Targeting the adenosinergic system: Ligand binding kinetics and label-free assays for the study of SLC29A1 transporter and A2B adenosine receptor

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CHAPTER 2

Equilibrative Nucleoside Transporter 1 and Adenosine Receptors: Partners in treatment

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Conventional medicine relies on the use of pharmaceuticals and biologics in order to alleviate symptoms and treat diseases. Traditional drug discovery is based on the development of molecules which bind in a potent and selective manner on the target protein, depending on the underlying pathophysiology. Membrane proteins, e.g. receptors, enzymes, transporters and ion channels, represent currently the largest category of druggable targets. Although it is believed that they comprise only about 27% of the human proteome[1], they are considered to be the target of approximately 60% of the FDA approved drugs[2]. The two largest families of membrane proteins are the G protein-coupled receptors (GPCRs) and the Solute Carriers (SLCs).

GPCRs

GPCRs are the largest protein family in the human genome with more than 800 members and are the target of about 34% of the currently marketed drugs[3]. This family is further classified into class A (rhodopsin), class B (secretin), class C (glutamate), frizzled/taste2 and adhesion GPCRs[4]. Structurally, all family members share an extracellular N-terminus, followed by a seven transmembrane domain (TMD) architecture, bridged by three extracellular and three intracellular loops, leading to a cytoplasmic C-terminus[4]. GPCRs regulate many physiological processes by recognizing stimuli/ligands from the extracellular space, undergo a conformational change and subsequently transduce the corresponding signal into the cell via a multitude of proteins, such as heterotrimeric G proteins, kinases and arrestins. Depending on the ligand and the activated GPCR a variety of intracellular signaling pathways can be activated.

Adenosine Receptors

Adenosine receptors (ARs) belong to the class A family of GPCRs and their endogenous ligand is adenosine. They are further divided into four subtypes, namely A_1, A_{2A}, A_{2B} and A_3 ARs and they are known to the greater audience for the stimulatory effect of coffee (caffeine), chocolate (theobromine) and tea (caffeine, theophylline), all resulting in antagonism of these receptors. Adenosine's affinity for each AR subtype is found to be higher and similar for A_1, A_{2A}, and A_3 ARs, while it presents a lower affinity for A_{2B}AR[5]. As far as the potency is concerned, adenosine is considered to be less efficacious on the A_{2B} receptor, however depending on the assay used nanomolar concentrations of adenosine could activate all subtypes[6]. Each AR subtype shows a distinctive pharmacological profile and tissue distribution. Activation of A_1 and A_3 receptors mainly has an inhibitory effect on adenylate cyclase (AC) through their interaction with a G_i protein, while A_{2A} and A_{2B} AR mainly interact with a G_S protein leading to stimulation of this enzyme. In addition to AC, other pathways have been found relevant to AR activation, including phospholipase C (PLC), Ca^{2+} and mitogen-activated protein kinases (MAPKs)[7].

Over the years, several attempts have been made to target these receptors therapeutically. With the exception of adenosine itself and the natural products caffeine and theophylline the clinical impact has been limited (Table 1, Figure 1). Currently only regadenoson, an A_{2A}AR agonist for diagnostic purposes and istradefylline, an A_{2A}AR antagonist for Parkinson's disease are on the market (Table 1, Figure 1). In addition, a few theophylline analogues are in the market e.g. doxofylline, for the treatment of asthma, however the mechanism of action is unclear and a direct AR implication is unlikely[8]. However, many clinical trials concerning AR agonists and antagonists are on-going as recently summarized by Jacobson *et al*.[9] and Borea *et al.*[10].

Table 1: Clinically approved drugs, targeting ARs.

Drug	Therapeutic use		
	Disease	**Mechanism of action**	**Ref.**
Adenosine[a]	paroxysmal supraventricular tachycardia myocardial perfusion imaging[b]	A_1AR agonist	11
Caffeine[a]	neonatal apnea in preterm infants	ARs antagonist weak PDE inhibitor	12
Theophylline[a]	asthma	A_1, A_{2A}, A_{2B}AR antagonist PDE-3, PDE-4 inhibitor	13
Regadenoson	myocardial perfusion imaging[b]	A_{2A}AR agonist	14
Istradefylline	Parkinson's disease as adjunctive treatment to levodopa / carbidopa in adults	A_{2A}AR antagonist	15

[a]non-selective AR ligands. [b]Used as diagnostic tool, not therapeutic compound.

The limited presence of AR targeting in the market is partly due to the ubiquitous presence of ARs in the human body and the adverse effects resulting from their non-selective activation or inhibition. 3D structures of the receptors are a great ally in understanding of ligand binding, hence assist the structure-based design of novel agonists or antagonists. Until now, the 3D structures of the A_1 and A_{2A} ARs bound to both agonists and antagonists have been elucidated[16-19], while structural studies on the A_{2B} and A_3 ARs have yet to be successful. Although emerging techniques and methods in structural biology have increased the number of solved protein structures, membrane proteins still prove challenging due to their hydrophobicity, low expression in native tissue, and their inherent flexibility and instability once extracted from the membrane[20].

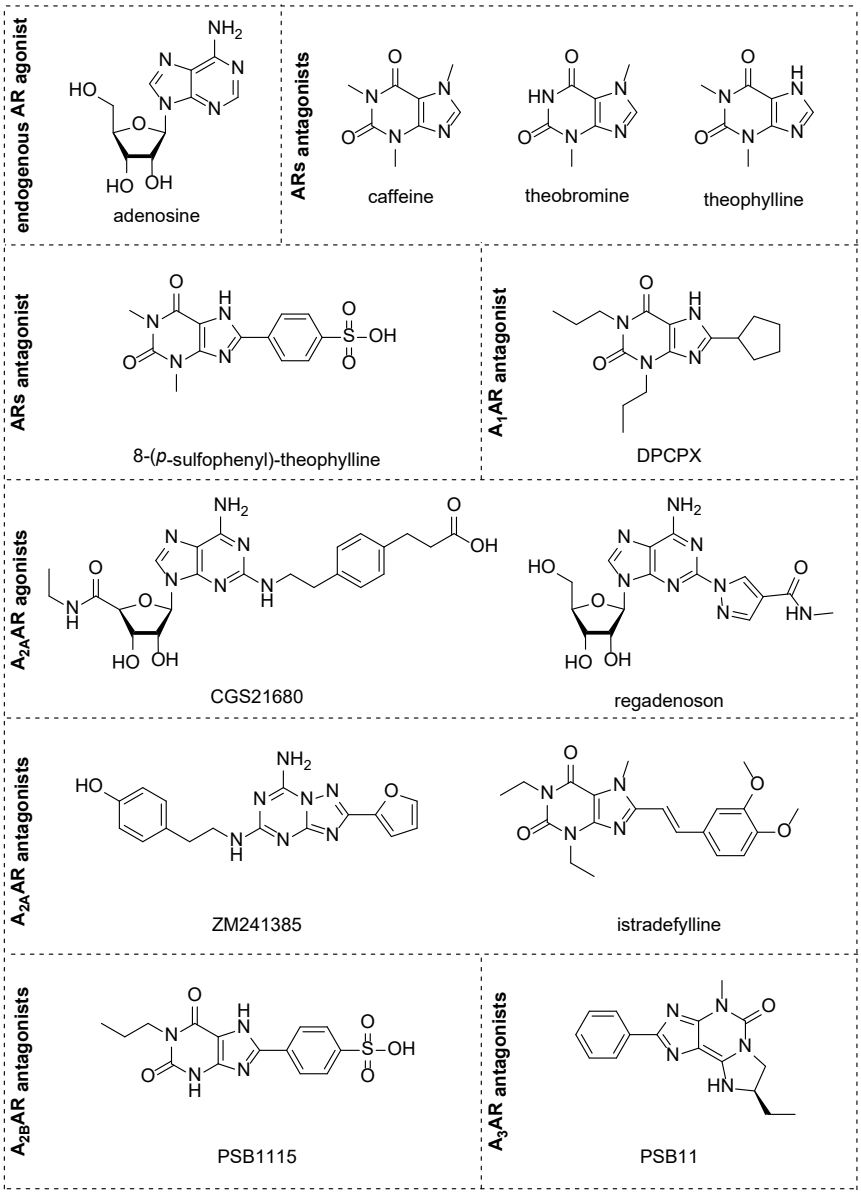

Figure 1: Structures of reference AR agonists and antagonists.

SLCs

SLCs compose the second largest family of membrane proteins and the largest family of membrane transporters in humans[21]. Membrane transporters are the cell

gatekeepers that regulate the translocation of small molecules, inorganic ions or other proteins across biological membranes. They are expressed throughout the body, prominently in the epithelia of major organs and in organs with barrier functions. It is considered that approximately 10% (~2000) of the human genes are transporter-related, highlighting the importance of transporters in human physiology and cell homeostasis[21]. Based on the IUPHAR classification system, membrane transporters can be divided into five groups; the solute carrier (SLC) family, the ATP-binding cassette transporter (ABC) family and the F-type, V-type and P-type ATPases. For the purposes of this review, SLCs will be further discussed.

Currently, there are 423 human genes encoding SLCs according to the HUGO Gene Nomenclature Committee (HGNC), grouped into more than 60 families based on their amino acid sequence, number of transmembrane α-helical domains (TMDs) and biological function[22,23]. By definition, members of each family have at least 20% or more amino acid sequence identity to at least one other family member, while they do not exhibit any significant sequence similarity to members of other families[24].

During the past 15 years, the SLC family has grown enormously, as 125 new SLC genes have been identified. The plethora of genes encoding SLCs and the vast cellular functions they are implicated to, highlight the importance of the family in human physiology. In addition, SLCs have been recognized as important therapeutic targets during pathophysiological conditions. More than 60 FDA approved agents are associated to SLCs[25]. Furosemide (targeting SLC12A1) and tricyclic antidepressants (targeting SLC6A4 or SLC6A2 or both) are examples of SLC *inhibitors*, which are hugely prescribed, while 6-mercaptopurine and acyclovir are SLC *substrates*, frequently used in anticancer and antiviral therapies, respectively.

SLC29

Nucleoside transporters (NTs) regulate the cellular uptake and efflux of nucleosides, mediating in that way the physiological process that nucleosides play a critical role in, *i.e.* synthesis of nucleic acids, energy metabolism, cAMP signaling pathway. Two structurally unrelated NT families exist in human and other mammalian cells and tissues: the SLC28 concentrative nucleoside transporter (CNT) and the SLC29 equilibrative nucleoside transporter (ENT) family. The two families are evolutionarily unrelated and provide a distinct mechanism of transportation[26]. The human ENT family mediates bidirectional Na^+-independent transport of the substrates and it has four family members: hENT1, hENT2, hENT3 and hENT4. Their action is responsible for the modulation of efficacy of more than 30 chemically diverse FDA approved drugs[27].

hENT1 is one of the major NTs on plasma membranes and it is encoded by the SLC29A1 gene. It is ubiquitously distributed while its expression levels vary between tissues[27], and it is known to mediate the facilitative diffusion of its substrates down their concentration gradients. hENT1 contains 456 amino-acid residues and its

topological structure consists of 11 TMDs with an intracellular amino terminus and an extracellular carboxyl terminus. Recently the structure of ENT1 in complex with two structurally distinct compounds has been resolved verifying the topology of the transporter[28]. Interestingly, a shared binding pocket between S-(4-Nitrobenzyl)-6-thioinosine (NBTI) and dilazep (Figure 2) was elucidated. However both compounds were also binding in one distinct pocket each, suggesting that more than one binding modes are possible in ENT1. The determination of the 3D structure of ENT1 with two bound inhibitors will likely facilitate the structure-based design of novel and selective inhibitors.

Table 2: Representative ENT1 inhibitors.

ENT1 inhibitors	Affinity (K_i) (nM)	Remarks	Affinity Ref.
NBTI	0.57 [a]	also named NBMPR	[33]
Dilazep	0.41 [a]	Clinically used ENT2 inhibitor	[33]
Dipyridamole	14 [a]	Clinically used ENT2-3 inhibitor PDE inhibitor	[33]
Lidoflazine	59 [b]	Calcium channel inhibitor	[34]
Soluflazine	1.8 [a]	Lidoflazine analogue	[35]
Mioflazine	17 [b]	Lidoflazine analogue	[34]
R75231	0.75 [b]	Lidoflazine analogue	[36]
Draflazine	0.94 [a]	Lidoflazine analogue (-)-R75231	[33]
Propentofylline	37000 [c]	PDE inhibitor weak A_1AR antagonist	[37]
KF24345	1.3 [a]	-	[38]

[^{3}H]NBTI binding assay on [a]erythrocytes, [b]calf lung tissue, [c]L1210/C2 cells.

ENT1 has been heavily studied as a drug target. Simulating the translocation of physiological nucleosides, synthetic analogs have been introduced as core strategy in the fight against cancer and viral infections[29]. Examples are gemcitabine and ribavirin. In addition to drug transportation, pharmacological inhibition of hENT1 is a promising therapeutic strategy for a variety of diseases. Increased ENT1 expression in some cancers, such as breast cancer[30] and pancreatic adenocarcinoma[31], support the use of ENT1 inhibitors as a potential anti-cancer therapy. In addition, ENT1 inhibition could serve as add-on cancer therapy in combination with anti-cancer nucleoside drugs transported by other NTs. Such combination therapy would potentially enhance the effects of anti-cancer nucleoside drugs by preventing cellular efflux. Lastly, ENT1 inhibition offers potential therapeutic effects by the modulation of

the extracellular concentration of adenosine and the subsequent AR signaling, which will be discussed further in the next paragraph[32].

