceptive reaction product peroxynitrite (Salvemini and Neumann, 2010) have been shown to play an important role in the development and maintenance of pain of several aetiologies including acute and chronic inflammation (Ndengele *et al.*, 2008), orofacial pain (Yeo *et al.*, 2008), opiate-induced hyperalgesia and antinociceptive tolerance (Muscoli *et al.*, 2007), nerve injury-induced pain (Rausaria *et al.*, 2011) and CIPN (Doyle *et al.*, 2012; Janes *et al.*, 2013). In a model of CIPN, the A_3 receptor agonist IB-MECA attenuated the spinal activation of NADPH oxidase, a source of superoxide as a precursor to peroxynitrite formation (Poderoso *et al.*, 1996; Janes *et al.*, 2014a). In prostate cancer cells, IB-MECA treatment has been shown to inhibit NADPH oxidase activation through inhibition of intracellular cAMP/PKA (Jajoo *et al.*, 2009) and by reducing the expression of NADPH oxidase subunits through inhibition of ERK1/2 activity (Jajoo *et al.*, 2009). As such, the downstream effects of the A_3 receptor on PKA activation and ERK phosphorylation could underlie the observed effect of IB-MECA on NADPH oxidase in the CNS during pain.

Given the role of adenosine in limiting excitatory neurotransmission, it is unsurprising that A_3 receptor activation impacts glutamatergic signalling. A_3 receptor agonists protect against the neurotoxic P2X7 receptor-mediated (Zhang *et al.*, 2006) or the glutamate- and NMDA-mediated rises in intracellular Ca^{2+} and neuronal excitability *in vitro* (Zhang *et al.*, 2010). Alterations in glutamatergic neurotransmission and increased neuronal excitability are widely observed in models of chronic pain (Hansson and Ronnback, 2004; Latremoliere and Woolf, 2009; Gwak *et al.*, 2012). A_3 receptor activation is associated with the inhibition of the post-translational nitration and inactivation of the glutamate transporter GLT-1 and glutamate synthase (Janes *et al.*, 2014a), together largely responsible for maintaining proper glutamatergic signalling and termination (Mao *et al.*, 2002). In addition, A_3 receptor agonists in neurons inhibit signalling through presynaptic metabotropic glutamate receptors (normally involved in reducing neurotransmission at glutamatergic synapses) (Macek *et al.*, 1998), which have been shown to be involved in the induction of several pain states (Fundytus, 2001).

Finally, activation of the innate immune receptor toll-like receptor 4 (TLR4) expressed on glial cells has been implicated in the development of neuropathic pain (Watkins *et al.*, 2009; Li *et al.*, 2014). Stimulation of the A_3 receptor with IB-MECA has been documented to decrease the TLR4-induced release of pro-inflammatory mediators including TNF and macrophage inflammatory protein-1α as well as to increase the production of the anti-inflammatory IL-10 (Hasko *et al.*, 1996; Sajjadi *et al.*, 1996; Szabo *et al.*, 1998). Suppression of pro-inflammatory mediators following TLR stimulation is lost in A_3 receptor knockout mice, suggesting that the A_3 receptor has a critical role in suppressing TLR4 responses (Salvatore *et al.*, 2000).

A_3 receptor-specific tools for the study of pain

For the benefit of future investigations of the A_3 receptor, we have provided a table of preclinically useful adenosine and A_3AR modulators, their pharmacological characteristics

Table 1

A_3 receptor-selective agents for use in the study of A_3-mediated antinociception

	K_i or K_D, nM (*or % inhibition at 10 μM*)								
	Human			Mouse			Rat		
Compound	**A_1**	**A_{2A}**	**A_3**	**A_1**	**A_{2A}**	**A_3**	**A_1**	**A_{2A}**	**A_3**
Agonists									
1 – IB-MECA	700 ± 270	6200 ± 100	**2.4 ± 0.5**	5.9	~700	**0.087**	54	56	**1.1**
2 – Cl-IB-MECA	220 ± 20	5400 ± 2500	**1.5 ± 0.2**	35	~10 000	**0.18**	820	470	**0.33**
3 – MRS1898	136 ± 22	784 ± 97	**1.51 ± 0.23**				83.9 ± 10.3	1660 ± 260	**0.17 ± 0.04**
4 – MRS5841	*16%*	*7%*	**1.90 ± 0.03**	*15%*	*1%*	**11.3 ± 1.9**			
5 – MRS5698	*6%*	*41%*	**3.49 ± 1.84**	*16%*	*27%*	**3.08 ± 0.23**			
6 – MRS5980	*6%*	*24%*	**0.70 ± 0.11**	*38%*	*7%*	**36.1 ± 4.7**			
Antagonists									
7 – MRS1523	>10 000	3660 ± 930	**18.9**	8000	>10 000	**731**	15 600	2050	**113**
8 – MRS1191	>10 000	>10 000	**31.4**				40 100	>10 000	**1850**

