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## Adenosine receptors as drug targets — what are the challenges?

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### Abstract

Adenosine signalling has long been a target for drug development, with adenosine itself or its derivatives being used clinically since the 1940s. In addition, methylxanthines such as caffeine have profound biological effects as antagonists at adenosine receptors. Moreover, drugs such as dipyridamole and methotrexate act by enhancing the activation of adenosine receptors. There is strong evidence that adenosine has a functional role in many diseases, and several pharmacological compounds specifically targeting individual adenosine receptors — either directly or indirectly — have now entered the clinic. However, only one adenosine receptor-specific agent — the adenosine A<sub>2A</sub> receptor agonist regadenoson (Lexiscan; Astellas Pharma) — has so far gained approval from the US Food and Drug Administration (FDA). Here, we focus on the biology of adenosine signalling to identify hurdles in the development of additional pharmacological compounds targeting adenosine receptors and discuss strategies to overcome these challenges.

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It is well known that adenosine is an important intermediary metabolite, acting as a building block for nucleic acids and a component of the biological energy currency ATP. In addition, adenosine functions as a signalling molecule through the activation of four distinct adenosine receptors — denoted A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>. These receptors are widely expressed and have been implicated in several biological functions, both physiological and pathological<sup>1,2</sup>. These include cardiac rhythm and circulation<sup>3,4</sup>, lipolysis<sup>5</sup>, renal blood flow<sup>6,7</sup>, immune function<sup>8</sup>, sleep regulation<sup>9,10</sup> and angiogenesis<sup>11</sup>, as well as inflammatory diseases<sup>12,13</sup>, ischaemia-reperfusion<sup>14</sup> and neurodegenerative disorders<sup>15</sup> (TABLE 1,2).

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#### FURTHER INFORMATION

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#### SUPPLEMENTARY INFORMATION

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The possibility of therapeutically targeting adenosine receptors is clear<sup>12,14,16</sup> and has been so for a long time (BOX 1). Adenosine itself is used clinically<sup>17</sup> (in the form of the generic drugs adenoscan and adenoscan) for the treatment of supraventricular tachycardia<sup>16,17</sup>, and many clinically used drugs (including dipyridamole and methotrexate) may exert their effects by altering extracellular adenosine concentrations and signalling. In addition, caffeine is used for treating premature apnoea in a clinical setting, and many people worldwide consume caffeine on a regular basis in doses that antagonize adenosine receptors. Selective adenosine receptor agonists and antagonists are available<sup>16</sup> and several trials are currently in progress (TABLE 1) (see the [ClinicalTrials.gov](http://ClinicalTrials.gov) website and REF. 18). However, although one A<sub>2A</sub> receptor agonist — regadenoson (Lexiscan; Astellas Pharma) — is approved by the US Food and Drug Administration (FDA) for myocardial perfusion imaging in patients with suspected coronary artery disease<sup>19</sup>, in general the translation of the abundant knowledge of adenosine biology to clinical progress has been slow.

The greatest challenge in developing adenosine receptor ligands for specific clinical applications is that adenosine signalling is so widespread. Adenosine itself is present ubiquitously, adenosine receptors are widely distributed throughout the body and adenosine acting at these receptors exerts a broad spectrum of physiological and pathophysiological functions<sup>20</sup>. Thus, demonstrating the effects of adenosine receptor activation or inactivation on specific systems under distinct experimental settings is not sufficient to suggest that adenosine can be delivered in a manner that is clinically effective and safe. The complexity of adenosine signalling contributes to the sometimes debilitating side effects of adenosine receptor agonists and antagonists, and was responsible for the failure of one of the largest clinical trials for an A<sub>1</sub> receptor antagonist so far<sup>21,22</sup>. Another challenge is that although the adenosine receptor antagonist caffeine is so commonly ingested in the normal diet, caffeine use has not been properly controlled in several previous clinical trials. In this article, we discuss the therapeutic potential of adenosine receptor modulators, focusing on the key biological factors limiting their clinical development and the hurdles that could and should be overcome. The important medicinal chemistry aspects have been extensively covered elsewhere in the literature (for example, see REF. 18) and are thus largely omitted here.

## Overview of adenosine receptors

Based on the competitive antagonism of adenosine activity by methylxanthines, the existence of adenosine receptors was postulated more than 40 years ago; 20 years later, four receptors were cloned from several mammalian species, including humans<sup>1</sup>, and identified as members of a large G protein-coupled receptor (GPCR) family<sup>1,2</sup> (TABLE 3).

### A<sub>1</sub> receptors

The A<sub>1</sub> receptor is the most conserved adenosine receptor subtype among species<sup>23</sup>, and it is widely expressed throughout the body with the highest levels found in the brain, especially at excitatory nerve endings<sup>24</sup>. Activation of the A<sub>1</sub> receptor inhibits adenylyl cyclase activity, activates potassium channels (including K<sub>ATP</sub> channels in neurons and the myocardium), blocks transient calcium channels and increases intracellular calcium and inositol-1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) levels by activating phospholipase C (PLC). A<sub>1</sub> receptors modulate neuronal activity by blocking neurotransmitter release and reducing the firing rate. A<sub>1</sub> receptors mediate negative chronotropic and inotropic effects in the heart<sup>25</sup> but they also exert effects in many other organs and cells, some of which are physiologically important, as discussed below (TABLE 2).

**A<sub>2A</sub> receptors**—High levels of the A<sub>2A</sub> receptor are found in the striatum of the brain, immune cells of the spleen, thymus, leukocytes and blood platelets, and intermediate levels are found in the heart, lung and blood vessels<sup>1,2</sup>. A<sub>2A</sub> receptor activation stimulates the

cyclic AMP–protein kinase A (PKA) pathway by coupling to G<sub>s</sub> protein<sup>23</sup> in peripheral tissues or G<sub>olf</sub> protein<sup>26,27</sup> in the brain. A<sub>2A</sub> receptors in the brain interact with several neurotransmitters to regulate motor activity, psychiatric behaviours, the sleep-wake cycle and neuronal cell death. In peripheral tissues, A<sub>2A</sub> receptors have a crucial role in the modulation of inflammation, myocardial oxygen consumption, coronary blood flow, angiogenesis and the control of cancer pathogenesis<sup>3</sup>.