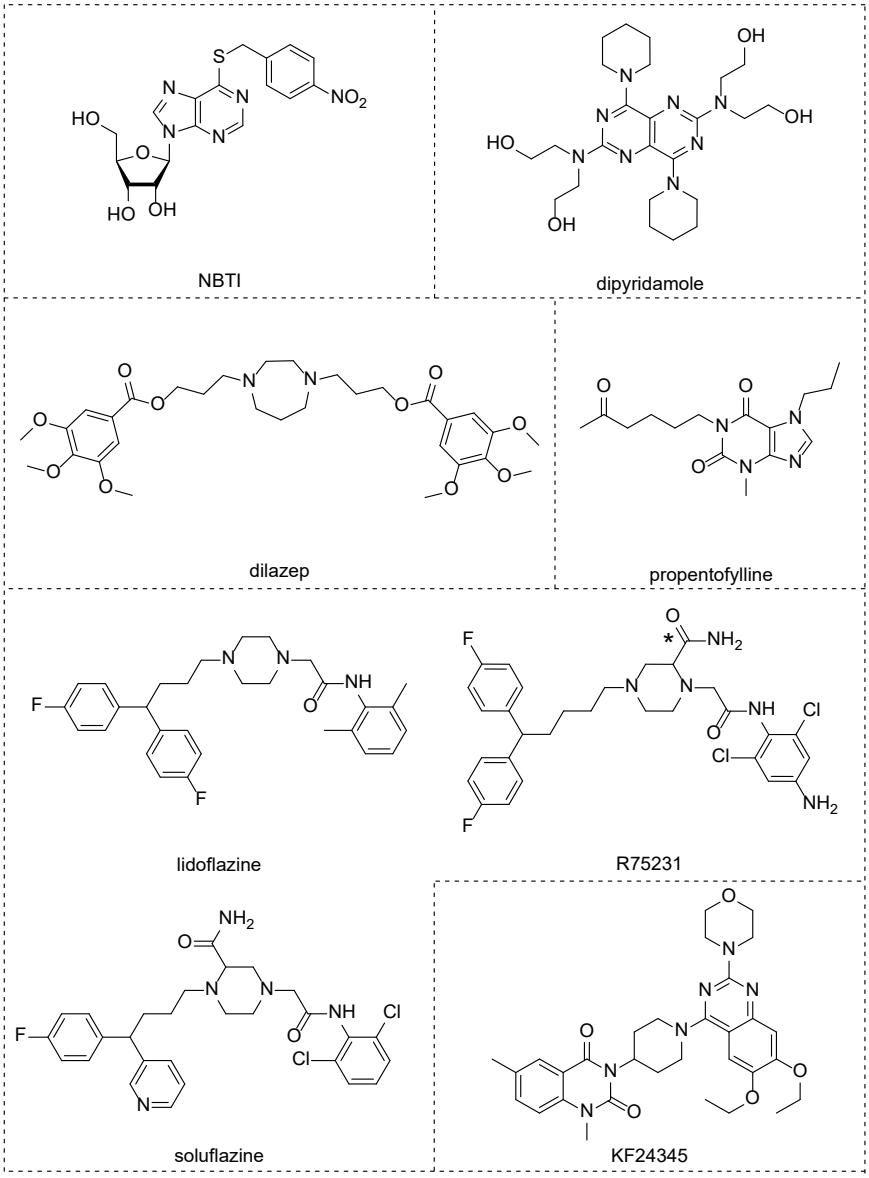

Figure 2: Structure of reference ENT1 inhibitors, representing various chemical scaffolds. Draflazine is (-)-R75231 and the asymmetric carbon atom is indicated by *.

Currently, a number of compounds from several chemical classes has been shown to inhibit ENT1 (Figure 2, Table 2). The most notable amongst them are the purine nucleoside analogues, such as NBTI, pyrimidopyrimidine analogs like dipyridamole, dilazep, and lidoflazine analogues represented by draflazine / R75231 and soluflazine. NBTI is one of the first ENT1 inhibitors studied. Based on the sensitivity of each ENT to NBTI, an initial separation between *es* (equilibrative, sensitive; ENT1) and *ei* (equilibrative, insensitive; ENT2) had been drawn. Dilazep and dipyridamole are the only two ENT1 inhibitors on the market as vasodilators, however they are not selective towards ENT1[39,40].

Interplay between SLCs and GPCRs

Transportation of endogenous and synthetic ligands, hence regulation of their concentration by SLCs, results in the modulation of their efficacy on a variety of other targets. A multitude of SLC and GPCR pairs that share an endogenous or synthetic ligand exist in the human body. Based on these pairs, two distinct therapeutic strategies could be followed:

- Efficacy enhancement of GPCR targeting drugs. Many GPCR modulators share structural characteristics with their endogenous ligands resulting in their translocation by an SLC (or other transporter). Elimination of an agonist / antagonist would negate their effect on the GPCR. A strategy where a GPCR ligand and an SLC inhibitor is combined, would prolong the availability of the GPCR ligand leading to a more pronounced effect. A prominent example is maraviroc. Maraviroc is a C-C chemokine receptor type 5 (CCR5) antagonist used in the treatment of HIV infection[41]. Maraviroc has also been identified as the substrate of multiple transporters including the SLCO1B1 which affects its pharmacokinetics[42]. A combination therapy of maraviroc with an SLCO1B1 inhibitor would potentially increase maraviroc's efficacy, as its uptake will be decreased and thus its plasma concentration and availability will be increased.
- Indirect targeting of a GPCR. In many cases direct activation of a GPCR with a synthetic ligand is not an option. An alternative approach is offered by inhibition of an SLC in close vicinity with the intended GPCR, resulting in a local increase of the endogenous ligand concentration, hence leading to an increased pharmacological effect via the GPCR. Several examples of drugs inhibiting an SLC and indirectly activating a GPCR are on the market. Citalopram, fluoxetine, fluvoxamine and paroxetine are selective serotonin reuptake inhibitors (SSRIs). By inhibiting SLC6A4 and as a result the presynaptic clearance of serotonin, a higher serotonin concentration is present in the synaptic cleft leading to an increased GPCR signaling[43]. Similarly, inhibition of other SLC6 family members (SLC6A2; norepinephrine transporter, SLC6A3; dopamine transporter) increase subsequent GPCR occupancy and signaling. However, the downstream

pathways by which monoamine reuptake inhibitors exert their antidepressant actions are not fully revealed yet[44]. Another example of SLC inhibition and concomitant GPCR activation is the use of tiagabine. Tiagabine increases the levels of GABA by the selective inhibition of SLC6A1 on presynaptic neurons and astrocytes, leading to an increased inhibitory neurotransmission within the CNS due to activation of $GABA_A$ receptors and eventually exerting its antiepileptic effect[45].

As mentioned earlier, ENT1 is one of the main regulators of adenosine's translocation, hence its inhibition offers great therapeutic opportunities for indirect activation of ARs. In combination with the limited number of ARs agonists in the market, and the clinically approved ENT1 inhibitors, such an approach starts getting increased attention. Based on the localization of the ARs as well as the disease mechanism, a broad spectrum of diseases can be targeted. The further focus of this review is to summarize efforts where ENT1 inhibition offers a therapeutic effect via indirect activation of ARs, as depicted in Figure 3.

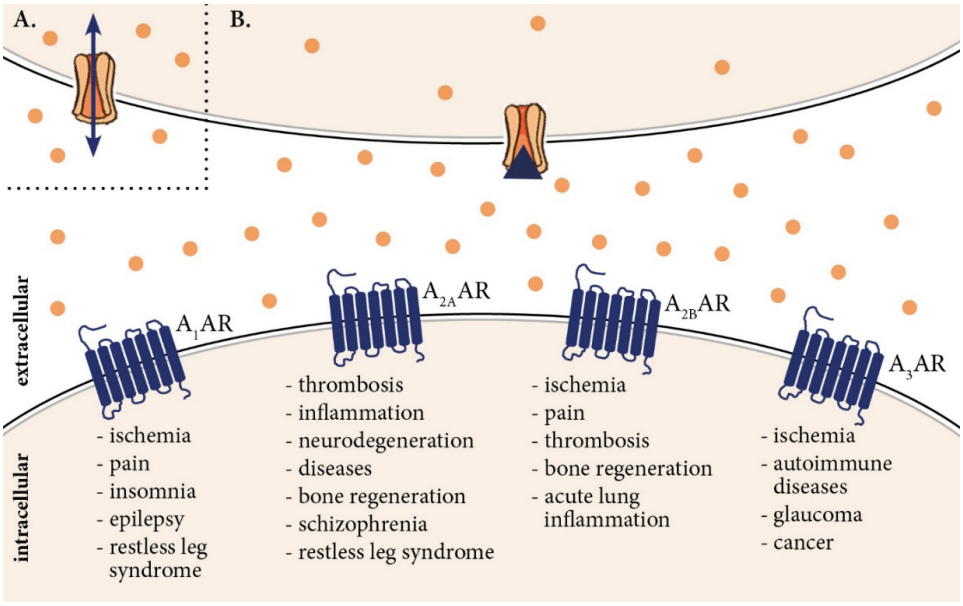

Figure 3: Graphic representation summarizing the therapeutic effects resulting from indirect activation of ARs due to ENT1 inhibition.
(A) Under physiological conditions, ENT1 equilibrates the concentration of adenosine (orange circles) across cell membranes. (B) ENT1 inhibitors (blue triangle) disrupt the physiological function of the transporter, hence modulate the concentration of endogenous adenosine. The increased extracellular concentration of adenosine results in activation of ARs, which could offer a multitude of therapeutic effects depending on the subtype.

ENT1 – A_1AR

Ischemia.

Ischemia is characterized by a restricted blood flow to a tissue, resulting in an oxygen deficiency, a lack of nutrients and an insufficient clearance of metabolic waste. Under ischemic conditions, an augmented adenosine production is witnessed[46]. In addition, adenosine plays an important role in ischemic pre- and post-conditioning. Pre-conditioning (PreC) is the mechanism of short periods of ischemia and reperfusion, protecting the organ from injury during a subsequent prolonged period of ischemia[47]. Similarly, post-conditioning (PostC) is the mechanism of short ischemia / reperfusion periods immediately after the long ischemic event. In all cases, adenosine is activating ARs leading to their increased signaling. Especially activation of A_1AR is generally accepted to play a neuroprotective role during ischemia and reperfusion injury. However, the use of selective A_1AR agonists has been proven a non-viable approach due to the undesirable peripheral secondary effects of hypotension, sedation and bradycardia[48,49]. In order to avoid these side effects, yet benefitting from protective A_1AR activation, the inhibition of ENT1, thus the decrease in endogenous adenosine elimination and indirect activation of A_1AR, was proposed as an alternative. Specifically, research on ENT1 inhibitors for cardiac and cerebral ischemia will be further discussed.

Cardiac ischemia. The effect of ENT1 inhibitors to enhance the protective role of adenosine in cardiac ischemia and reperfusion has long been established. Many different ENT1 inhibitors have been tested *in vivo* on a variety of animal models, and in the clinic. Treatment with dilazep (0.2 mg/kg, iv infusion) on PreC rabbit hearts, prolonged the infarct size-limiting effect of PreC when ischemia lasted for 30 min, while it failed to retrieve PreC protection in the 50 min occlusion group[50], showing a time limiting cardioprotective effect. Perfused hearts from guinea pigs treated with dipyridamole (4 mg/kg/day, p.o.) for 2, 3 or 6 weeks showed a sustained protection against ischemia-reperfusion injury, as assessed by the recovery of left ventricular developed pressure, left ventricular end-diastolic pressure and creatine kinase release[51]. Treatment with the selective A_1AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 200 nM) reversed the positive effect of dipyridamole, a reversal not observed with the A_{2A}/A_{2B}AR antagonist, 3,7-dimethyl-1-propargylxanthine (10 mM). Lidoflazine (1 mg/kg, iv), administered before the event of global ischemia in dogs, increased the myocardial adenosine levels 3.5 times and assisted significantly in the functional recovery compared to the control group[52]. The protective effect of lidoflazine was significantly attenuated by its co-administration with aminophylline (7 mg/kg, iv), an AR antagonist. Several analogues of lidoflazine, such as soluflazine, R75231 and draflazine have also been identified to cause cardioprotection[53-55]. It should be noted that although the effects of lidoflazine and its analogues are predominantly exerted via ENT1 inhibition, there are studies reporting that cardioprotective effects of lidoflazine and R75231 are mediated by their interaction with calcium channels[56].

Next to only ENT1 inhibitor administration, a combination of ENT1 and adenosine deaminase (AD) inhibitors has been tested in order to diminish adenosine catabolism and removal, hence even further elevating its extracellular levels. In a study performed on open-chest dogs undergoing a 15-minute coronary artery occlusion and 4 hours of reperfusion, administration of NBTI and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; an AD inhibitor) lead to a significant and sustained attenuation of postischemic myocardial dysfunction. The enhanced recovery was attributed to myocardial adenosine as its levels were significantly increased, while no significant difference was measured in ATP levels, indicating that ATP repletion is not responsible for the positive effect[57]. Further studies on dogs treated with the same combination of ENT1 and AD inhibitors tested under various conditions (such as PreC, PostC, hypothermic and warm cardioplegia), showed a protective effect associated with elevation of adenosine concentrations[58-60].

ENT1's contribution to cardioprotection in *in vivo* animal models, has also been evaluated via its removal. ENT1-null mouse hearts were examined in response to ischemia (30 min coronary occlusion followed by 2 h reperfusion) and found to have significantly less myocardial infarction compared to wild-type (WT) littermates[61]. Subsequently, cardiomyocytes isolated from ENT1-null adult mice and from WT littermates were evaluated on their expression on ENTs and ARs. Similar expression profiles were found for all proteins, except for ENT1 as expected, between the two cell lines, hence no compensatory mechanism was activated on ENT1-null mice[61].

The effect of ENT1 inhibition on cardioprotection was also evaluated in man. Lidoflazine's myocardial protection after ischemia has been shown in many cases[62,63]. Hence it has been used in clinical centers for its myocardial protective effects during coronary artery surgery[64]. A low dose of dipyridamole (15 mg in 30 min, iv infusion) given prior to exercise, increased the exercise tolerance in patients with chronic stable angina, as evaluated in a double-blind, placebo controlled study. The effect was attributed to PreC of the myocardium and hypothesized to be mediated via adenosine / A_1AR[65]. In addition, pretreatment with dipyridamole (0.5 mg/kg, intracoronary infusion) of patients undergoing percutaneous transluminal coronary angioplasty significantly preserved systolic and diastolic ventricular performance, hence reduced the risk of the angioplasty procedure[66]. However, in a recent trial in patients undergoing elective coronary artery bypass surgery, dipyridamole did not limit myocardial ischemia and reperfusion injury, as evaluated via postoperative plasma hs-troponin I release[67].