Sources: Jacobson *et al.*, 1992; Ge *et al.*, 2006; Melman *et al.*, 2008; Franchetti *et al.*, 2009; Liang *et al.*, 2010; Tosh *et al.*, 2012; Paoletta *et al.*, 2013; and unpublished data.

(Table 1) and their structures (Figure 4). Adenosine itself is a native, nonselective adenosine receptor agonist, while its metabolite inosine, generated following the action of ADA, weakly activates the A_3 receptor (Gao *et al.*, 2011). Preclinically successful A_3 receptor agonists (compounds **1–6**) have been generated through substitutions at the C2, N^6 and 5′ positions, most favorably including: N^6-benzyl (compounds **1–6**) or small alkyl (compound **6**); C2-arylethynyl (compounds **4–6**) or aryltriazolyl substitutions. IB-MECA (compound **1**) and Cl-IB-MECA (compound **2**) are widely used in pharmacological probes of nM affinity with CI-IB-MECA being more selective for the A_3 receptor (K_D 2.4 nM versus 1.5 nM respectively), and both of these agents have been employed in clinical trials, such that they represent clinically available avenues for targeting the A_3 receptor. Compounds **3–6** contain a conformationally constrained bicyclic (*N*-methanocarba) ring in place of ribose, which adds to the A_3 receptor selectivity (Tosh *et al.*, 2012; 2014; 2015), and these agents are specific for the A_3 receptor with a selectivity of 0.7–3.5 nM. MRS5481 (compound **4**) is a peripherally restricted agonist that is highly A_3-selective (K_D 1.9 nM) (Paoletta *et al.*, 2013). MRS5698 (compound **5**) is balanced in affinity at the human and mouse A_3 receptors (K_D 3 nM) with insignificant activity at A_1 and A_{2A} receptors at 10 μM. Compound **6** displays a long duration of action *in vivo* when administered p.o.

An alternative strategy for the study of the A_3 receptor is the use of compounds that enhance extracellular adenosine concentrations such as inhibitors of ADA (e.g. compound **9,** pentostatin) and inhibitors of adenosine kinase (e.g. compound **10** 5-iodotubercidin and compound **11** ABT-702) used in tandem with selective A_3 receptor antagonists to elucidate A_3-mediated effects (Figure 4). As an ADK inhibitor, compound **11** decreases both chronic and acute pain with peripheral or central administration (Kowaluk *et al.*, 2000), and compound **12** (TRR496) is a selective A_1 receptor allosteric enhancer that suppresses pain in a manner comparable and additive with morphine in formalin and writhing tests and has anti-allodynic effects in the streptozotocin-induced model of diabetic neuropathic pain (Vincenzi *et al.*, 2014). An effective and moderately selective A_3 receptor antagonist for use in mouse and rat is compound **8** (MRS1523) (Li *et al.*, 1998), although species differences in affinity should be taken into consideration when using selective adenosine receptor ligands, as heterocyclic antagonists of the A_3 receptor often have much higher affinity at the human than the murine A_3 receptor.

Concluding remarks

Although early studies investigating the A_3 receptor presumed a peripheral pro-nociceptive role in pain—in large part