**A<sub>2B</sub> receptors**—A<sub>2B</sub> receptors are widely expressed, but mostly in low abundance. Although A<sub>2B</sub> receptors stimulate mitogen-activated protein kinase (MAPK) activity at a similar affinity as A<sub>2A</sub> receptors in cultured cells<sup>28</sup>, the A<sub>2B</sub> receptor is the most adenosine-insensitive receptor among all four adenosine receptors, requiring micromolar adenosine concentrations — which are only rarely achieved under physiological conditions. During conditions in which adenosine levels are elevated, such as hypoxia, ischaemia or inflammation, functional roles of A<sub>2B</sub> receptor signalling have been described in genetic and pharmacological studies; these roles include tissue adaptation to hypoxia<sup>8,29</sup>, increased ischaemia tolerance<sup>6,30</sup> or attenuation of acute inflammation<sup>31-33</sup>.

**A<sub>3</sub> receptors**—There is considerable variation in the pharmacology and distribution — and hence function — of A<sub>3</sub> receptors among species. In mice, A<sub>3</sub> receptor signalling has been linked to mast cell degranulation<sup>34</sup>, but the situation may be different in humans. Despite the low level of A<sub>3</sub> receptor expression in most cells and tissues, its expression was upregulated in blood cells from patients with rheumatoid arthritis, Crohn's disease<sup>35</sup> and colon cancer<sup>36</sup> when compared to healthy individuals, correlating with the upregulation of nuclear factor-κB (NF-κB) signalling and the phosphoinositide 3-kinase (PI3K)-PKB-AKT signalling pathways. Indeed, preclinical studies have demonstrated anti-inflammatory, anticancer and cytoprotective effects of A<sub>3</sub> receptor agonists<sup>37</sup> (as discussed below).

### Sources of adenosine

Adenosine is the only important agonist for the three key adenosine receptors — A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> — and the major, full agonist ligand for the A<sub>3</sub> receptor (for which inosine is an incomplete agonist). The concentration of adenosine in the extracellular compartment is the consequence of many biological processes, including extracellular adenosine production, adenosine transport, adenosine formation from intracellular adenosine sources (for example, via the *S*-adenosylhomocysteine pathway) and adenosine metabolism to inosine or AMP (see FIG. 1; upper panel).

Extracellular adenosine comes from two sources. First, it may be derived from the external transport of intracellularly generated adenosine. However, adenosine is involved in several different metabolic pathways, and its intracellular concentration can never be zero. Therefore, most — if not all — cells possess equilibrative adenosine transporters, which allow adenosine to quickly cross the cell membrane<sup>38</sup>. Consequently, there will be — by necessity — a finite level of adenosine in the extracellular space, even under the most basal conditions. From the baseline level, adenosine concentrations can increase substantially. Notably, very minor changes in steady-state ATP levels in the cell (normally ~5 mM) will translate into major changes in intracellular adenosine concentrations (normally around 100,000 times higher). Second, extracellular adenosine may also be formed from the extracellular hydrolysis of adenine nucleotides. In many instances, extracellular adenosine is derived from the breakdown of extracellular nucleotides, particularly ATP and ADP. The generation of extracellular adenosine from ATP is predominantly controlled through a two-step enzymatic reaction: first, the conversion of ATP or ADP to AMP by ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1; also known as CD39), which is followed by AMP hydrolysis to adenosine by ecto-5'-nucleotidase (NT5E; also known as CD73).

ATP can be released from various cell types by multiple mechanisms: it can be co-released from storage vesicles together with other hormones (such as neurotransmitters), it can be released via a 'kiss and run' mechanism<sup>39</sup> (a type of synaptic vesicle release where the vesicle opens and closes transiently) or it can be released from the lysosome by exocytosis<sup>40</sup>. ATP release mechanisms include uncontrolled leakage from necrotic cells<sup>4</sup>, leakage from cells undergoing other forms of cell death, controlled release through pannexin hemichannels<sup>41,42</sup> as well as release from inflammatory cells or vascular endothelia through connexin hemichannels and channels such as P2X purinergic receptor 7 (REFS 43-45). The basal physiological level of extracellular adenosine from both sources has been estimated to be in the range of 30-200 nM<sup>46</sup>. The processes that lead to increased intracellular adenosine formation often affect several cells or a whole tissue, thereby causing considerably widespread and enduring changes in adenosine concentration. By contrast, the release of adenine nucleotides may be limited in quantity and spatially restricted. Thus, the two modes of changes in adenosine concentration can have considerably different consequences.

### Adenosine receptor functions

Adenosine receptors have been implicated in several key physiological processes, ranging from neuromodulation to immune regulation, and from vascular function to metabolic control. Adenosine has been postulated to have a role as a danger signal involved in homeostasis. One approach that has proved to be particularly effective in uncovering the normal physiological roles of adenosine receptors is genetic knockout. Genetic knockout mouse models for all four adenosine receptors (*Adora1*, *Adora2a*, *Adora2b* and *Adora3*, which encode A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors, respectively) have now been generated by the targeted deletion of either of the two critical exons of the adenosine receptors<sup>20,47</sup>. Although detection of the pathophysiological roles of adenosine signalling has been more difficult, as this requires models of disease in the genetically modified organism, the available genetic knockout mouse models have provided some insights. A list of some of the key physiological and pathophysiological roles of the adenosine receptors (derived mainly from studies with genetic knockout models) is given in TABLE 2, and some of these roles are discussed in more detail below.

Two lines of *Adora1*-knockout mice have been generated<sup>7,48</sup>, and *Adora1*-knockout mice exhibit decreased fertility, a significant decrease in lifespan<sup>49</sup> and an increased risk of seizures<sup>48</sup>. In the kidney, knockout studies have confirmed the pharmacological finding that stimulating the A<sub>1</sub> receptor on the glomerular afferent arteriole reduces renal blood flow and the glomerular filtration rate, and stimulation of the A<sub>1</sub> receptor on the proximal tubules increases sodium and water reabsorption<sup>7</sup>. These studies provide the rationale for developing A<sub>1</sub> receptor antagonists to control renal dysfunction in patients with acute heart failure.

A<sub>2A</sub> receptors are expressed at high levels in the dorsal striatum, a critical basal ganglia structure involved in motor control, where they are colocalized with dopamine D<sub>2</sub> receptors; they inhibit D<sub>2</sub> receptor binding in the striatum and immediate-early gene expression<sup>50-52</sup>. The behavioural actions of A<sub>2A</sub> receptor antagonists partly overlap with those of D<sub>2</sub> receptor agonists, which has implications for the treatment of Parkinson's disease (as discussed below) and also influences working memory<sup>53,54</sup>, reversal learning<sup>53</sup> and goal-oriented behaviour<sup>55</sup>, while leaving spatial reference memory, motor function and anxiety-like behaviours intact.