In the majority of the cases, ENT1 inhibition resulted in a significant protective effect for the ischemic tissue. The effect was proven to be adenosine-related and mediated via adenosine receptors. Based on the current knowledge on AR and ischemic protection it is well supported that the increased extracellular adenosine levels mainly activate A_1AR in cardio-ischemic injury. However, it would be beneficial to follow a more pharmacological approach, using selective AR antagonists, to study the positive effect of ENT1 inhibitors and its underlying mechanism. Although A_1AR

is mediating the initial responses of adenosine during ischemia, one should not neglect the protective role of the other ARs[68]. Activation of A_{2A}AR offers an additional protection via inhibition of platelet aggregation and attenuation of inflammation (see in ENT1-A_{2A}AR section), hence potentially (further) decreasing post-ischemic damage. In addition, adenosine activation of both A_{2A}AR and A_{2B}AR results in vasodilation ameliorating post-ischemic blood-flow[69]. Experiments in ENT1 knockout mice support the hypothesis of A_{2A} / A_{2B}AR's protective role via an increase of cAMP levels after hypoxic challenge of cardiomyocytes[70]. Finally, A_3AR together with A_1AR contributes to preconditioning by enhancing the activity of K_{ATP} channels[71,72].

Cerebral ischemia. Similar to cardiac ischemia, in cerebral ischemia adenosine exhibits protective effects, mainly attributed to A_1AR. Activation of A_1AR results in the inhibition of Ca^{2+} influx, and a lower presynaptic release of neurotransmitters, including glutamate. The latter leads to overstimulation of NMDA receptors, and a reduction of neuronal excitability by increasing the K^+ and Cl^- ion concentrations[48]. However, recently A_{2A}AR has also been associated with therapeutic effects in cerebral ischemia. In the initial phase of ischemia, A_{2A}AR antagonists may be protective via controlling precocious excitotoxicity, while in a later stage activation of peripheral A_{2A}AR is offering protection by controlling immune blood cell infiltration and inflammation[48].

Indirect activation of A_1AR via ENT1 inhibition has been studied and found protective in cases of cerebral ischemia. Data supportive to the beneficial effect of neuronal ENT1 has been presented in the study of Zhang and colleagues[73]. Initial experiments showed that exogenous adenosine (10 – 100 μM; simulating hypoxic conditions) produced a significantly higher inhibitory effect in hippocampal slices from WT mice than transgenic mice with neuronal expression of hENT1 (ENT1-rich). The presence of DPCPX (1 μM) reversed adenosine's inhibitory effects in both WT and ENT1-rich slices, validating the A_1AR-mediated effects. Under hypoxic / oxygen-glucose deprived conditions the same trend was monitored. WT slices presented a higher inhibition of excitatory neurotransmission than ENT1-rich mice, while treatment with NBTI (100 nM) abrogated these differences. Altogether these data indicate that neuronal ENT1 reduces the hypoxia/ischemia-induced increase in extracellular adenosine concentrations and that its inhibition restores the beneficial effects of adenosine in cerebral ischemia. Similar results were observed in a different study using WT and ENT1-rich mice[74]. Endothelin-1 (1 μL; 400 pmol; intracortical injection) induced greater ischemic injury in ENT1-rich mice compared to WT, as measured by cerebral blood flow and infarct size, indicating that hENT1-mediated adenosine removal reduces the protective effect of adenosine. Finally, in a recent study the effects of PreC on neuroprotection were investigated on a mouse model (C57BL/6)[75]. After five identical episodes of hypoxic exposure every 3 days no neuroprotection was observed. However a reduced cerebral blood flow in the ischemic region and a significant increase in ENT1 expression was measured resulting in a decreased extracellular adenosine concentration. Inhibition of ENT1 with propentofylline (10 mg/kg and 20 mg/kg; i.p.) increased the cerebral blood flow

and re-established neuroprotection, highlighting the increased therapeutic effect that ENT1 inhibitors could have. By increasing adenosine uptake, propentofylline lead to increased adenosine levels, which subsequently activated primarily A_1AR, although A_{2A}AR was still implicated.

It is interesting to note that there are indications of a compensation / protection mechanism in cerebral ischemia models. A study on hypoxia and glucose deprivation has revealed that these conditions can modulate expression of NTs in rat astrocytes in primary culture[76]. One hour of hypoxia and glucose deprivation resulted in an increase in adenosine and ATP concentrations in culture medium, as well as a decrease in rENT1 expression in astrocytes. In addition, when hypoxia and glucose deprivation was followed by 1 h recovery period, both rENT1 and rENT2 expression were decreased, resulting in lower uptake of [^{3}H]adenosine by equilibrative mechanisms compared to cultures from the control group. Hence, administration of ENT1 inhibitors, or dual inhibitors of ENT1 and ENT2, could support this compensatory mechanism and potentially enhance further the protective effect of adenosine during cerebral ischemia.

Pain

The role of adenosine in nociception has been well established over the last decades. Adenosine has been found to modulate pain in the periphery, spinal cord and brain primarily via A_1AR. Additional direct and indirect effects on pain transmission both in periphery and CNS have been described in recent years, implicating A_{2A}, A_{2B} and A_3 ARs[77]. Hence activation of ARs represents a non-opiate target for pain management.

Several examples of ENT1 inhibition and pain-inhibiting actions are described in the literature. Dilazep (25 nmol, i.t.) induced antinociception in mice, as evaluated in the mouse tail-flick assay[78]. In the same study, co-administration of NBTI (12.5 nmol), dipyridamole (5 nmol) or dilazep (10 nmol) with adenosine hemisulfate (110 nmol) significantly potentiated and prolonged the antinociceptive effect of adenosine. Moreover, the analgesic activity of ENT1 inhibition is supported by experiments with propentofylline (10 mg, i.t) in nerve-injured rats[79], and dipyridamole (5 mg/kg, i.p.) upon chronic stress-induced hyperalgesia in rats[80]. In the latter case, involvement of the adenosinergic system was proved by DPCPX (0.8 mg/kg, i.p.) reversing the positive effect. Pertinent to previously published data, draflazine, dilazep, dipyridamole, lidoflazine, soluflazine, and KF24345 were found to be efficient in a thermal hyperalgesia model of inflammatory pain in guinea pigs[81]. Further investigation of draflazine (10 mg/kg, subcutaneous administration) in other thermal and mechanical hyperalgesia models, completely abolished hypersensitivity. These draflazine-induced antihyperalgesic effects were reversed by A_1AR, A_2AR and non-selective AR antagonists, (cyclopentyl-theophylline, 40 mg/kg; 3,7-dimethyl-1-propargylxanthine, 10 mg/kg; caffeine, 40 mg/kg respectively, i.p.), revealing an adenosine-mediated analgesic effect[81]. Except for well described ENT1 inhibitors, amitriptyline[82] and

prostatic acid phosphatase[83] have been found to produce antinociception at least partially by increasing endogenous adenosine levels. In both cases, the effect was suppressed by adenosine antagonists.

Although there is a limited number of clinical trials on the analgesic effects of ENT1 inhibitors, dipyridamole has shown a beneficial profile in clinical settings. When evaluated for its analgesic effect in an open label trial, dipyridamole was effective in seven out of fifteen patients with chronic pain, opening the road for further investigation[84]; while its combination with a low dose of prednisolone ameliorated pain and functionality in patients with osteoarthritis[85]. Overall, there are plenty of data indicating that ENT1 inhibitors have therapeutic potential in pain management by potentiating adenosine's analgesic effects, hence, enabling a desirable decrease in the use of opiates.

Epilepsy

Epilepsy is a common and chronic neurological disorder characterized by recurrent seizures, and affects around 50 million people worldwide[86]. Multiple antiepileptic drugs are available, however about 20-30% of the patient population remains unresponsive to existing pharmacotherapy[87]. Adenosine is known to be an inhibitory neuromodulator that exhibits antiepileptic effects in the central nervous system (CNS) mainly via A_1AR[88]. Activation of A_1AR is known to inhibit glutamate release and subsequently suppress neuronal excitability and epileptic seizures[89-91]. ENT1 has also been shown to critically modulate the glutamatergic synaptic transmission by regulating the concentration of adenosine[92]. Hence, the increase of extracellular adenosine concentrations offers an interesting approach for drug resistant epilepsy.

Multiple initial studies using ENT1 inhibitors to subsequently activate adenosine receptors have been performed. Dipyridamole has been found to inhibit excitatory transmission in slices of rat hippocampus[93-95], while soluflazine decreased electrophysiological activity in slices of guinea pig hippocampus[96]. Dilazep (0.5 nmol, focal administration) has been shown to enhance the adenosine induced anti-convulsant effect in rats[97]. In addition, dilazep (0.5-50 nmol, focal administration) provided a potent and efficacious protection against seizures without the co-administration of exogenous adenosine. The use of the AR antagonist, 8-(p-sulfophenyl)-theophylline, had the opposite profile, by showing a pro-convulsant effect[97]. In all cases the effect was attributed to the elevated endogenous adenosine concentration resulting from ENT1 inhibition. However, based on current knowledge, the controls or the concentrations used cannot confirm an A_1AR specific signal in all cases, rather than a general AR mediated one. More recent efforts using NBTI confirm that ENT1 inhibition mimics the effects of adenosine on reducing the epileptiform discharge on the hippocampus of epileptic rats[98]. This protective effect was partially antagonized by the selective A_1AR antagonist DPCPX. In a similar study, NBTI also attenuated seizure severity and prolonged onset latency[99]. Finally, further research has also shown that ENT1 expression on patient and rat epileptic

brain is increased[99,100], while A_1AR expression is decreased[100], supporting the hypothesis that targeting the purinergic system could be a viable solution to achieve an antiepileptic effect.

Insomnia

It is well established that adenosine is one of the neuromodulators governing sleep[101]. Increased extracellular adenosine levels in the basal forebrain (BF) have been found to promote sleep, while after its initiation adenosine levels decrease. In addition, during prolonged periods of sleep deprivation, an increase in adenosine levels is monitored leading to the initiation of a new period of sleep, while AR antagonists are extensively used to maintain wakefulness. The most well-known example is coffee consumption. Accumulated findings support that mainly A_1 and A_{2A} ARs are involved in sleep induction. In animal models, activation of A_1AR in BF, tuberomammillary nucleus, and lateral hypothalamus induces sleep, whereas its activation in the lateral preoptic area promotes wakefulness[102]. Similarly, A_{2A}AR agonists administered to the brain induce sleep, while the wakefulness inducing effect of caffeine was seen in A_1R KO, but not in A_{2A}R KO mice[102]. Hence, ARs activation by AR agonists, or by increased extracellular adenosine levels, *i.e.* inhibition of adenosine metabolism and clearance, seems a viable approach to treat insomnia or other sleep disorders.

With respect to the role of NTs in sleep regulation, studies in rats subjected to 6 h sleep deprivation showed that *ENT1* mRNA levels in the cortex and BF were unchanged but NBTI binding was reduced in the latter[103]. According to the authors, the effect is consistent with a site-specific decrease in adenosine transport, suggesting a unique role of BF in the regulation of vigilance state during prolonged wakefulness. In addition, a dramatic reduction in rat cortical *CNT2* mRNA has been monitored during sleep deprivation, suggesting a transcriptional regulation of CNT2 expression as a modulator of adenosine uptake[104].

Pharmacological modulation of ENT1 activity has shown results consistent with the adenosinergic hypothesis of sleep regulation. NBTI injection (1 μM) into the BF and thalamus of cats increased adenosine levels, and induced both slow wave and rapid eye movement (REM) sleep[105]. In another rat model, the same compound (10 μM) decreased the discharge rate of BF neurons during waking and non-rapid eye movement sleep in rats, in the same manner as adenosine (300 μM)[106]. When administered in the lateral preoptic area, NBTI exerted the opposite effect. The awakening effect of NBTI was hypothesized to be mediated via the activation of A_{2A}AR[107]. Such studies indicate that the pharmacological targeting of ENT1 in specific brain regions could result in possible restoration of a normal sleep–wake pattern, and thus offer a viable treatment for sleep-related conditions such as insomnia and narcolepsy. Next to NBTI, lidoflazine analogues have also shown sleep inducing effects. Soluflazine (50 nmol, i.c.v. injection) and mioflazine (2.5 mg/kg, p.o.) increased sleep in rats, and significantly decreased wakefulness and increased slow

wave sleep in dogs, respectively[108,109]. In the latter case, the subsequent activation of ARs and mediation to the sleep-inducing effect was verified by the use of caffeine (2.5 and 10 mg/kg, p.o.). Finally, pre-treatment with dipyridamole (40 mg/kg, intravenous administration) produced significantly faster onset and longer duration of sleep in mice, compared to only anesthetics (thiopentone, 50 mg/kg or propofol, 150 mg/kg or midazolam, 100 mg/kg, intravenously) or anesthetics combined with adenosine (10 mg/kg, intravenously) or 2-chloroadenosine(0.25 mg/kg, intravenously)[110].

Restless leg syndrome (RLS)

RLS, also called Willis-Ekbom Disease is a neurological sensorimotor disorder characterized by a restlessness feeling and urge to move (akathisia), and periodic leg movements during sleep (PLMS)[111]. On moderate and severe cases of RLS, an enhanced arousal state is also present. It is considered a prevalent disorder as about 5-10% of the general population suffers from RLS symptoms[112,113]. RLS is associated with abnormalities in brain iron homeostasis (brain iron deficiency; BID) which appear to cause a hyperglutamatergic and hyperdopaminergic state, involved in the enhanced arousal and in akathisia and PLMS, respectively[114]. However, recent studies reveal a possible third mechanism that could link BID with the aforementioned states: a hypoadenosinergic state. A recent review by Ferré *et al*.[115] discusses the regulation of dopaminergic and glutamatergic system by A_1 and A_{2A} ARs, as well as experimental data showing downregulation of A_1ARs in BID animal models, offering the link for a putative unified pathophysiological mechanism for akathisia, PLMS and arousal state of RLS. In order to restore the adenosinergic state, researchers have focused on inhibiting ENTs, thus increasing extracellular adenosine and subsequently cause an increase in tonic A_1AR activation[116].