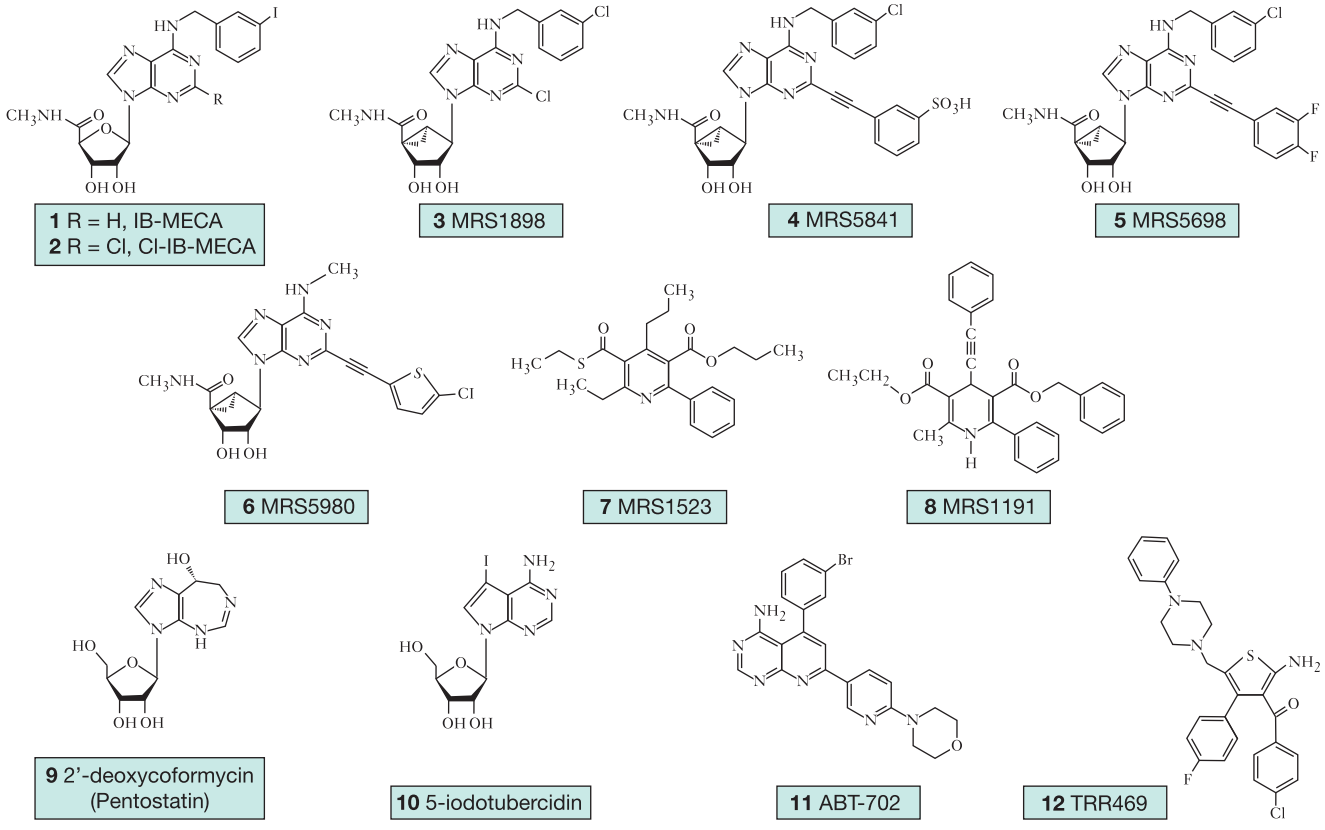

Figure 4

Pharmacological agents useful for the study of A_3 receptor-mediated antinociception. Preclinically useful selective A_3 receptor agonists (**1–6**), A_3 receptor antagonists (**7–8**) and adenosine modulators (**9–12**).

due to the use of nonselective agonists—the development of selective pharmacological tools targeting the A_3 receptor has now uncovered the robust antinociceptive properties of A_3 receptor agonists in a variety of pathological pain states. Emerging evidence suggests that harnessing the endogenous antinociceptive A_3 receptor pathway yields effective pain relief without altering normal protective nociception and without producing reward effects associated with abuse potential. Noteworthy, the cardiovascular side effects marring the usefulness of adenosine-targeted therapies is reduced in A_3 receptor-mediated strategies, and A_3 receptor agonists do not display complications in on-going phase II/III clinical trials for non-pain conditions. We propose that A_3 receptor activation may be a safe and successful strategy for exploiting the potent analgesic actions of adenosine to provide a breakthrough non-opioid treatment for patients suffering from chronic pain.

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Author contributions

All authors contributed to writing and reviewing sections of the manuscript and approved the final version.

Conflict of interest

The authors declare no conflicts of interest.

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