Three strains of *Adora2a*-knockout mice have been generated (*Adora2a* knockout in CD1 mice<sup>56</sup>, in mixed 129sv mice crossed with C57BL/6 mice<sup>57</sup> and in congenic C57BL/6 mice<sup>58</sup>). *Adora2a*-knockout mice exhibit reduced exploratory behaviour and score higher in anxiety tests, with male mice being much more aggressive towards intruders<sup>56</sup>. Because of

their reduced activity they gain weight, especially in the form of fat. Their response to acute pain stimuli is slower and their blood pressure and heart rate are elevated<sup>56</sup>. Genetic *Adora2a*-knockout models have uncovered complex roles of the A<sub>2A</sub> receptor in tissue protection: that is, A<sub>2A</sub> receptor activation confers tissue protection in peripheral organs<sup>59,60</sup>, whereas its inactivation confers neuroprotection against brain injury<sup>57,61,62</sup>. Genetic knockout models have shown that both A<sub>1</sub> and A<sub>2A</sub> receptors are involved in mediating the sleep-promoting properties of adenosine in the brain<sup>63</sup>. Moreover, the arousal effects of caffeine seen in wild-type animals are blunted in *Adora2a*-knockout mice<sup>10</sup>. It is therefore conceivable that adenosine receptor ligands could be used as normal cognitive enhancers or sleep promoters.

Several strains of *Adora2b*-knockout mice have also been generated<sup>64,65</sup>. Given that A<sub>2B</sub> receptors are generally only expressed at low levels and they typically exhibit low affinity for adenosine in most assays<sup>66</sup>, it is surprising that *Adora2b*-knockout mice have very strong phenotypes, especially in relation to the vasculature<sup>64</sup>. *Adora2b*-knockout mice show low-grade inflammation of the vasculature at the baseline<sup>64</sup> and suffer from increased vascular leakiness in several organs<sup>29</sup>. A major reason for this could be that local adenine nucleotide signalling is very important in this compartment and thus adenosine levels can locally and transiently be considerably high<sup>29</sup>. Moreover, an increased susceptibility to ischaemic and inflammatory injuries is typically observed in the intestine<sup>67</sup>, liver<sup>68</sup>, kidney<sup>6,69</sup>, lung<sup>70</sup> and heart<sup>30,65</sup> of *Adora2b*-knockout mice, with conditions characterized by elevations in extracellular adenosine levels. Under such conditions, studies in *Adora2b*-knockout mice have shown that A<sub>2B</sub> receptor signalling is implicated in attenuating hypoxia-driven inflammation and in the adaptation of tissues to conditions of limited oxygen availability.

Conversely, activation of the A<sub>2B</sub> receptor has been shown to promote bone cell differentiation<sup>71,72</sup>, control glucose homeostasis<sup>73</sup> and regulate hyperlipidaemia and atherosclerosis<sup>74</sup>. However, some harmful effects of A<sub>2B</sub> receptor activation have also been reported — including promotion of tumour growth in the bladder and breast<sup>75</sup>, renal fibrogenesis<sup>76</sup> and inflammatory damage — in an experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis<sup>77</sup>. It is crucial to clarify whether these different and often opposing biological effects of the A<sub>2B</sub> receptor, as revealed by these knockout mouse models, are in part attributed to the confounding effect of different genetic backgrounds, developmental compensation or distinct biological effects under pathological conditions.

Similarly, knockout of the A<sub>3</sub> receptor in mice was surprising, resulting in marked phenotypes even at locations where the receptors are very sparse (for example, the brain) and where antagonists (or agonists) have little effect<sup>78,79</sup>. The reason for this remains unknown, but a possible developmental role for A<sub>3</sub> receptors has been postulated<sup>78</sup>. Mice deficient in the genes encoding ENTPD1 or NT5E (enzymes that mediate the generation of extracellular adenosine from ATP) also appear to be healthy and reproduce normally in a pathogen-free environment, but they have subtle defects in the vascular barrier function of several organs<sup>80,81</sup>. *Entpd1*-knockout mice have a prolonged bleeding time owing to a defect in their platelet function, which does not appear to be associated with a change in adenosine receptor signalling; rather, it appears to be related to desensitization of P2Y purinergic receptor 1 (caused by elevated ATP levels) through which adenine nucleotides signal and which is crucial for platelet activation<sup>104</sup>.

Together, these findings in *Adora*-knockout mice as well as *Entpd1*- and *Nt5e*-knockout mice indicate that long-term treatment with antagonists should be tolerated without having very serious consequences. The relatively minor differences between adenosine receptor knockout mice and wild-type mice under physiological conditions could theoretically be due

to major compensatory changes. Although there is little evidence for the existence of such compensatory changes (TABLE 3), it should be acknowledged that this has not yet been systematically examined. For example, studies in mice with concomitant deletion of all four adenosine receptors have not been yet published; it would be interesting to see whether these mice are viable and whether they show a phenotype at the baseline.

### Adenosine signalling in pathological conditions

Adenosine signalling is not very prominent under physiological conditions in most tissues, but aberrant adenosine signalling has been implicated as a common disease mechanism underlying inflammatory and ischaemic tissue damage<sup>3,59,82,83</sup>. This includes excessive inflammatory tissue damage such as that seen in acute liver injury, ischaemic kidney injury, acute lung injury, traumatic brain injury, ischaemic brain injury, epilepsy and certain neurodegenerative disorders such as Parkinson's disease and Huntington's disease<sup>20,61,84</sup>. As such, enhanced adenosine signalling is essential for the resolution of these pathological conditions associated with tissue inflammation and remodelling.

**Adenosine levels**—Intracellular adenosine formation is increased whenever ATP consumption exceeds ATP synthesis, which consequently leads to an increase in levels of AMP — the precursor of adenosine. ATP-dependent adenosine signalling occurs typically during conditions that are associated with the release of ATP from intracellular stores, and can occur during injurious conditions such as ischaemia and reperfusion<sup>14</sup>, hypoxia<sup>45</sup> (FIG. 1) or acute inflammation<sup>3</sup>. The surge in extracellular adenosine in response to pathological conditions is accompanied by increased levels of local inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumour necrosis factor (TNF), which leads to a delayed (~24-hour), marked and sustained increase in adenosine receptor expression on tissues and inflammatory cells<sup>32,33,85</sup>. Indeed, many studies have demonstrated that cellular responses to hypoxia are characterized by robust increases in extracellular adenosine production<sup>80,86</sup> and signalling events through adenosine receptors<sup>3</sup>.