In initial experiments in a reserpinized mouse model[115], systemically administered SKF81297 (5 mg/kg, i.p.; D_1R agonist) and quinpirole (5 mg/kg i.p.; D_2R agonist) simulate the hyperdopaminergic state. Dipyridamole (30 mg/kg and 100 mg/kg, i.p.), used as a corrector of the hypoadenosinergic state, significantly decreased the locomotor activating effect of the dopamine receptor agonists, while caffeine (30 mg/kg, i.p.) significantly potentiated the locomotor activation induced by either agonist. In all cases, the dipyridamole effect was abrogated by caffeine, showing the direct implication of ARs, specifically A_1 and A_{2A} ARs, as they form heteromers with D_1R and D_2R respectively[115]. Next to *in vivo* mice experiments, dipyridamole's efficacy in idiopathic RLS on humans was evaluated in a clinical trial[116]. Dipyridamole (initially 100 mg, uptitration to 400 mg when necessary, once per day for eight weeks) showed significant therapeutic effects on sensory and motor symptoms, as well as sleep, supporting the hypothesis of an underlying adenosinergic mechanism in RLS.

ENT1 – A_{2A}AR

Platelet aggregation / Thrombosis

Platelet aggregation is an important process of hemostasis, resulting in a formation of blood clot (thrombus) at the site of injury in order to stop bleeding. However, under pathophysiological conditions a thrombus can form in a non-injured vessel, or it could get released (embolus) and travel in the body causing thrombosis in a distant site from its point of origin. Many diseases, including stroke, pulmonary embolism, heart and peripheral vascular disease are characterized by pathophysiological thrombosis. Adenosine blocks platelet aggregation via inhibition of platelet activation. By activating AR, mainly A_{2A}AR, adenosine leads to stimulation of AC in platelets, hence elevation of the intracellular concentration of the potent platelet activation inhibitor and second messenger, cyclic adenosine monophosphate (cAMP)[117]. In addition to A_{2A}AR, evidence that A_{2B}AR is implicated in the antiplatelet effect of adenosine has been found. *In vivo* experiments have shown that platelet A_{2B}AR expression is upregulated under stress, e.g. after injury and systemic inflammation, contributing to adenosine-mediated platelet aggregation[118]. Due to the high expression of ENT1 on erythrocytes, adenosine is quickly removed from blood, resulting in the decrease of adenosine's effect. Cognizant of that, ENT1 inhibitors have been studied as potential anti-platelet aggregation therapy[119].

Dilazep was found to block the expression and activity of tissue factor on human umbilical vein endothelial cells (HUVECs) and monocytes[120]. Tissue factor is important in the initiation of thrombin formation. The inhibitory effect of dilazep was partially reversed by the AR antagonist 8-(p-sulfophenyl)-theophylline, supporting the hypothesis that the anticoagulant effect was partly mediated by adenosine and ARs. Based on current knowledge this antagonist is not very potent on A_{2A}AR, offering an alternative explanation for the partial reverse of the dilazep effect. Dilazep and NBTI exhibited their antiplatelet effect in more studies, however no action was taken to prove an AR-mediated effect[121,122].

Dipyridamole was initially found to increase extracellular levels of adenosine. When tested in healthy volunteers, an 80% inhibition of adenosine uptake in platelets was monitored after dipyridamole (100 mg, instant form, four times daily and 200 mg, slow release preparation, twice daily, for 3 days) treatment[123], while a 60% increase in plasma adenosine level was measured in a similar set-up (after dipyridamole (100 mg, four times daily, for 5 days, p.o.) treatment)[124]. At the same time, many studies demonstrated that dipyridamole inhibits platelet aggregation[125,126]. Gresele *et al.* showed that dipyridamole's antiplatelet effect was reversed by AD and partially reversed by 5'-deoxy-5'-methylthioadenosine and theophylline, two AR antagonists, supporting the hypothesis that NT inhibition leads to an antiplatelet effect via the increase of extracellular adenosine and the subsequent signaling of AR[119]. An *in vitro* study on whole blood of healthy volunteers showed the antiplatelet effect of dipyridamole, an effect that was abolished by ZM 241385, verifying that A_{2A}AR is

mediating the inhibitory effect on platelet aggregation[127]. Recently the antithrombotic effect of dipyridamole has also been demonstrated in antiphospholipid syndrome, an immune system disorder that causes an increased risk of blood clots[128]. Dipyridamole mitigated venous thrombosis in mice and phenocopies the effect of CGS 21680 (an A_{2A}AR agonist) via activation of the adenosine A_{2A} receptor. However, it should be noted that dipyridamole is not a very selective ENT inhibitor[129]. It also inhibits various phosphodiesterase (PDE), leading to an alternative or additional way to increase intracellular levels of cAMP in platelets to exert its antiplatelet effect[130]. An effect similar to the one of dipyridamole was obtained by the combination of RE 102 BS, a dipyridamole derivative, with a PDE inhibitor (MX-MB 82 or enprofylline)[119].

Inflammatory diseases

Adenosine is involved in the regulation of the immune system by calibrating the activity of various immune cells and finally assisting in the resolution of inflammation[131]. As reviewed by Antonioli *et al.*[132], pharmacological modulation of all AR subtypes with selective agonists or antagonists, has been found a viable tool against various immune and / or inflammatory diseases. However, compelling evidence illustrates A_{2A}AR to be the primary target of the adenosinergic system to promote the anti-inflammatory response in specific cells, as proven in various models of inflammation[132,133]. Over the years many efforts have made into using A_{2A}AR specific agonists for various inflammatory disorders, including asthma, chronic obstructive pulmonary disease (COPD), glomerulonephritis and ischemia-reperfusion in kidney[132].

An alternative approach to the activation of A_{2A}AR has been tested by many researchers. Elevation of endogenous extracellular adenosine concentration via the inhibition of ENT1, has been proposed for the reduction of inflammatory responses. In inflammatory retinopathy such an alternative could be a viable approach. (−)-Cannabidiol (CBD) in nanomolar concentrations has been found to inhibit ENT1 in murine microglia and macrophages[134]. When lipopolysaccharide (LPS)-treated mice were treated with a low dose of CBD, a decrease in the proinflammatory Tumor Necrosis Factor-α (TNFα) production was measured. This effect was counteracted with administration of ZM 241385 (10 mg/kg, i.p.) and abolished in $A_{2A}AR^{-/-}$ mice, while no change was monitored with the administration of DPCPX (3 mg/kg, i.p.), indicating an A_{2A} and not an A_1 AR effect[134]. Similar results have been reported by Liou *et al.* in the retinas of LPS-treated rats and retinal microglial cells[135], showing that the effect is adenosine-related and not cannabinoid receptor-mediated[136].

When tested for another inflammation-related model, *i.e.* murine collagen-induced arthritis, CBD appeared to exert immunosuppressive and anti-inflammatory actions as well. CDB-treated mice (optimal effects at 5 mg/kg per day i.p. or 25 mg/kg per day, p.o.) were protected against severe damage of the joints, while decreased type II collagen and IFN-γ production was found in *ex vivo* draining lymph node cells from CBD-treated mice[137]. When the study was performed there was no knowledge of CDB inhibiting ENT1, hence the adenosinergic hypothesis was not tested. With

the current knowledge one would hypothesize that these effects are adenosine- and A_{2A}AR-related.

KF24245 is a potent ENT1 inhibitor and causes an A_{2A}AR-mediated anti-inflammatory response. Noji *et al.*, administered KF24245 (10 mg/kg, p.o.) to LPS-treated mice and a significant reduction in TNFα in serum was monitored. The effect was reversed only by ZM 241385 (3 mg /kg)[138]. Once the same ENT1 inhibitor was tested on acute pancreatitis, a decrease in mortality and severity of the disease was recorded. Two different experimental models of acute pancreatitis in mice were used, *i.e.* induced by choline-deficient and ethionine-supplemented diet[139] and cerulein-induced[140]. In the former study, KF24245 (10 mg/kg, p.o.) showed protection against hyperamylasemia, acinar cell injury and serum tumor necrosis factor-α elevation and eventually decreased mortality, which was abolished by ZM 241385 (3 mg/kg, p.o.)[139]. The latter study confirmed the therapeutic potential of the ENT1 inhibitor for acute pancreatitis, as the cerulean-induced increase of serum amylase and lipase, interstitial edema, polymorphonuclear cell infiltration, and acinar cell necrosis in the pancreas were reversed by KF24345 (10 mg/kg p.o.)[140]. A similar decrease of serum amylase was observed for draflazine (R75231; 300 mg/kg, p.o.) and dipyridamole (1,000 mg/kg, p.o.)[139]. Except for animal-based inflammation models, the dipyridamole response has also been studied during experimental human endotoxemia. The subjects, receiving dipyridamole (200 mg, slow release, twice daily), had an increased circulating endogenous adenosine concentration due to ENT1 blockade on erythrocytes, resulting in an anti-inflammatory response and a faster decline in proinflammatory cytokines[141].

However, ENT1 inhibition is not a viable strategy for all inflammatory diseases. In cases of chronic lung diseases as asthma or COPD, a selective A_{2A}AR activation is needed. It has been well documented that inhalation of adenosine in these diseases causes severe dyspnea and bronchospasm[142]. In addition, inhaled dipyridamole induces increased airway sensitivity in asthmatic patients, while intravenous administration results in bronchospasm or intolerable dyspnoea in emphysema and COPD patients[143].

Huntington's disease (HD)

HD is an inherited neurodegenerative disease caused by a CAG (cytosine-adenine-guanine) trinucleotide expansion in the huntingtin gene and phenotypically characterized by progressive chorea, cognitive impairments and emotional disturbances. One of the many disturbances observed in the brains affected by HD is the impairment of the adenosine homeostasis[144]. Extracellular adenosine levels measured in the brain of rodent models simulating HD have been found to be abnormal[145,146]. Hence, the adenosinergic system and especially A_{2A}AR attracted the interest of many research teams investigating HD. The high A_{2A}AR expression in the striatum[147], a brain region affected in HD, the link between at least one A_{2A}AR polymorphism (rs5751876) with an early onset of the disease[148],

as well as the decline in motor performances and survival of a mouse model once A_{2A}AR is genetically removed[149], constitute A_{2A}AR a relevant target. Although A_{2A}AR antagonists show promising results towards neuroprotection, with istradefylline used clinically in Parkinson's disease, their use in HD seems controversial, while agonists show promising results[150]. Due to the lack of A_{2A}AR agonists in the clinic ENT1 inhibition was proposed as an alternative approach. Both targets are located in the striatum[147,151], hence blocking ENT1 would elevate extracellular levels of adenosine which subsequently will activate A_{2A}AR. Such an approach is also supported by the significantly upregulated expression of ENT1 in the striatum of HD mice models, as well as in postmortem prefrontal cortex from HD human patients with a grade 2 Vonsattel neuropathological severity, but not in more severe HD stages (Vonsattel severity score 3 and 4)[145]. Thus inhibition of ENT1, especially in the early stages of the disease, appears to be an interesting intervention to restore the low adenosine tone in HD and possibly reduce the progression of the disease or to significantly delay the age of onset[152].

Studies performed on an HD mouse model (R6/2) have shown that intrastriatal administration of NBTI (10 μM) and co-administration of NBTI (10 μM) and dipyridamole (10 μM), increased extracellular adenosine levels in the striatum and lead to a prolonged adenosine increase, respectively[153], proving that elevation of adenosine levels via ENT1 inhibition is a viable. Further genetic removal of the target enhanced the survival of R6/2 mice by 7.9%, while chronic inhibition (JM 1907; a low affinity ENT1 inhibitor; 0.11 mg/kg/day for 6 weeks) resulted in both enhancement of survival by 5.7% and amelioration of motor coordination. Except for ENT1 inhibition and the resulted A_{2A}AR activation, many researchers are focusing their efforts on molecules with a dual action on both targets[154-156]. A typical example is N^6-(4-hydroxybenzyl)adenine riboside (T1-11). T1-11 binds to the binding pockets of both A_{2A}AR and ENT1 with a low affinity, and its *in vivo* effects are consistent with A_{2A}AR activation and ENT1 inhibition[154]. Interestingly, when added (0.05 mg/ml) to the drinking water of an HD mouse model (R6/2), reduction in the formation of striatal Htt aggregates, improvement in the progressive deterioration in motor coordination and increase in the level of brain derived neurotrophic factor (BDNF) were monitored[154]. Although, based on theoretical reasoning, administration of a selective (T1-11) and non-selective (adenosine) agonist competing for the same target (A_{2A}AR) is not a logical intervention, it has been proven to be effective in the case of these dual-action compounds.

Other

Except for the aforementioned pathophysiological conditions, there are other examples of diseases where ENT1 inhibition and subsequent A_{2A}AR activation could be beneficial.

Bone regeneration. The global burden of bone defects worldwide has been calculated to be immense. Fractures occurring as a result of osteoporosis, defects

from bone tumors, facial fractures and dental pathology resulting in loss of the bone that structurally supports dentition / teeth, are some of the diseases making the need for bone regeneration treatment imperative[157]. Recently, ARs (A_1 and A_{2B}) activation has gained attention regarding bone metabolism and formation, with A_{2A}AR to be the main target for regeneration[158]. Direct activation of A_{2A}AR with CGS21680 (1 μM), as well as indirect activation, *i.e.* with increased adenosine concentration resulting from treatment with dipyridamole (1 μM), promoted bone regeneration in a C57Bl/6 mice[159]. Antagonism with ZM 241385 (1 μM) or depletion of A_{2A}AR abrogated the regenerative effect, validating in all cases an A_{2A}AR-mediated response. In addition, dipyridamole significantly augmented the repair and regeneration of craniofacial bone[160], critical-sized long bone[161] and calvarial bone[162] by 3D-printed bioactive ceramic scaffolds (immersed in 100 μM dipyridamole) in rabbits and sheep, respectively.