Several studies have indicated that extracellular adenosine levels can rise from the baseline (20-300 nM)<sup>42,46,87-89</sup> to the low micromolar range in conditions of extreme physiology such as strenuous exercise or subsistence at high altitude and hence low ambient oxygen levels<sup>46,87,88</sup>. In ischaemic areas or after massive tissue trauma leading to cell death by necrosis, extracellular adenosine levels can increase to ~30  $\mu$ M<sup>88-90</sup>. As noted above, this increase could be due to increased intracellular formation of adenosine or to increased release of nucleotides. Recent research has focused on the latter possibility.

Although the ability of adenosine to stimulate adenosine receptors is dependent on the number of adenosine receptors<sup>2,15,91</sup>, and this number can change (that is, increase) under some circumstances (see below), it is more likely that an increase in adenosine receptor signalling in pathological conditions largely depends on increased levels of adenosine. Numerous reports have indicated that increased ATP-dependent adenosine signalling (for example, as a result of hypoxia exposure, acute inflammation, and so on) can profoundly alter disease susceptibility in both animal models of disease and patients<sup>4,92</sup>.

**Adenosine receptor expression and activity**—Together with dramatic increases in extracellular adenosine levels, conditions of tissue hypoxia are associated with enhanced expression of adenosine receptors — particularly the A<sub>2A</sub> receptor<sup>93</sup> and A<sub>2B</sub> receptor<sup>29,31,67,80,94,95</sup> — and a marked induction of enzymes that are responsible for ATP-dependent adenosine signalling, such as the ectonucleotidases ENTPD1 (REFS 80,86,96-99) and NT5E<sup>31,65,81,100</sup>, as these pathways are tightly controlled on a transcriptional level.

During ischaemia, activation of A<sub>2B</sub> receptors on the organs has been shown to promote ischaemia tolerance, improve oxygen-efficient metabolism<sup>30</sup> and protect against ischaemia-reperfusion injury of the heart<sup>30,65,94</sup>, kidneys<sup>6,69</sup> or the intestine<sup>31,67</sup>. Activation of A<sub>2B</sub> receptors can attenuate the activation and transmigration of inflammatory cells into post-ischaemic tissues and protect against sepsis-induced mortality by dampening excessive inflammation<sup>101</sup>. Moreover, activation of A<sub>2A</sub> receptors on inflammatory cells that have invaded the tissue can be an efficient treatment for reperfusion injury<sup>102-103</sup>. For example, activation of A<sub>2A</sub> receptors expressed on T cells<sup>102</sup> or dendritic cells<sup>296</sup> has been implicated in the attenuation of organ injury during hepatic or renal ischaemia, respectively.

The complex interplay between adenosine and adenine nucleotide signalling is highlighted in ENTPD1-deficient mice; these mice not only have elevated circulating nucleotide levels and related pathology<sup>104,105</sup> but also a deficiency in extracellular adenosine signalling, which is associated with increased vascular leakage during hypoxia<sup>80</sup>, or more severe tissue injury during lung inflammation or ischaemia and reperfusion<sup>96-99,106,107</sup>. In addition, protective and anti-inflammatory adenosine signalling against ischaemic and inflammatory injury is absent in the heart<sup>65</sup>, lung<sup>106</sup>, liver<sup>100</sup>, kidney<sup>108</sup>, intestine<sup>31</sup> and blood<sup>109</sup> of mice lacking ENTPD1.

A<sub>1</sub> receptors are downregulated by hypoxia in C6 glioma cells<sup>110</sup> but are upregulated in human temporal lobe epilepsy<sup>111</sup> and in mice in which seizures were induced by pentylenetetrazole (PTZ)<sup>112</sup>. Adenosine receptor expression may also be altered in cancer cells, but the significance of this is unclear. Hypoxia in solid tumours is associated with increased levels of ENTPD1- and NT5E-dependent adenosine, causing A<sub>2A</sub> receptor-mediated attenuation of the immune response against cancer. For example, genetic deletion of NT5E<sup>113</sup>, ENTPD1 (REF. 114) or the A<sub>2A</sub> receptor<sup>115</sup> in the host mouse is associated with the rejection of established immunogenic tumours via the regulation of T cell function and pathological angiogenesis. Conversely, A<sub>2A</sub> receptor inactivation in the brain has been consistently associated with protection against brain damage after ischaemia<sup>57</sup>, excitotoxicity<sup>116</sup>, traumatic brain injury<sup>117,118</sup> and neurodegeneration in Parkinson's disease<sup>119</sup> and Alzheimer's disease<sup>120</sup>. In some cases, both activation and inactivation of the A<sub>2A</sub> receptor have been shown to have a protective effect, including in animal models of Huntington's disease<sup>121,122</sup> and spinal cord injury<sup>123</sup>. Moreover, *ADORA3* mRNA expression is upregulated in hepatocellular carcinoma tissues in comparison with adjacent normal tissues<sup>124</sup> and is also upregulated in peripheral blood mononuclear cells (PBMCs) derived from patients with hepatocellular carcinoma compared to healthy individuals<sup>36</sup>; this indicates that the A<sub>3</sub> receptor in PBMCs may be a potential biomarker of hepatocellular carcinoma, reflecting the A<sub>3</sub> receptor status in remote tumours.

Several transcriptional mechanisms contribute to the induction of A<sub>2A</sub> receptor, A<sub>2B</sub> receptor and ectonucleotidase expression in response to stress, hypoxia and inflammation and other pathological insults as well as a local increase in adenosine levels. Hypoxia is associated with a transcriptional programme that results in the induction of ENTPD1 (REFS 80,96,97,99) and NT5E<sup>31,65,81,106</sup>, thereby elevating the capacity of different tissues for extracellular adenosine production<sup>13,14</sup>. Moreover, ENTPD1 and NT5E have been implicated in the conversion of ATP and ADP to adenosine on regulatory T cells, thereby providing an autocrine feedback loop to enhance the anti-inflammatory functions of this subset of T cells<sup>125</sup>.

Other studies have provided evidence that hypoxia also attenuates extracellular adenosine uptake<sup>126-128</sup> and its subsequent metabolic breakdown in the intracellular compartment, through the activity of the transcription factor hypoxia-inducible factor (HIF)<sup>129,130</sup>. The mechanism underlying the transcriptional induction of the A<sub>2A</sub> receptor in inflammatory

cells by IL-1 $\beta$  and TNF may involve activation of the transcription factor NF- $\kappa$ B<sup>85,131</sup>, whereas transcriptional pathways under the control of HIF have been described for the induction of the A<sub>2A</sub> receptor<sup>93</sup> and A<sub>2B</sub> receptor<sup>31,80,94,95</sup>. Similarly, in T cells, a system involving the regulation of adenosine, haem oxygenase 1, carbon monoxide and the A<sub>2A</sub> receptor leads to resolution of the inflammatory response<sup>132</sup>. The adenosine signal is amplified through a feed-forward mechanism with both a surge in extracellular ATP and/ or adenosine and the coordinated induction of the A<sub>2A</sub> receptor by local inflammatory cytokines.

Knockout studies have revealed that IL-6 is crucial in mediating A<sub>1</sub> receptor upregulation to amplify the A<sub>1</sub> receptor-mediated protection against PTZ-induced seizures<sup>112</sup>. Interestingly, extracellular ATP and adenosine signalling frequently control opposing biological effects<sup>133,134</sup> but they can also act synergistically<sup>43</sup>. Hypoxia shifts the balance from ATP towards adenosine signalling by enhancing the hydrolysis of adenosine precursor nucleotides. This shift from ATP towards adenosine signalling involves: increased extracellular adenosine levels by ATP release; induction of ENTPD1 expression by the transcription factor SP1 and of NT5E expression by HIF $\alpha$ ; induction of A<sub>2A</sub> receptor expression by HIF2 $\alpha$  and of A<sub>2B</sub> receptor expression by HIF1 $\alpha$ ; inhibition of adenosine kinase activity by HIF1 $\alpha$ ; and suppression of ENT1 or ENT2 activity by HIF1 $\alpha$ <sup>3</sup> (see FIG. 1; lower panel).

### Targeting adenosine receptors

Numerous articles have indicated the potential of adenosine receptors as therapeutic targets<sup>3,12,16,135</sup>. The introduction of useful radioligands for adenosine receptors has aided the drug discovery process. Over the past 20 years, medicinal chemistry efforts have generated agonists and antagonists with high affinity (a dissociation constant ( $K_d$ ) at low nanomolar concentrations) and high selectivity (>100- to 200-fold higher than other adenosine receptor subtypes) for the human variants of each of the four receptors<sup>1</sup>. Moreover, both agonist and antagonist ligands containing positron-emitting radio-isotopes have been developed to monitor the *in vivo* occupancy of adenosine receptors in humans<sup>18</sup>. As such, the lack of selective ligands is not a limiting factor for research and drug development on adenosine receptors, as has been the case for some other GPCRs. Moreover, there are continued medicinal chemistry efforts to develop novel adenosine ligands with refined structure-activity relationships, improved *in vivo* biodistribution and tissue selectivity, which is crucial to druggability (reviewed in REF. 18).

A bigger problem is the broad distribution of the receptors, and a possible approach for achieving tissue selectivity could be the use of partial agonists that would predominantly act where there is a high number of so-called 'spare' receptors. For example, it is well known that adipocytes have a large 'receptor reserve', and partial A<sub>1</sub> receptor agonists might therefore selectively activate those A<sub>1</sub> receptors<sup>136</sup>. Based on decades of preclinical studies, there have been numerous attempts to develop drugs and many clinical trials are now underway; for some recent examples, see TABLE 1. The clinical indications for drugs that are in advanced clinical trials targeting adenosine receptors include Parkinson's disease, chronic heart failure as well as inflammatory and autoimmune disorders. Some of the most recent developments and challenges in these key therapeutic areas are discussed below.

**Parkinson's disease**—Based on the concentrated striatal expression of A<sub>2A</sub> receptors, the antagonistic A<sub>2A</sub>-D<sub>2</sub> receptor interaction and preclinical studies demonstrating motor benefit in rodent and non-human primate models of Parkinson's disease<sup>50,52,137,138</sup>, A<sub>2A</sub> receptor antagonists have emerged as leading non-dopaminergic drugs for the treatment of Parkinson's disease. Over the past 8 years, a total of 25 clinical trials have been conducted



(see TABLE 1 and Supplementary information S1 (table)). Six double-blind, placebo-controlled clinical Phase IIb and Phase III trials of istradefylline (KW-6002), involving a total of >2,000 patients with advanced Parkinson's disease, and one Phase IIb trial with prelad enant (SCH420814), involving 253 patients with advanced Parkinson's disease, have been reported<sup>139</sup>. These clinical Phase IIb and Phase III trials have shown a modest but significant reduction in the average 'off-time' by about 1.7 hours compared to the optimal l-DOPA (1-3,4-dihydroxyphenylalanine; also known as levodopa) dose regimen<sup>138</sup>. However, another similar Phase III clinical trial with istradefylline in patients with Parkinson's disease did not demonstrate a significant reduction in the 'off-time' compared to placebo<sup>140</sup>.

These relatively modest motor effects differed from the preclinical studies with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-treated primates, which unambiguously demonstrated a marked motor benefit. Clearly, further clinical investigation of A<sub>2A</sub> receptor antagonism is warranted to understand its full potential as a treatment for Parkinson's disease. An encouraging and consistent finding in the clinical trials of A<sub>2A</sub> receptor antagonists for Parkinson's disease is that istradefylline and preladenant had an excellent safety profile<sup>138,139</sup>. In April 2007, Kyowa Hakko Kirin Pharma filed a new drug application (NDA) for istradefylline for the treatment of advanced Parkinson's disease; however, the FDA issued a non-approval letter in 2008, citing the need for additional efficacy data. Currently, several Phase IIb and Phase III trials for A<sub>2A</sub> receptor antagonists (other than istradefylline) are still underway and these agents remain one of the leading non-dopaminergic treatment candidates for Parkinson's disease<sup>141</sup>.

**Chronic heart failure**—Impaired renal function is common in patients with acute heart failure; it directly contributes to deterioration of the heart and is associated with an adverse outcome, including increased mortality. Local adenosine production in the kidney is increased in patients with heart failure as a result of hypoxia caused by reduced renal perfusion and by stimulation with diuretics<sup>142</sup>. Based on our understanding of the mechanisms associated with renal dysfunction and the demonstrated control of renal function via A<sub>1</sub> receptors, A<sub>1</sub> receptor antagonists were developed. These antagonists reduced the risk of persistent worsening renal failure by >50% in a Phase IIb study involving 301 patients with acute heart failure, and improved renal plasma flow in 63 ambulatory patients with chronic heart failure<sup>143</sup>.

Based on these promising results, a placebo-controlled, randomized Phase III trial involving 2,033 patients with acute heart failure (the PROTECT study) was carried out with the A<sub>1</sub> receptor antagonist rolofylline; this was the largest study to date involving the use of A<sub>1</sub> receptor antagonists to target renal function. Unfortunately, the results were disappointing and rolofylline did not prevent persistent worsening renal function<sup>21,144</sup>. The reason for this absence of renoprotective effects is likely to be due to an enhanced diuretic effect (as evident by more pronounced weight loss) in the rolofylline group, which may have offset the effects of rolofylline on the preservation of renal function<sup>22</sup>. Moreover, pharmacological and genetic studies have clearly demonstrated that A<sub>1</sub> receptors mediate protective effects against ischaemic kidney injury and brain injury, which is consistent with the increased frequency of stroke and seizure activity in clinical trials of A<sub>1</sub> receptor antagonists<sup>22</sup>. Thus, the development of A<sub>1</sub> receptor antagonists for the treatment of disorders associated with impaired fluid retention, such as congestive heart failure, should proceed with caution.