Schizophrenia. Schizophrenia is a chronic mental illness affecting about 1% of the general population. Management of its positive, negative and cognitive symptoms is an unmet goal. The predominant concept on the genesis of schizophrenia symptoms is focusing on abnormalities in the dopaminergic and glutamatergic system, with an increased interest on GABAergic dysfunctions. Adenosine has been proposed as a potential modulator that could normalize both dopaminergic and glutamatergic transmission via A_1 and A_{2A} ARs[163]. Special attention has been drawn on activation of A_{2A}AR pre-synaptically in addition to the manipulation of extracellular levels of adenosine via adenosine kinase[164]. The approach examining the therapeutic effect of extracellular adenosine has been tested in a clinical set up, not via adenosine kinase but by inhibition of ENT1 with dipyridamole. An 8 week clinical study by Akhondzadeh *et al.* has shown that a combination of dipyridamole (75 mg/day) and haloperidol (20 mg/day) results in a further significant decrease in positive and general psycho-pathological symptoms, as well as in PANSS total scores compared to haloperidol alone[165]. The therapeutic effect has been attributed to the antagonistic effects of adenosine on the dopaminergic system, especially on D_2 receptor. However this theory was challenged by Brunstein *et al*.[166]. The alternative theory was also adenosine-mediated, but adenosine's action was considered to be via A_1AR and another neurotransmitter system, possibly glutamate, as the dopaminergic activity should have been abolished by haloperidol. In any case, the availability of ENT1 inhibitors in the clinic facilitates the investigation of adenosine's therapeutic effects and offers great opportunities to understand its involvement in the disease.

ENT1 – A_{2B}AR

Although A_{2B} is the least studied of the ARs, its activation as a result of ENT1 inhibition has been described to lead to additive positive effects in a number of diseases, as discussed in the cases of cardiac ischemia, pain, platelet aggregation and bone regeneration. Next to these pathophysiological conditions, A_{2B}AR has

been found to play an important and primary role in renal and hepatic ischemia and reperfusion injury as well as in acute lung inflammation.

Renal and Hepatic ischemia and reperfusion injury

As in the case of cardiac ischemia, adenosine also exhibits protective effects against renal and hepatic ischemia. Currently, there are emerging data showing that adenosine signaling through A_{2B}AR plays a protective role in renal, hepatic and intestinal ischemia in animal models[167-169]. Grenz *et al*. studied the potential protection of all four ARs in mice renal ischemia and only in the cases of A_{2B}AR knock out (KO) mice or selective A_{2B}AR antagonism, the protection from ischemia was abolished. In agreement with that, selective A_{2B}AR activation after ischemia showed improved renal function[168]. Similarly, Choukèr *et al*. using KO mice, demonstrated hepatotoxic protection via the A_{2B}AR. Their observations were verified by the use of a selective agonist and antagonist, concluding A_{2B}AR offers a protective role in warm liver ischemia and reperfusion injury[169].

Cognizant of the above, ENT1 inhibitors were evaluated for their renal and ischemic protective role. Mice pre-treated with dipyridamole (0.25mg/25g) experienced less severe hepatic injury after ischemia due to elevated adenosine levels. Further investigation revealed that the ENT1-dependent liver protection was mediated via A_{2B}AR[170]. Next to the traditional dipyridamole use, rapadocin, a novel ENT1 inhibitor, protected against ischemic reperfusion injury *in vivo*. Mice administered with rapadocin (4 mg/kg) had significantly lower levels of creatinine and urea nitrogen in blood compared to vehicle treated mice, indicating the protective role of rapadocin[171]. Further investigation revealed that the adenosine protective effect was A_{2B}AR specific, as co-administration of rapadocin and PSB 1115, an A_{2B}AR antagonist, abrogated the protective effect[171]. Other AR subtypes, namely A_1 and A_{2A}, have been described to mediate the dipyridamole (10 and 30 mg/kg, i.p.) effect in ischemia reperfusion-induced acute kidney injury (AKI) in rats[172].

Acute lung inflammation

As stated under the inflammatory diseases in the ENT1-A_{2A}AR section, an increase in extracellular adenosine concentrations and subsequent A_{2A}ARs activation is beneficial in inflammation, repair, and remodeling processes. However, such an approach is not viable in chronic respiratory diseases. Long term elevated adenosine levels would activate A_1, A_{2B} and A_3 ARs, promoting a pro-inflammatory state that contributes to the development and progression of chronic lung diseases[173]. To the contrary, in a case of acute lung injury adenosine has an anti-inflammatory, tissue-protective role mainly via activation of A_{2A} and A_{2B} ARs[174].

In a mechanical-induced acute lung injury (ALI) model in mice, dipyridamole (1 mg/kg, i.p.) was associated with significant increases in ALI survival time (395 min vs 277 min for their littermates)[175]. The protective effect was abolished in mice with

alveolar epithelial A_{2B} gene deletion, indicating that the lung anti-inflammatory result was A_{2B}AR-mediated. Interestingly, the protective effect under ALI was repeated only in case of ENT2 depletion, as it was evaluated by the improved gas exchange during ALI in conjunction with elevated adenosine levels in the bronchoalveolar fluid. ENT1 depletion did not result in similar effects. Hence in alveolar space ENT1 inhibition is not relevant or enough to increase adenosine levels and induce the protective A_{2B}AR effects. Additionally, on a *Pseudomonas aeruginosa* infection-induced ALI mice model, NBTI treatment (2 mg/kg) resulted in elevated lung adenosine levels, leading to attenuation of ALI, as assessed by lung wet-to-dry weight ratio, bronchoalveolar lavage (BAL) protein levels, BAL inflammatory cell counts, pro-inflammatory cytokines, and pulmonary function[176]. However, ENT1 knock-out only partially attenuated the disease. Finally, the protective effected of NBTI was counteracted when specific A_{2A} and A_{2B} ARs antagonists, ZM 241385 and PSB 1115, respectively, indicating that the protective effect is resulting from the activation of these specific ARs[176].

ENT1 – A_3AR

A_3 is an AR subtype that is less studied and its actions are not yet fully understood compared to A_1 and A_{2A} subtypes. Part of its "mystery" is that A_3AR presents a dual nature under different pathophysiologic conditions. Depending on the investigated system, it appears to have a protective and harmful role under ischemic conditions, both a pro- and anti-inflammatory character, as well as a pro- and anti-tumoral effect[177]. As its role was not conclusive, the most challenging task for scientists has been to decipher in which cases a selective A_3AR agonist or antagonist would be the optimal choice. Currently, there is a strong inclination towards agonists, with some of them being under clinical studies and showing potential in the treatment of autoimmune diseases (rheumatoid arthritis, psoriasis), glaucoma and cancer[177].

Since the role of the receptor itself is still under investigation, clinical applications of ENT1 inhibitors as indirect activators of A_3AR have yet to be realized. As mentioned earlier, A_3AR is implicated in PreC in ischemia[178] and in pain regulation. However to our knowledge, indirect activation of A_3AR via an increase in the levels of extracellular adenosine following ENT1 inhibition, has not been investigated.

Conclusions and Future Prospects

GPCRs and SLCs are the two largest membrane protein families expressed ubiquitously in the human body, exerting a multitude of actions to support human physiology. Modulating their actions in a diseased state offers treatment to many

pathophysiological conditions, as proven by the increased number of drugs targeting them. However, in many cases direct targeting of GPCRs is not the most efficacious solution or not even a viable one due to the lack of suitable drugs, *i.e.* safe, effective and selective small molecules. Taken into account the numerous SLC and GPCR pairs that share an endogenous or synthetic ligand, two concepts have raised; the "enhancement of GPCR targeting drugs' efficacy" and the "indirect targeting of a GPCR". The latter one, has been further explored and discussed in this chapter.

Targeting ENT1 for a subsequent effect through ARs is a characteristic example of such an indirect targeting. The two approved ENT1 inhibitors, dilazep and dipyridamole, exert their vasodilator actions via ARs. During the last thirty years, accumulating evidence shows that ENT1 inhibitors could have therapeutic applications in diverse diseases, ranging from cardiovascular to neurodegenerative and psychiatric diseases. In this review the ameliorating effects of ENT1 inhibitors in cardiac/cerebral/renal and hepatic ischemia, pain, epilepsy, insomnia, RLS, thrombosis, acute/chronic inflammatory diseases and HD were described as the consequence of an increase in extracellular adenosine concentrations in the target tissue leading to a concomitant activation of ARs expressed there.

Given the positive results arising from ENT1 inhibition in the examined cases and the lack of AR drugs on the market, one could be as bold as arguing that all diseases implicating ARs could be revised and re-examined with ENT1 inhibitors. In the majority of cases more than one AR subtype is mediating the therapeutic effect, showing the cooperativity between various mechanisms. However, in those cases where a specific AR effect needs to be achieved in order to avoid possible side effects resulting from the other ARs, the example of research on muscarinic receptors could be followed[179]. Thus, ENT1 inhibitors could be administered in combination with selective antagonists to block activation of the unwanted ARs. In such an effort, special attention has to be paid to the implications of A_{2B} and A_3 AR involvement, as indirect activation of these receptors is still an unexplored territory.

References

1 Almén, M. S., Nordström, K. J. V., Fredriksson, R. & Schiöth, H. B. Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. *BMC Biol* 7, 50-50, doi:10.1186/1741-7007-7-50 (2009).

2 Yıldırım, M. A., Goh, K.-I., Cusick, M. E., Barabási, A.-L. & Vidal, M. Drug—target network. *Nature Biotechnology* 25, 1119-1126, doi:10.1038/nbt1338 (2007).

3 Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B. & Gloriam, D. E. Trends in GPCR drug discovery: new agents, targets and indications. *Nature Reviews Drug Discovery* (2017).

4 Alexander, S. P. H. *et al.* THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: G protein-coupled receptors. *British Journal of Pharmacology* 176, S21-S141, doi:10.1111/bph.14748 (2019).

5 Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Linden, J. & Müller, C. E. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine Receptors—An Update. *Pharmacological Reviews* 63, 1-34, doi:10.1124/pr.110.003285 (2011).

6 Schulte, G. & Fredholm, B. B. Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol Pharmacol* 58, 477-482 (2000).

7 Jacobson, K. A. & Gao, Z.-G. Adenosine receptors as therapeutic targets. *Nature Reviews Drug Discovery* 5, 247, doi:10.1038/nrd1983 (2006).

8 van Mastbergen, J., Jolas, T., Allegra, L. & Page, C. P. The mechanism of action of doxofylline is unrelated to HDAC inhibition, PDE inhibition or adenosine receptor antagonism. *Pulmonary pharmacology & therapeutics* 25, 55-61, doi:10.1016/j.pupt.2011.10.007 (2012).

9 Jacobson, K. A., Tosh, D. K., Jain, S. & Gao, Z.-G. Historical and Current Adenosine Receptor Agonists in Preclinical and Clinical Development. *Front Cell Neurosci* 13, 124-124, doi:10.3389/fncel.2019.00124 (2019).

10 Borea, P. A., Gessi, S., Merighi, S., Vincenzi, F. & Varani, K. Pharmacology of Adenosine Receptors: The State of the Art. *Physiological reviews* 98, 1591-1625, doi:10.1152/physrev.00049.2017 (2018).

11 Ferguson, J. D. & DiMarco, J. P. Contemporary management of paroxysmal supraventricular tachycardia. *Circulation* 107, 1096-1099, doi:10.1161/01.cir.0000059743.36226.e8 (2003).

12 Comer, A. M., Perry, C. M. & Figgitt, D. P. Caffeine citrate: a review of its use in apnoea of prematurity. *Paediatric drugs* 3, 61-79, doi:10.2165/00128072-200103010-00005 (2001).

13 Barnes, P. J. Theophylline. *American journal of respiratory and critical care medicine* 188, 901-906, doi:10.1164/rccm.201302-0388PP (2013).

14 Hage, F. G. *et al.* The prognostic value of regadenoson myocardial perfusion imaging. *Journal of nuclear cardiology : official publication of the American Society of Nuclear Cardiology* 22, 1214-1221, doi:10.1007/s12350-014-0050-y (2015).

15 U.S. Food & Drug Administration. Drug Approval Package: NOURIANZ; https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/022075Orig1s000TOC.cfm. (Accessed on 18-Nov-2019).

16 Glukhova, A. *et al.* Structure of the Adenosine A1 Receptor Reveals the Basis for Subtype Selectivity. *Cell* 168, 867-877.e813, doi:10.1016/j.cell.2017.01.042 (2017).

17 Jaakola, V. P. *et al.* The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322, 1211-1217, doi:10.1126/

science.1164772 (2008).

18 Draper-Joyce, C. J. *et al.* Structure of the adenosine-bound human adenosine A1 receptor-Gi complex. *Nature* 558, 559-563, doi:10.1038/s41586-018-0236-6 (2018).

19 Xu, F. *et al.* Structure of an agonist-bound human A2A adenosine receptor. *Science* 332, 322-327, doi:10.1126/science.1202793 (2011).

20 Carpenter, E. P., Beis, K., Cameron, A. D. & Iwata, S. Overcoming the challenges of membrane protein crystallography. *Curr Opin Struct Biol* 18, 581-586, doi:10.1016/j.sbi.2008.07.001 (2008).

21 César-Razquin, A. *et al.* A Call for Systematic Research on Solute Carriers. *Cell* 162, 478-487, doi:https://doi.org/10.1016/j.cell.2015.07.022 (2015).

22 Hediger, M. A., Clemencon, B., Burrier, R. E. & Bruford, E. A. The ABCs of membrane transporters in health and disease (SLC series): introduction. *Mol Aspects Med* 34, 95-107, doi:10.1016/j.mam.2012.12.009 (2013).