**Inflammatory diseases, autoimmune disorders and cancer**—Given the high levels of expression of all four subtypes of adenosine receptors in cells of the immune system and the dynamic modification of their expression within inflammatory and tumour environments, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors are being actively pursued as therapeutic targets for autoimmune diseases, chronic inflammatory disorders and cancer<sup>145</sup>. For

example, based on preclinical pharmacology and encouraging safety data in Phase I studies, the A<sub>3</sub> receptor agonists CF101 (also known as IB-MECA) and CF102 have been tested in several Phase II trials for rheumatoid arthritis<sup>146</sup>. CF101 treatment was associated with a 20% improvement in disease symptoms according to the classification of rheumatoid arthritis responses by the American College of Rheumatology. Based on anecdotal findings from this trial indicating that CF101 also improved indicators of dry eye syndrome, a follow-up Phase II trial (randomized, double-blind and placebo-controlled) was carried out, which determined that CF101 improved the clearance of corneal staining, tear break-up time and tear meniscus height<sup>147</sup>. Notably, orally administered A<sub>3</sub> receptor agonists (given at doses that are effective for treating dry eye syndrome) do not cause cardiovascular and other side effects<sup>147</sup>.

The efficacy and safety of CF101 was also tested in a Phase II trial of moderate to severe chronic plaque-type psoriasis<sup>37</sup>; it was found to be effective and thus advanced to Phase III trials for these indications as an anti-inflammatory agent. In addition, active Phase II clinical trials are underway to test the efficacy of A<sub>3</sub> receptor agonists for the treatment of hepatocellular carcinoma and hepatitis<sup>37</sup>. Furthermore, experimental studies in mice suggest a possible use of A<sub>3</sub> receptor agonists in suppressing melanoma growth by inducing T cell-mediated adoptive immunity<sup>148</sup> and in the control of chronic neuropathological pain<sup>37,149</sup>. These therapeutic effects of CF101 are believed to be mediated by its inhibition (via cAMP and calcium signalling) of the oxidative burst and its anti-inflammatory activity<sup>150</sup>. However, it should be noted that both pro-inflammatory and anti-inflammatory effects of A<sub>3</sub> receptor activation have been demonstrated, depending on the cell type and animal species being studied<sup>151</sup>.

**Sickle cell disease**—Patients with sickle cell disease have periodic episodes of vaso-occlusive crisis and, in some cases, life-threatening pulmonary vaso-occlusion. Historically, microvascular occlusion was attributed to rigid sickled erythrocytes. Recently, ischaemia-reperfusion injury with resultant white cell activation has been implicated as a crucial contributor to the pathophysiology of sickle cell disease<sup>152</sup>. Like ischaemia-reperfusion injury, sickle cell disease is associated with increased levels of adenosine. An experimental study has provided strong evidence that A<sub>2B</sub> receptor activation on erythrocytes promotes sickling in patients with sickle cell disease<sup>153</sup>. However, treatment with an A<sub>2A</sub> receptor agonist has been indicated to attenuate sterile inflammation and T cell activation in this disorder<sup>152</sup>. A clinical trial in patients with sickle cell disease is currently being conducted using the FDA-approved A<sub>2A</sub> receptor agonist regadenoson (ClinicalTrials.gov identifier: NCT01085201)<sup>154,297</sup>. Thus, different adenosine receptors (A<sub>2A</sub> or A<sub>2B</sub> receptors) can mediate opposing effects in a single disease<sup>155</sup> — a finding that may be of considerable significance when developing adenosine receptor-targeting agents.

### Challenges in targeting adenosine signalling

Despite the clear potential of adenosine receptors as therapeutic targets, only one agent has so far reached the clinic. From a medicinal chemistry perspective, standard pharmacological assays of selectivity and efficacy may not provide sufficient information on the different bio-distribution and pharmacokinetics of adenosine ligands with subtle structural differences. Understanding differences in local drug distribution are important for the prediction of potential side effects, given the ubiquitous presence of adenosine and the widespread distribution of its receptors. Despite the large numbers of selective adenosine receptor agonists and antagonists reported in the literature, the clinical application of adenosine ligands is lacking. Below, we discuss the various challenges that are likely to be hampering the success of drugs targeting adenosine receptors.

**Measurement of adenosine levels**—As mentioned above, determining the local levels of adenosine is crucial to understanding its biology and pharmacology. Therefore, the fact that local adenosine concentrations rapidly fluctuate and are difficult to measure represents a major challenge for this field. Direct biosensor-based measurements of adenosine and microdialysis probes coupled with electrochemical detectors are commonly used to probe or sample the extracellular concentration of adenosine and various bodily fluids in different tissues under physiological and pathological conditions<sup>156</sup>. However, the microdialysis technique is known to destroy some cells when the probe is inserted into a tissue, which results in the release of ATP that is converted to adenosine, thereby elevating local adenosine levels. In addition, when the microdialysis probe is left in a tissue for an extended period of time, it is covered by cells such as glial cells, which metabolize adenosine and reduce adenosine levels before they can be measured.

Moreover, adenosine can be very rapidly formed during sampling — for example, the ATP hydrolysis that occurs when a tissue is extracted generates much higher levels of adenosine in a few seconds than were initially present. Thus, adenosine levels can only be accurately determined in tissues via the freeze-clamping technique, but this obviously precludes any finer structural resolution. When sampling blood, it is difficult to avoid platelet destruction and the subsequent release of ADP and ATP, which are rapidly broken down to adenosine. This means that the accurate determination of adenosine levels locally under physiological and pathophysiological conditions rarely occurs, and unfortunately the literature is replete with incorrect estimates.

Another major challenge relates to the difficulty in distinguishing between the various sources of extracellular adenosine under physiological and pathological conditions. For example, despite excellent evidence that ATP released from astrocytes is an important signal under numerous circumstances<sup>39</sup>, a recent study provides compelling evidence that neuronal adenosine release — and not astrocytic ATP release — mediates feedback inhibition of excitatory activity in seizure models<sup>157</sup>. Therefore, to determine the unique adenosine signals (and therefore adenosine receptors) that are associated specifically with disease status, it is crucial to develop strategies that are capable of detecting and characterizing the changes in extracellular adenosine levels in different definable extracellular domains within the brain parenchyma (that is, neuronal and/or synaptic, astrocytic, microglial or vascular domains).

**Measurement of the number of adenosine receptors**—Although we do not know how receptor distribution in patients varies in different diseases, recent studies indicate that this could potentially be studied by monitoring receptors using *in vivo* imaging methods. Indeed, two positron emission tomography (PET) ligands — the A<sub>2A</sub> receptor antagonist ligand [<sup>11</sup>C]-SCH442416 and the A<sub>2A</sub> receptor agonist ligand [<sup>11</sup>C]-TMSX — were recently developed and have been successfully used to measure the distribution of A<sub>2A</sub> receptors in the striatum of patients with Parkinson's disease using PET imaging<sup>158,159</sup>. These studies have provided two important insights: first, that the number of A<sub>2A</sub> receptors in the putamen is increased in patients with Parkinson's disease who have dyskinesia, indicating a possible involvement of A<sub>2A</sub> receptors in the pathogenesis of l-DOPA-induced dyskinesia; and second, that there are more A<sub>2A</sub> receptors in drug-naïve patients with Parkinson's disease than in controls, which possibly compensates for the depletion of dopamine<sup>158,159</sup>.

These A<sub>2A</sub> receptor ligands will probably also be very useful in the direct assessment of drug-A<sub>2A</sub> receptor interactions in disease pathogenesis and the development of unwanted side effects. They should also aid in improving the design of clinical trials of A<sub>2A</sub> receptor antagonists for Parkinson's disease, as one would be able to adequately monitor receptor occupancy over time. In addition, as A<sub>2A</sub> receptor expression in human tissues has been

shown to correlate with disease progression, such as in Huntington's disease<sup>160,161</sup>, these ligands may be used as potential biomarkers to monitor the disease course during clinical trials. Furthermore, PET ligands for other adenosine receptors could be used to monitor disease progression in ischaemia and cancer by assessing the expression of adenosine receptors in patients suffering from these conditions.

**Complexity of adenosine signalling during disease course**—In acute injury settings, hypoxia-driven elevations in extracellular adenosine levels activate pathways that promote tissue adaptation to conditions of limited oxygen availability and dampen hypoxia-driven inflammation<sup>13,15</sup>. These pathways include those involved in restoring normal oxygen levels<sup>153</sup>, enhancing metabolic ischaemia tolerance<sup>30</sup> and dampening hypoxia-induced inflammation<sup>3,8,82</sup>. Indeed, preclinical studies have shown that adenosine signalling is beneficial in acute injury of the lungs<sup>70,162</sup> and ischaemic injury of the kidneys<sup>69,60</sup>, the heart<sup>65</sup>, the gastrointestinal tract<sup>163</sup> and the liver<sup>164</sup>. However, if elevated adenosine levels are sustained beyond the acute injury phase, hypoxic adenosine responses can become detrimental owing to the activation of pathways that promote tissue injury and fibrosis<sup>165</sup>. For example, chronic elevations of adenosine levels during hypoxia can contribute to tissue fibrosis in different organs, including the lungs<sup>166,167</sup>, liver<sup>168</sup>, skin<sup>169</sup> and penis<sup>170,171</sup>. Therefore, under conditions of chronically elevated adenosine levels, blockade of adenosine signalling appears to be beneficial.

An interesting example of the opposing effects of adenosine signalling in a single disease model comes from studies of bleomycin-induced lung injury. Studies in *Nt5e*-knockout mice<sup>172</sup>, which are unable to convert extracellular AMP to adenosine and thus develop a more severe degree of lung injury during the acute phase of bleomycin-induced lung disease, have shown that extracellular adenosine production is implicated in lung protection during acute lung injury. Subsequent studies in other models of lung injury — such as ventilator-induced lung injury<sup>70</sup>, lipopolysaccharide-induced lung inflammation<sup>32</sup> or lung inflammation in adenosine deaminase-deficient mice<sup>173</sup> — have implicated A<sub>2B</sub> receptor signalling in adenosine-dependent protection from acute lung injury<sup>92</sup>. However, there is contrasting evidence that A<sub>2B</sub> receptor signalling can be detrimental in more chronic forms of lung injury<sup>174</sup>. As such, there were substantial reductions in pulmonary fibrosis in mice following the genetic removal of *Adora2b* (which encodes the A<sub>2B</sub> receptor) during the chronic phase of bleomycin-induced lung injury, which indicates a profibrotic role for this receptor. These studies highlight the opposing roles of A<sub>2B</sub> receptor signalling during acute versus chronic stages of bleomycin-induced lung injury<sup>175</sup>.

Similarly, both acute activation<sup>176</sup> or prolonged inactivation of A<sub>2A</sub> receptors can partially protect against sepsis<sup>177</sup> via the immunosuppressive cytokine IL-10 (REF. 178). Moreover, both protective and detrimental effects of A<sub>2A</sub> receptor activation are observed at different stages of liver injury<sup>59,179</sup>, traumatic brain injury<sup>117</sup>, ischaemic spinal cord injury<sup>123</sup> and Huntington's disease<sup>180</sup>. Finally, as discussed above, the opposing effects of A<sub>1</sub> receptor antagonists in renal function may have contributed to the failure of the first Phase III clinical trial of A<sub>1</sub> receptor antagonists in acute heart failure<sup>144</sup>. The opposing effects of adenosine signalling at different stages of disease clearly represent a major challenge for drug development.

The causes of such opposing and/or time-dependent effects are one of the central questions in this field. The opposite effects of adenosine receptor activation at different stages of various disorders could reflect the complexity of adenosine receptor signalling on various cell types; each of these receptors can have a detrimental or protective effect depending on the nature of the tissue injury and associated pathological conditions. A major challenge in developing effective therapeutic strategies targeting adenosine receptors is to decipher these

complex actions of adenosine receptors at the level of cellular and tissue specificity as well as disease progression, and to define the specific interactions between adenosine receptors and other neurotransmitter receptors. Moreover, other signalling molecules — such as other neurotransmitters in the brain — can further complicate this interaction. In the case of opposite effects on lung injury by *Adora2b* knockout and *Nt5e* knockout, A<sub>2B</sub> receptor-dependent regulation of IL-6 production was identified as a potential mechanism involved in the diminished pulmonary fibrosis seen in *Adora2b*-knockout mice<sup>175</sup>.