23 HUGO Gene Nomenclature Committee (HGNC). https://www.genenames.org/data/genegroup/#!/group/752. (Accessed on 16-Oct-2019).

24 Schlessinger, A., Khuri, N., Giacomini, K. M. & Sali, A. Molecular modeling and ligand docking for Solute Carrier (SLC) transporters. *Curr Top Med Chem* 13, 843-856 (2013).

25 Rask-Andersen, M., Masuram, S., Fredriksson, R. & Schioth, H. B. Solute carriers as drug targets: current use, clinical trials and prospective. *Mol Aspects Med* 34, 702-710, doi:10.1016/j.mam.2012.07.015 (2013).

26 Young, J. D., Yao, S. Y. M., Baldwin, J. M., Cass, C. E. & Baldwin, S. A. The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. *Molecular Aspects of Medicine* 34, 529-547, doi:https://doi.org/10.1016/j.mam.2012.05.007 (2013).

27 Boswell-Casteel, R. C. & Hays, F. A. Equilibrative nucleoside transporters-A review. *Nucleosides, nucleotides & nucleic acids* 36, 7-30, doi:10.1080/15257770.2016.1210805 (2017).

28 Wright, N. J. & Lee, S.-Y. Structures of human ENT1 in complex with adenosine reuptake inhibitors. *Nature Structural & Molecular Biology* 26, 599-606, doi:10.1038/s41594-019-0245-7 (2019).

29 Baldwin, S. A. *et al.* The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch* 447, 735-743, doi:10.1007/s00424-003-1103-2 (2004).

30 Lane, J., Martin, T. A., McGuigan, C., Mason, M. D. & Jiang, W. G. The differential expression of hCNT1 and hENT1 in breast cancer and the possible impact on breast cancer therapy. *Journal of experimental therapeutics & oncology* 8, 203-210 (2010).

31 Greenhalf, W. *et al.* Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. *Journal of the National Cancer Institute* 106, djt347, doi:10.1093/jnci/djt347 (2014).

32 Molina-Arcas, M., Casado, F. J. & Pastor-Anglada, M. Nucleoside transporter

proteins. *Current vascular pharmacology* 7, 426-434 (2009).

33 Vlachodimou, A., Konstantinopoulou, K., Ijzerman, A. P. & Heitman, L. H. Affinity, binding kinetics and functional characterization of draflazine analogues for human equilibrative nucleoside transporter 1 (SLC29A1). *Biochemical Pharmacology* 172, 113747, doi:https://doi.org/10.1016/j.bcp.2019.113747 (2020).

34 IJzerman, A. P., Thedinga, K. H., Custers, A. F., Hoos, B. & Van Belle, H. Inhibition of nucleoside transport by a new series of compounds related to lidoflazine and mioflazine. *Eur J Pharmacol* 172, 273-281, doi:10.1016/0922-4106(89)90057-6 (1989).

35 Hammond, J. R. Interaction of a series of draflazine analogues with equilibrative nucleoside transporters: species differences and transporter subtype selectivity. *Naunyn Schmiedebergs Arch Pharmacol* 361, 373-382, doi:10.1007/s002100000214 (2000).

36 IJzerman, A. P., Kruidering, M., van Weert, A., van Belle, H. & Janssen, C. [3H] R75231 — a new radioligand for the nitrobenzylthioinosine sensitive nucleoside transport proteins Characterization of (±)-[3H]R75231 binding to calf lung membranes, stereospecificity of its two stereoisomers, and comparison with [3H]nitrobenzylthioinosine binding. *Naunyn-Schmiedeberg's Arch Pharmacol* 345, 558-563, doi:10.1007/bf00168949 (1992).

37 Parkinson, F. E., Paterson, A. R., Young, J. D. & Cass, C. E. Inhibitory effects of propentofylline on [3H]adenosine influx. A study of three nucleoside transport systems. *Biochem Pharmacol* 46, 891-896, doi:10.1016/0006-2952(93)90499-m (1993).

38 Hammond, J. R. & Archer, R. G. E. Interaction of the Novel Adenosine Uptake Inhibitor 3-[1-(6,7-Diethoxy-2-morpholinoquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(1H,3H)-quinazolinedione Hydrochloride (KF24345) with the es and ei Subtypes of Equilibrative Nucleoside Transporters. *Journal of Pharmacology and Experimental Therapeutics* 308, 1083-1093, doi:10.1124/jpet.103.060434 (2004).

39 Khalil, A., Belal, F. & Al-Badr, A. A. Dipyridamole: comprehensive profile. *Profiles of drug substances, excipients, and related methodology* 31, 215-280, doi:10.1016/s0099-5428(04)31007-5 (2005).

40 Deguchi, H. *et al.* Dilazep, an antiplatelet agent, inhibits tissue factor expression in endothelial cells and monocytes. *Blood* 90, 2345-2356 (1997).

41 Latinovic, O., Kuruppu, J., Davis, C., Le, N. & Heredia, A. Pharmacotherapy of HIV-1 Infection: Focus on CCR5 Antagonist Maraviroc. *Clin Med Ther* 1, 1497-1510, doi:10.4137/cmt.s2365 (2009).

42 Siccardi, M. *et al.* Maraviroc is a substrate for OATP1B1 in vitro and maraviroc plasma concentrations are influenced by SLCO1B1 521 T>C polymorphism. *Pharmacogenetics and genomics* 20, 759-765, doi:10.1097/FPC.0b013e3283402efb (2010).

43 Nutt, D. J. *et al.* Mechanisms of action of selective serotonin reuptake inhibitors

in the treatment of psychiatric disorders. *European Neuropsychopharmacology* 9, S81-S86, doi:https://doi.org/10.1016/S0924-977X(99)00030-9 (1999).

44 Haase, J. & Brown, E. Integrating the monoamine, neurotrophin and cytokine hypotheses of depression — A central role for the serotonin transporter? *Pharmacology & Therapeutics* 147, 1-11, doi:https://doi.org/10.1016/j.pharmthera.2014.10.002 (2015).

45 Madsen, K. K., White, H. S. & Schousboe, A. Neuronal and non-neuronal GABA transporters as targets for antiepileptic drugs. *Pharmacology & Therapeutics* 125, 394-401, doi:https://doi.org/10.1016/j.pharmthera.2009.11.007 (2010).

46 Ganesana, M. & Venton, B. J. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PloS one* 13, e0196932, doi:10.1371/journal.pone.0196932 (2018).

47 de Jong, J. W., de Jonge, R., Keijzer, E. & Bradamante, S. The role of adenosine in preconditioning. *Pharmacology & Therapeutics* 87, 141-149, doi:https://doi.org/10.1016/S0163-7258(00)00044-9 (2000).

48 Melani, A., Pugliese, A. M. & Pedata, F. in *International Review of Neurobiology* Vol. 119 (ed Akihisa Mori) 309-348 (Academic Press, 2014).

49 Glukhova, A. *et al.* Structure of the Adenosine A1 Receptor Reveals the Basis for Subtype Selectivity. *Cell* 168, 867-877.e813, doi:https://doi.org/10.1016/j.cell.2017.01.042 (2017).

50 Yamasaki, K., Tanaka, M., Yokota, R., Fujiwara, H. & Sasayama, S. Dilazep dihydrochloride prolongs the infarct size-limiting effect of ischemic preconditioning in rabbits. *Heart Vessels* 15, 227-232, doi:10.1007/s003800070012 (2000).

51 Figueredo, V. M., Diamond, I., Zhou, H. Z. & Camacho, S. A. Chronic dipyridamole therapy produces sustained protection against cardiac ischemia-reperfusion injury. *The American journal of physiology* 277, H2091-2097, doi:10.1152/ajpheart.1999.277.5.H2091 (1999).

52 Chang-Chun, C. *et al.* Nucleoside transport inhibition mediates lidoflazine-induced cardioprotection during intermittent aortic crossclamping. *The Journal of thoracic and cardiovascular surgery* 104, 1602-1609 (1992).

53 Van Belle, H., Goossens, F. & Wynants, J. Biochemical and functional effects of nucleoside transport inhibition in the isolated cat heart. *Journal of Molecular and Cellular Cardiology* 21, 797-805, doi:https://doi.org/10.1016/0022-2828(89)90719-0 (1989).

54 Sommerschild, H. T. *et al.* Importance of Endogenous Adenosine During Ischemia and Reperfusion in Neonatal Porcine Hearts. *Circulation* 96, 3094-3103, doi:doi:10.1161/01.CIR.96.9.3094 (1997).

55 Martin, B. J., Lasley, R. D. & R. M. Mentzer, J. Infarct size reduction with the nucleoside transport inhibitor R-75231 in swine. *American Journal of Physiology-Heart and Circulatory Physiology* 272, H1857-H1865, doi:10.1152/ajpheart.1997.272.4.H1857 (1997).

56 Grover, G. J. & Sleph, P. G. The cardioprotective effects of R 75231 and lidoflazine are not caused by adenosine A1 receptor activation. *J Pharmacol*

Exp Ther 268, 90-96 (1994).

57 Zughaib, M. E. *et al.* Augmentation of endogenous adenosine attenuates myocardial 'stunning' independently of coronary flow or hemodynamic effects. *Circulation* 88, 2359-2369, doi:10.1161/01.cir.88.5.2359 (1993).

58 Abd-Elfattah, A. S. *et al.* Hot shot induction and reperfusion with a specific blocker of the es-ENT1 nucleoside transporter before and after hypothermic cardioplegia abolishes myocardial stunning in acutely ischemic hearts despite metabolic derangement: Hot shot drug delivery before hypothermic cardioplegia. *The Journal of thoracic and cardiovascular surgery* 146, 961-970. e963, doi:https://doi.org/10.1016/j.jtcvs.2012.10.054 (2013).

59 Abd-Elfattah, A. S., Aly, H., Hanan, S. & Wechsler, A. S. Myocardial protection in beating heart cardiac surgery: I: Pre- or postconditioning with inhibition of es-ENT1 nucleoside transporter and adenosine deaminase attenuates post-MI reperfusion-mediated ventricular fibrillation and regional contractile dysfunction. *The Journal of thoracic and cardiovascular surgery* 144, 250-255.e253, doi:https://doi.org/10.1016/j.jtcvs.2011.10.095 (2012).

60 Abd-Elfattah, A. S., Ding, M., Jessen, M. E. & Wechsler, A. S. On-pump inhibition of es-ENT1 nucleoside transporter and adenosine deaminase during aortic crossclamping entraps intracellular adenosine and protects against reperfusion injury: Role of adenosine A1 receptor. *The Journal of thoracic and cardiovascular surgery* 144, 243-249.e241, doi:https://doi.org/10.1016/j.jtcvs.2011.09.073 (2012).

61 Rose, J. B. *et al.* Equilibrative nucleoside transporter 1 plays an essential role in cardioprotection. *American Journal of Physiology-Heart and Circulatory Physiology* 298, H771-H777, doi:10.1152/ajpheart.00711.2009 (2009).

62 Flameng, W. *et al.* Cardioprotective effects of lidoflazine in extensive aorta-coronary bypass grafting. *The Journal of thoracic and cardiovascular surgery* 85, 758-768 (1983).

63 Akpinar, B., Vanerman, H. & Wellens, F. Lidoflazine and myocardial protection. *The Journal of thoracic and cardiovascular surgery* 109, 1013-1014, doi:https://doi.org/10.1016/S0022-5223(95)70330-6 (1995).

64 De Hert, S. G. *et al.* Effects of lidoflazine on left ventricular function in patients. *Journal of Cardiothoracic and Vascular Anesthesia* 11, 42-48, doi:https://doi.org/10.1016/S1053-0770(97)90251-2 (1997).

65 Tommasi, S., Carluccio, E., Bentivoglio, M., Corea, L. & Picano, E. Low-dose dipyridamole infusion acutely increases exercise capacity in angina pectoris: A double-blind, placebo controlled crossover stress echocardiographic study. *Journal of the American College of Cardiology* 35, 83-88, doi:https://doi.org/10.1016/S0735-1097(99)00534-3 (2000).

66 Strauer, B. E., Heidland, U. E., Heintzen, M. P. & Schwartzkopff, B. Pharmacologic myocardial protection during percutaneous transluminal coronary angioplasty by intracoronary application of dipyridamole: impact on hemodynamic function and left ventricular performance. *J Am Coll Cardiol* 28, 1119-1126, doi:10.1016/s0735-1097(96)00307-5 (1996).

67 El Messaoudi, S. *et al.* Effect of dipyridamole on myocardial reperfusion injury: A double-blind randomized controlled trial in patients undergoing elective coronary artery bypass surgery. *Clinical Pharmacology & Therapeutics* 99, 381-389, doi:10.1002/cpt.106 (2016).

68 McIntosh, V. J. & Lasley, R. D. Adenosine receptor-mediated cardioprotection: are all 4 subtypes required or redundant? *Journal of cardiovascular pharmacology and therapeutics* 17, 21-33, doi:10.1177/1074248410396877 (2012).

69 Morrison, R. R., Talukder, M. A., Ledent, C. & Mustafa, S. J. Cardiac effects of adenosine in A(2A) receptor knockout hearts: uncovering A(2B) receptors. *American journal of physiology. Heart and circulatory physiology* 282, H437-444, doi:10.1152/ajpheart.00723.2001 (2002).

70 Rose, J. B. *et al.* Absence of equilibrative nucleoside transporter 1 in ENT1 knockout mice leads to altered nucleoside levels following hypoxic challenge. *Life Sciences* 89, 621-630, doi:https://doi.org/10.1016/j.lfs.2011.08.007 (2011).

71 Wan, T. C., Tampo, A., Kwok, W. M. & Auchampach, J. A. Ability of CP-532,903 to protect mouse hearts from ischemia/reperfusion injury is dependent on expression of A3 adenosine receptors in cardiomyoyctes. *Biochem Pharmacol* 163, 21-31, doi:10.1016/j.bcp.2019.01.022 (2019).

72 Kirsch, G. E., Codina, J., Birnbaumer, L. & Brown, A. M. Coupling of ATP-sensitive K+ channels to A1 receptors by G proteins in rat ventricular myocytes. *The American journal of physiology* 259, H820-826, doi:10.1152/ajpheart.1990.259.3.H820 (1990).

73 Zhang, D., Xiong, W., Albensi, B. C. & Parkinson, F. E. Expression of human equilibrative nucleoside transporter 1 in mouse neurons regulates adenosine levels in physiological and hypoxic-ischemic conditions. *Journal of neurochemistry* 118, 4-11, doi:10.1111/j.1471-4159.2011.07242.x (2011).

74 Soylu, H. *et al.* Intracortical injection of endothelin-1 induces cortical infarcts in mice: effect of neuronal expression of an adenosine transporter. *Experimental & Translational Stroke Medicine* 4, 4, doi:10.1186/2040-7378-4-4 (2012).

75 Cui, M. *et al.* Decreased Extracellular Adenosine Levels Lead to Loss of Hypoxia-Induced Neuroprotection after Repeated Episodes of Exposure to Hypoxia. *PloS one* 8, e57065, doi:10.1371/journal.pone.0057065 (2013).

76 Redzic, Z. B., Malatiali, S. A., Al-Bader, M. & Al-Sarraf, H. Effects of Hypoxia, Glucose Deprivation and Recovery on the Expression of Nucleoside Transporters and Adenosine Uptake in Primary Culture of Rat Cortical Astrocytes. *Neurochemical Research* 35, 1434-1444, doi:10.1007/s11064-010-0203-6 (2010).

77 Sawynok, J. Adenosine receptor targets for pain. *Neuroscience* 338, 1-18, doi:https://doi.org/10.1016/j.neuroscience.2015.10.031 (2016).

78 Keil, G. J. & Delander, G. E. Time-dependent antinociceptive interactions between opioids and nucleoside transport inhibitors. *Journal of Pharmacology and Experimental Therapeutics* 274, 1387-1392 (1995).

79 Raghavendra, V., Tanga, F., Rutkowski, M. D. & DeLeo, J. A. Anti-hyperalgesic

and morphine-sparing actions of propentofylline following peripheral nerve injury in rats: mechanistic implications of spinal glia and proinflammatory cytokines. *Pain* 104, 655-664, doi:10.1016/s0304-3959(03)00138-6 (2003).

80 da Silva Torres, I. L. *et al.* Effect of drugs active at adenosine receptors upon chronic stress-induced hyperalgesia in rats. *European Journal of Pharmacology* 481, 197-201, doi:https://doi.org/10.1016/j.ejphar.2003.09.045 (2003).

81 Maes, S. S., Pype, S., Hoffmann, V. L., Biermans, M. & Meert, T. F. Antihyperalgesic activity of nucleoside transport inhibitors in models of inflammatory pain in guinea pigs. *Journal of pain research* 5, 391-400, doi:10.2147/jpr.S35108 (2012).

82 Sawynok, J., Reid, A. R., Xue Jun, L. & Parkinson, F. E. Amitriptyline enhances extracellular tissue levels of adenosine in the rat hindpaw and inhibits adenosine uptake. *European Journal of Pharmacology* 518, 116-122, doi:https://doi.org/10.1016/j.ejphar.2005.06.030 (2005).

83 Chen, L. *et al.* Antinociceptive effect of prostatic acid phosphatase in a rat model of cancer-induced bone pain. *Pain physician* 16, 533-546 (2013).

84 Merskey, H. & Hamilton, J. T. An open label trial of the possible analgesic effects of dipyridamole. *Journal of Pain and Symptom Management* 4, 34-37, doi:https://doi.org/10.1016/0885-3924(89)90062-6 (1989).

85 Kvien, T. K. *et al.* Efficacy and safety of a novel synergistic drug candidate, CRx-102, in hand osteoarthritis. *Annals of the Rheumatic Diseases* 67, 942-948, doi:10.1136/ard.2007.074401 (2008).

86 World Health Organization. *Epilepsy: a public health imperative, https://www.who.int/mental_health/neurology/epilepsy/report_2019/en/*. 171 (2019).

87 Janmohamed, M., Brodie, M. J. & Kwan, P. Pharmacoresistance – Epidemiology, mechanisms, and impact on epilepsy treatment. *Neuropharmacology*, 107790, doi:https://doi.org/10.1016/j.neuropharm.2019.107790 (2019).

88 Fredholm, B. B., Chen, J.-F., Masino, S. A. & Vaugeois, J.-M. Actions of adenosine at its receptors in the CNS: Insights from Knockouts and Drugs. *Annual Review of Pharmacology and Toxicology* 45, 385-412, doi:10.1146/annurev.pharmtox.45.120403.095731 (2005).

89 Avsar, E. & Empson, R. M. Adenosine acting via A1 receptors, controls the transition to status epilepticus-like behaviour in an in vitro model of epilepsy. *Neuropharmacology* 47, 427-437, doi:https://doi.org/10.1016/j.neuropharm.2004.04.015 (2004).

90 Lupica, C. R., Proctor, W. R. & Dunwiddie, T. V. Presynaptic inhibition of excitatory synaptic transmission by adenosine in rat hippocampus: analysis of unitary EPSP variance measured by whole-cell recording. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 12, 3753-3764 (1992).

91 Gouder, N., Fritschy, J. M. & Boison, D. Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. *Epilepsia* 44, 877-885, doi:10.1046/j.1528-1157.2003.03603.x (2003).

92 Ackley, M. A. *et al.* Control of glutamatergic neurotransmission in the rat spinal

dorsal horn by the nucleoside transporter ENT1. *The Journal of physiology* 548, 507-517, doi:10.1113/jphysiol.2002.038091 (2003).

93 Mitchell, J. B., Lupica, C. R. & Dunwiddie, T. V. Activity-dependent release of endogenous adenosine modulates synaptic responses in the rat hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 13, 3439-3447, doi:10.1523/JNEUROSCI.13-08-03439.1993 (1993).

94 Tancredi, V., D'Antuono, M., Nehlig, A. & Avoli, M. Modulation of epileptiform activity by adenosine A1 receptor-mediated mechanisms in the juvenile rat hippocampus. *J Pharmacol Exp Ther* 286, 1412-1419 (1998).

95 Narimatsu, E. & Aoki, M. Transient depression of excitatory synaptic transmission induced by adenosine uptake inhibition in rat hippocampal slices. *Brain Research* 862, 284-287, doi:https://doi.org/10.1016/S0006-8993(00)02123-5 (2000).

96 Ashton, D., Willems, R., De Prins, E., Van Belle, H. & Wauquier, A. The nucleoside-transport inhibitor soluflazine (R 64 719) increases the effects of adenosine in the guinea-pig hippocampal slice and is antagonized by adenosine deaminase. *European Journal of Pharmacology* 142, 403-408, doi:https://doi.org/10.1016/0014-2999(87)90079-3 (1987).

97 Zhang, G., Franklin, P. H. & Murray, T. F. Manipulation of endogenous adenosine in the rat prepiriform cortex modulates seizure susceptibility. *J Pharmacol Exp Ther* 264, 1415-1424 (1993).

98 Huang, H. *et al.* Nitrobenzylthioinosine mimics adenosine to attenuate the epileptiform discharge of hippocampal neurons from epileptic rats. *Oncotarget* 8 (2017).

99 Xu, Z. *et al.* ENT1 inhibition attenuates epileptic seizure severity via regulation of glutamatergic neurotransmission. *Neuromolecular medicine* 17, 1-11, doi:10.1007/s12017-014-8338-2 (2015).

100 Zhang, Y. N. *et al.* Nrf2-ARE signaling pathway regulates the expressions of A1R and ENT1 in the brain of epileptic rats. *European review for medical and pharmacological sciences* 22, 6896-6904, doi:10.26355/eurrev_201810_16159 (2018).

101 Lazarus, M., Oishi, Y., Bjorness, T. E. & Greene, R. W. Gating and the Need for Sleep: Dissociable Effects of Adenosine A1 and A2A Receptors. *Frontiers in neuroscience* 13, doi:10.3389/fnins.2019.00740 (2019).

102 Huang, Z.-L., Zhang, Z. & Qu, W.-M. in *International Review of Neurobiology* Vol. 119 (ed Akihisa Mori) 349-371 (Academic Press, 2014).

103 Alanko, L., Stenberg, D. & Porkka-Heiskanen, T. Nitrobenzylthioinosine (NBMPR) binding and nucleoside transporter ENT1 mRNA expression after prolonged wakefulness and recovery sleep in the cortex and basal forebrain of rat. *Journal of Sleep Research* 12, 299-304, doi:10.1046/j.0962-1105.2003.00372.x (2003).

104 Guillén-Gómez, E. *et al.* Distribution of CNT2 and ENT1 transcripts in rat brain: selective decrease of CNT2 mRNA in the cerebral cortex of sleep-deprived rats. *Journal of neurochemistry* 90, 883-893, doi:10.1111/j.1471-4159.2004.02545.x (2004).

105 Porkka-Heiskanen, T. *et al.* Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science (New York, N.Y.)* 276, 1265-1268, doi:10.1126/science.276.5316.1265 (1997).

106 Alam, M. N., Szymusiak, R., Gong, H., King, J. & McGinty, D. Adenosinergic modulation of rat basal forebrain neurons during sleep and waking: neuronal recording with microdialysis. *J Physiol* 521 Pt 3, 679-690, doi:10.1111/j.1469-7793.1999.00679.x (1999).

107 Methippara, M. M., Kumar, S., Alam, M. N., Szymusiak, R. & McGinty, D. Effects on sleep of microdialysis of adenosine A1 and A2a receptor analogs into the lateral preoptic area of rats. *American journal of physiology. Regulatory, integrative and comparative physiology* 289, R1715-1723, doi:10.1152/ajpregu.00247.2005 (2005).

108 Wauquier, A., Van Belle, H., Van den Broeck, W. A. & Janssen, P. A. Sleep improvement in dogs after oral administration of mioflazine, a nucleoside transport inhibitor. *Psychopharmacology (Berl)* 91, 434-439, doi:10.1007/bf00216007 (1987).

109 O'Connor, S. D., Stojanovic, M. & Radulovacki, M. The effect of soluflazine on sleep in rats. *Neuropharmacology* 30, 671-674, doi:10.1016/0028-3908(91)90089-t (1991).

110 Kaputlu, İ., Şadan, G. & Özdem, S. Exogenous adenosine potentiates hypnosis induced by intravenous anaesthetics. *Anaesthesia* 53, 496-500, doi:10.1046/j.1365-2044.1998.00330.x (1998).

111 Ferré, S., Quiroz, C., Rea, W., Guitart, X. & García-Borreguero, D. in *Advances in Pharmacology* Vol. 84 (eds Stefan Clemens & Imad Ghorayeb) 3-19 (Academic Press, 2019).

112 Allen, R. P. *et al.* Restless Legs Syndrome Prevalence and Impact: REST General Population Study. *Archives of Internal Medicine* 165, 1286-1292, doi:10.1001/archinte.165.11.1286 (2005).

113 Hening, W. *et al.* Impact, diagnosis and treatment of restless legs syndrome (RLS) in a primary care population: the REST (RLS epidemiology, symptoms, and treatment) primary care study. *Sleep medicine* 5, 237-246, doi:https://doi.org/10.1016/j.sleep.2004.03.006 (2004).

114 Ferre, S., Garcia-Borreguero, D., Allen, R. P. & Earley, C. J. New Insights into the Neurobiology of Restless Legs Syndrome. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 25, 113-125, doi:10.1177/1073858418791763 (2019).

115 Ferre, S. *et al.* Pivotal Role of Adenosine Neurotransmission in Restless Legs Syndrome. *Frontiers in neuroscience* 11, 722, doi:10.3389/fnins.2017.00722 (2017).

116 Garcia-Borreguero, D. *et al.* Treatment of restless legs syndrome/Willis-Ekbom disease with the non-selective ENT1/ENT2 inhibitor dipyridamole: testing the adenosine hypothesis. *Sleep medicine* 45, 94-97, doi:10.1016/j.sleep.2018.02.002 (2018).

117 Johnston-Cox, H. A., Yang, D. & Ravid, K. Physiological implications of adenosine receptor-mediated platelet aggregation. *Journal of cellular physiology* 226, 46-51, doi:10.1002/jcp.22379 (2011).

118 Yang, D. *et al.* A new role for the A2b adenosine receptor in regulating platelet function. *Journal of Thrombosis and Haemostasis* 8, 817-827, doi:10.1111/j.1538-7836.2010.03769.x (2010).

119 Gresele, P., Arnout, J., Deckmyn, H. & Vermylen, J. Mechanism of the Antiplatelet Action of Dipyridamole in Whole Blood: Modulation of Adenosine Concentration and Activity. *Thromb Haemost* 55, 012-018, doi:10.1055/s-0038-1661437 (1986).

120 Deguchi, H. *et al.* Dilazep, an Antiplatelet Agent, Inhibits Tissue Factor Expression in Endothelial Cells and Monocytes. *Blood* 90, 2345-2356, doi:10.1182/blood.V90.6.2345 (1997).

121 Dawicki, D. D., Agarwal, K. C. & Parks, R. E. Potentiation of the antiplatelet action of adenosine in whole blood by dipyridamole or dillazep and the camp phosphodiesterase inhibitor, RA 233. *Thrombosis Research* 43, 161-175, doi:https://doi.org/10.1016/0049-3848(86)90057-5 (1986).

122 Dawicki, D. D., Agarwal, K. C. & Parks, R. E., Jr. Role of adenosine uptake and metabolism by blood cells in the antiplatelet actions of dipyridamole, dilazep and nitrobenzylthioinosine. *Biochem Pharmacol* 34, 3965-3972, doi:10.1016/0006-2952(85)90373-9 (1985).

123 Dresse, A. *et al.* Pharmacokinetics of oral dipyridamole (Persantine®) and its effect on platelet adenosine uptake in man. *European journal of clinical pharmacology* 23, 229-234, doi:10.1007/BF00547559 (1982).

124 German, D. C., Kredich, N. M. & Bjornsson, T. D. Oral dipyridamole increases plasma adenosine levels in human beings. *Clinical Pharmacology & Therapeutics* 45, 80-84, doi:10.1038/clpt.1989.12 (1989).

125 Saniabadi, A. R., Tomiak, R. H. H., Lowe, G. D. O., Barbenel, J. C. & Forbes, C. D. Dipyridamole inhibits red cell-induced platelet activation. *Atherosclerosis* 76, 149-154, doi:https://doi.org/10.1016/0021-9150(89)90098-1 (1989).

126 Gresele, P. *et al.* Dipyridamole inhibits platelet aggregation in whole blood. *Thromb Haemost* 50, 852-856 (1983).

127 Nylander, S. *et al.* Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y12 antagonism. *Journal of Thrombosis and Haemostasis* 11, 1867-1876, doi:10.1111/jth.12360 (2013).

128 Ali, R. A. *et al.* Adenosine receptor agonism protects against NETosis and thrombosis in antiphospholipid syndrome. *Nature Communications* 10, 1916, doi:10.1038/s41467-019-09801-x (2019).

129 Khalil, A., Belal, F. & Al-Badr, A. A. in *Profiles of Drug Substances, Excipients and Related Methodology* Vol. 31 (ed Harry G. Brittain) 215-280 (Academic Press, 2005).

130 Mills, D. C. & Smith, J. B. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3':5'-cyclic monophosphate in platelets.

Biochem J 121, 185-196, doi:10.1042/bj1210185 (1971).

131 Haskó, G., Linden, J., Cronstein, B. & Pacher, P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nature Reviews Drug Discovery* 7, 759-770, doi:10.1038/nrd2638 (2008).

132 Antonioli, L. *et al.* Adenosine and inflammation: what's new on the horizon? *Drug discovery today* 19, 1051-1068, doi:https://doi.org/10.1016/j.drudis.2014.02.010 (2014).

133 Haskó, G. & Pacher, P. A2A receptors in inflammation and injury: lessons learned from transgenic animals. *Journal of Leukocyte Biology* 83, 447-455, doi:10.1189/jlb.0607359 (2008).

134 Carrier, E. J., Auchampach, J. A. & Hillard, C. J. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proceedings of the National Academy of Sciences of the United States of America* 103, 7895-7900, doi:10.1073/pnas.0511232103 (2006).

135 Liou, G. I. *et al.* Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A2A adenosine receptor. *Investigative ophthalmology & visual science* 49, 5526-5531, doi:10.1167/iovs.08-2196 (2008).

136 Liou, G. I., Ahmad, S., Naime, M., Fatteh, N. & Ibrahim, A. S. Role of adenosine in diabetic retinopathy. *J Ocul Biol Dis Infor* 4, 19-24, doi:10.1007/s12177-011-9067-5 (2011).

137 Malfait, A. M. *et al.* The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proceedings of the National Academy of Sciences* 97, 9561-9566, doi:10.1073/pnas.160105897 (2000).

138 Noji, T. *et al.* KF24345, an adenosine uptake inhibitor, suppresses lipopolysaccharide-induced tumor necrosis factor-alpha production and leukopenia via endogenous adenosine in mice. *J Pharmacol Exp Ther* 300, 200-205, doi:10.1124/jpet.300.1.200 (2002).

139 Noji, T. *et al.* KF24345, an adenosine uptake inhibitor, ameliorates the severity and mortality of lethal acute pancreatitis via endogenous adenosine in mice. *European Journal of Pharmacology* 454, 85-93, doi:https://doi.org/10.1016/S0014-2999(02)02476-7 (2002).

140 Noji, T. *et al.* Adenosine uptake inhibition ameliorates cerulein-induced acute pancreatitis in mice. *Pancreas* 25, 387-392, doi:10.1097/00006676-200211000-00011 (2002).

141 Ramakers, B. P. *et al.* Dipyridamole augments the antiinflammatory response during human endotoxemia. *Crit Care* 15, R289-R289, doi:10.1186/cc10576 (2011).

142 Brown, R. A., Spina, D. & Page, C. P. Adenosine receptors and asthma. *British Journal of Pharmacology* 153, S446-S456, doi:10.1038/bjp.2008.22 (2008).

143 Rosenbrier Ribeiro, L. & Ian Storer, R. A semi-quantitative translational

pharmacology analysis to understand the relationship between in vitro ENT1 inhibition and the clinical incidence of dyspnoea and bronchospasm. *Toxicology and Applied Pharmacology* 317, 41-50, doi:https://doi.org/10.1016/j.taap.2016.12.021 (2017).

144 Lee, C.-f. & Chern, Y. in *International Review of Neurobiology* Vol. 119 (ed Akihisa Mori) 195-232 (Academic Press, 2014).

145 Guitart, X. *et al.* Equilibrative nucleoside transporter ENT1 as a biomarker of Huntington disease. *Neurobiology of disease* 96, 47-53, doi:10.1016/j.nbd.2016.08.013 (2016).

146 Gianfriddo, M., Melani, A., Turchi, D., Giovannini, M. G. & Pedata, F. Adenosine and glutamate extracellular concentrations and mitogen-activated protein kinases in the striatum of Huntington transgenic mice. Selective antagonism of adenosine A2A receptors reduces transmitter outflow. *Neurobiology of disease* 17, 77-88, doi:10.1016/j.nbd.2004.05.008 (2004).

147 Ribeiro, J. A., Sebastião, A. M. & de Mendonça, A. Adenosine receptors in the nervous system: pathophysiological implications. *Progress in Neurobiology* 68, 377-392, doi:https://doi.org/10.1016/S0301-0082(02)00155-7 (2002).

148 Dhaenens, C. M. *et al.* A genetic variation in the ADORA2A gene modifies age at onset in Huntington's disease. *Neurobiology of disease* 35, 474-476, doi:10.1016/j.nbd.2009.06.009 (2009).

149 Mievis, S., Blum, D. & Ledent, C. A2A receptor knockout worsens survival and motor behaviour in a transgenic mouse model of Huntington's disease. *Neurobiology of disease* 41, 570-576, doi:https://doi.org/10.1016/j.nbd.2010.09.021 (2011).

150 Franco, R. & Navarro, G. Adenosine A(2A) Receptor Antagonists in Neurodegenerative Diseases: Huge Potential and Huge Challenges. *Front Psychiatry* 9, 68-68, doi:10.3389/fpsyt.2018.00068 (2018).

151 Anderson, C. M. *et al.* Distribution of Equilibrative, Nitrobenzylthioinosine-Sensitive Nucleoside Transporters (ENT1) in Brain. *Journal of neurochemistry* 73, 867-873, doi:10.1046/j.1471-4159.1999.0730867.x (1999).

152 Guitart, X., Chern, Y. & Ferre, S. Targeting the equilibrative nucleoside transporter ENT1 in Huntington disease. *Oncotarget* 8, 12550-12551, doi:10.18632/oncotarget.15111 (2017).

153 Kao, Y. H. *et al.* Targeting ENT1 and adenosine tone for the treatment of Huntington's disease. *Human molecular genetics* 26, 467-478, doi:10.1093/hmg/ddw402 (2017).

154 Huang, N.-K. *et al.* A New Drug Design Targeting the Adenosinergic System for Huntington's Disease. *PloS one* 6, e20934, doi:10.1371/journal.pone.0020934 (2011).

155 Chen, J.-B. *et al.* Design and Synthesis of Novel Dual-Action Compounds Targeting the Adenosine A2A Receptor and Adenosine Transporter for Neuroprotection. *ChemMedChem* 6, 1390-1400, doi:10.1002/cmdc.201100126 (2011).

156 Lin, Y.-L., Chern, Y., Fang, J.-M., Lin, J.-H. & Huang, N.-K. Dual-action compounds targeting adenosine A2a receptor and adenosine transporter for prevention and treatment of neurodegenerative diseases. WO2012064340A1. (2010).

157 Lopez, C. D. *et al.* Local delivery of adenosine receptor agonists to promote bone regeneration and defect healing. *Advanced Drug Delivery Reviews* 146, 240-247, doi:https://doi.org/10.1016/j.addr.2018.06.010 (2019).

158 Mediero, A., Wilder, T., Shah, L. & Cronstein, B. N. Adenosine A2A receptor (A2AR) stimulation modulates expression of semaphorins 4D and 3A, regulators of bone homeostasis. *The FASEB Journal* 32, 3487-3501, doi:10.1096/fj.201700217R (2018).

159 Mediero, A., Wilder, T., Perez-Aso, M. & Cronstein, B. N. Direct or indirect stimulation of adenosine A2A receptors enhances bone regeneration as well as bone morphogenetic protein-2. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 29, 1577-1590, doi:10.1096/fj.14-265066 (2015).

160 Lopez, C. D. *et al.* Dipyridamole Augments Three-Dimensionally Printed Bioactive Ceramic Scaffolds to Regenerate Craniofacial Bone. *Plastic and reconstructive surgery* 143, 1408-1419, doi:10.1097/prs.0000000000005531 (2019).

161 Witek, L. *et al.* Repair of Critical-Sized Long Bone Defects Using Dipyridamole-Augmented 3D-Printed Bioactive Ceramic Scaffolds. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 37, 2499-2507, doi:10.1002/jor.24424 (2019).

162 Bekisz, J. M. *et al.* Dipyridamole enhances osteogenesis of three-dimensionally printed bioactive ceramic scaffolds in calvarial defects. *Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery* 46, 237-244, doi:10.1016/j.jcms.2017.11.011 (2018).

163 Rial, D., Lara, D. R. & Cunha, R. A. in *International Review of Neurobiology* Vol. 119 (ed Akihisa Mori) 395-449 (Academic Press, 2014).

164 Boison, D., Singer, P., Shen, H. Y., Feldon, J. & Yee, B. K. Adenosine hypothesis of schizophrenia--opportunities for pharmacotherapy. *Neuropharmacology* 62, 1527-1543, doi:10.1016/j.neuropharm.2011.01.048 (2012).

165 Akhondzadeh, Shasavand, Jamilian, Shabestari & Kamalipour. Dipyridamole in the treatment of schizophrenia: adenosine–dopamine receptor interactions. *Journal of clinical pharmacy and therapeutics* 25, 131-137, doi:10.1046/j.1365-2710.2000.00273.x (2000).

166 Brunstein, M. G., Belmonte-de-Abreu, P., Souza, D. O. & Lara, D. R. Therapeutic benefit of adjunctive dipyridamole in schizophrenia is probably due to adenosine–glutamate interactions. *Journal of clinical pharmacy and therapeutics* 26, 155-156, doi:10.1046/j.1365-2710.2001.00335.x (2001).

167 Hart, M. L., Jacobi, B., Schittenhelm, J., Henn, M. & Eltzschig, H. K. Cutting Edge: A2B Adenosine receptor signaling provides potent protection during

intestinal ischemia/reperfusion injury. *Journal of immunology (Baltimore, Md. : 1950)* 182, 3965-3968, doi:10.4049/jimmunol.0802193 (2009).

168 Grenz, A. *et al.* The reno-vascular A2B adenosine receptor protects the kidney from ischemia. *PLoS Med* 5, e137-e137, doi:10.1371/journal.pmed.0050137 (2008).

169 Chouker, A. *et al.* In vivo hypoxic preconditioning protects from warm liver ischemia-reperfusion injury through the adenosine A2B receptor. *Transplantation* 94, 894-902, doi:10.1097/TP.0b013e31826a9a46 (2012).

170 Zimmerman, M. A. *et al.* Equilibrative nucleoside transporter (ENT)-1-dependent elevation of extracellular adenosine protects the liver during ischemia and reperfusion. *Hepatology (Baltimore, Md.)* 58, 1766-1778, doi:10.1002/hep.26505 (2013).

171 Guo, Z. *et al.* Rapamycin-inspired macrocycles with new target specificity. *Nature Chemistry* 11, 254-263, doi:10.1038/s41557-018-0187-4 (2019).

172 Puri, N. *et al.* Dipyridamole attenuates ischemia reperfusion induced acute kidney injury through adenosinergic A1 and A2A receptor agonism in rats. *Naunyn Schmiedebergs Arch Pharmacol* 389, 361-368, doi:10.1007/s00210-015-1206-2 (2016).

173 Le, T.-T. T. *et al.* Purinergic Signaling in Pulmonary Inflammation. *Frontiers in Immunology* 10, doi:10.3389/fimmu.2019.01633 (2019).

174 Eckle, T., Koeppen, M. & Eltzschig, H. K. Role of Extracellular Adenosine in Acute Lung Injury. *Physiology* 24, 298-306, doi:10.1152/physiol.00022.2009 (2009).

175 Eckle, T. *et al.* Crosstalk between the equilibrative nucleoside transporter ENT2 and alveolar Adora2b adenosine receptors dampens acute lung injury. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27, 3078-3089, doi:10.1096/fj.13-228551 (2013).

176 Chambers, E. D. *et al.* Blockade of equilibrative nucleoside transporter 1/2 protects against Pseudomonas aeruginosa–induced acute lung injury and NLRP3 inflammasome activation. *The FASEB Journal* 34, 1516-1531, doi:10.1096/fj.201902286R (2020).

177 Borea, P. A. *et al.* The A3 adenosine receptor: history and perspectives. *Pharmacol Rev* 67, 74-102, doi:10.1124/pr.113.008540 (2015).

178 Chaudary, N., Naydenova, Z., Shuralyova, I. & Coe, I. R. The adenosine transporter, mENT1, is a target for adenosine receptor signaling and protein kinase Cepsilon in hypoxic and pharmacological preconditioning in the mouse cardiomyocyte cell line, HL-1. *J Pharmacol Exp Ther* 310, 1190-1198, doi:10.1124/jpet.104.067157 (2004).

179 Karuna Pharmaceuticals. *A Study to Assess Safety and Efficacy of KarXT in Adult Patients With Schizophrenia*, <https://ClinicalTrials.gov/show/NCT03697252> (ClinicalTrials.gov Identifier: NCT03697252).