In the brain, a switch between a protective versus damaging effect of A<sub>2A</sub> receptors has been shown to be associated with the local interactions between adenosine and glutamate. In response to various brain injuries, extracellular levels of adenosine as well as glutamate increase rapidly (within minutes) and dramatically (up to 100-fold) owing to their presynaptic release from neurons and to the exocytosis and possible reversal of glutamate uptake from astrocytes<sup>181,182</sup>. Remarkably, increasing the local levels of glutamate redirected A<sub>2A</sub> receptor signalling from the PKA to the PKC pathway, thus switching the effect of A<sub>2A</sub> receptor activation from anti-inflammatory to pro-inflammatory<sup>117</sup>. This glutamate-A<sub>2A</sub> receptor interaction can also be demonstrated *in vivo* in a cortical impact model of traumatic brain injury in mice. Thus, extrasynaptic glutamate levels can control the effect of A<sub>2A</sub> receptor activation both *in vivo* and *in vitro*, switching it from anti-inflammatory and neuroprotective to pro-inflammatory and cytotoxic<sup>117</sup>. Such findings may explain — at least in part — the opposing effects of A<sub>2A</sub> receptor ligands on tissue injury by demonstrating that the effects of A<sub>2A</sub> receptors in brain injury are context-dependent as they can be influenced by local glutamate levels.

**Tolerance to adenosine receptor ligands**—Another finding, possibly related to the cases described above, is that repeated exposure to adenosine receptor ligands (particularly to A<sub>1</sub> receptor ligands and caffeine) leads to the rapid development of tolerance, which is evident in both motor and cardiovascular responses<sup>183,184</sup>. In some cases, repeated or prolonged exposure even results in the development of opposite effects, as seen with caffeine exposure in various neuronal injury models; although acute caffeine exposure exacerbates tissue injury, chronic caffeine administration usually has a protective effect<sup>183,184</sup>. The mechanism underlying this desensitization is not clear.

Similar paradoxical effects of acute versus chronic treatment have been reported for A<sub>1</sub> receptor agonists and antagonists<sup>185</sup>. Acute treatment with A<sub>1</sub> receptor agonists has a protective effect in brain injury, whereas chronic treatment increases ischaemic brain injury in rodents<sup>185-187</sup>. A<sub>2A</sub> receptor agonists also induce desensitization after prolonged treatment in cultured cells, as a result of the binding of the carboxyl terminus of the A<sub>2A</sub> receptor to F-actin crosslinking protein (also known as  $\alpha$ -actinin), which promotes A<sub>2A</sub> receptor internalization<sup>188,189</sup>. Conversely, unlike caffeine, selective A<sub>2A</sub> receptor antagonists do not induce the rapid development of tolerance; motor responses to A<sub>2A</sub> receptor antagonists did not decline even after 1 week of repeated treatments in an animal model of Parkinson's disease<sup>190,191</sup>. This finding is encouraging for the current development of A<sub>2A</sub> receptor antagonists for neurodegenerative disorders, which would require chronic treatment for several years.

#### **Distinct effects of adenosine signalling in different cellular elements**—

Targeting the same receptor in different cells within the same tissue can induce fundamentally different outcomes. One approach that can be applied to identify distinct functions of adenosine receptors in different cells within a tissue is to develop cell-specific conditional knockout models of individual adenosine receptor-encoding genes using the Cre-*loxP* system<sup>6</sup>. Brain-region-specific as well as cell-specific *Adora*-knockout mice are currently available (reviewed in REF. 47). Regional deletion of *Adora2a* genes has been

achieved in the entire forebrain (that is, the striatum, cortex and hippocampus)<sup>192,193</sup> or only in the striatum<sup>194</sup>. In addition, local deletion of the *Adora1* gene in hippocampal CA1 or CA3 neurons and the *Adora2a* gene in the nuclear accumbens has been attained by the local injection of adeno-associated virus (AAV) vectors containing the *Cre* transgene into the brains of mice expressing adenosine receptor-encoding genes in which a critical exon is flanked by *loxP* sites<sup>195</sup>. This strategy allowed for a temporal and regional specificity that has uncovered previously under-explored or under-appreciated functions of adenosine receptors. For example, using local infection with AAV vectors carrying short hairpin RNA targeted to produce site-specific silencing of the *Adora2a* gene, we examined the specific role of A<sub>2A</sub> receptors in the basal ganglia in the modulation of the sleep-wake cycle and demonstrated that the arousal effect of caffeine is mediated by A<sub>2A</sub> receptors in the nuclear accumbens shell<sup>10</sup>.

Similarly, conditional knockout of *Adora2b* genes using the *Cre-loxP* system has been developed — for example, with selective deletion of the *Adora2b* gene in vascular endothelial cells<sup>6</sup>. In addition, several other recent technological advances allow systems-level study of GPCR function in freely behaving animals. These include specific local modulation of neuronal activity using genetically engineered optical switches (for example, channel rhodopsin)<sup>196,197</sup> as well as reversible silencing (for example, non-mammalian chloride channels)<sup>198</sup> and activation (for example, stimulatory GPCRs)<sup>199,200</sup> of neurons. Applying these technical advances to the study of adenosine receptors will provide a new level of understanding of adenosine receptor function and could facilitate the development of adenosine receptor-targeting drugs.

**Different effects in developing and mature individuals**—Another potential complication is the differential effects of adenosine receptor activation (or inactivation) at different stages in development. For example, studies in immature mice (7 days old) exposed to hypoxic brain injury indicate that A<sub>2A</sub> receptor signalling has a protective role<sup>201</sup>, whereas studies in adult mice in which the *Adora2a* gene is knocked out suggest that this receptor has a harmful effect in brain ischaemia during adult-hood<sup>57</sup>. Indeed, many studies in adult mice support the use of A<sub>2A</sub> receptor antagonists to treat brain ischaemia<sup>62,202,203</sup>. There is some evidence for similar differential effects of A<sub>1</sub> receptors. Thus, in newborn mice, A<sub>1</sub> receptor agonism appears to have a deleterious effect in response to hypoxia-induced brain (white matter) injury<sup>204</sup>, whereas in adult mice it has a protective effect. The underlying reason for this could be that there are differing mechanisms mediating neuronal damage in the immature and mature brain.

**Adenosine receptor heterodimerization**—There are reports showing that adenosine receptors — like other class A GPCRs — can undergo homo- and heterodimerization or even oligomerization. For example, a heterodimer composed of an A<sub>2A</sub> receptor and a D<sub>2</sub> receptor has been observed in cultured cells as well as in the striatum of intact animals<sup>2,205-207</sup>. It has been suggested that some of the pharmacological differences in A<sub>2A</sub> receptor antagonists (that were tested for anti-parkinsonian effects in an animal model of Parkinson's disease) may be correlated with differences in receptor heterodimerization (A<sub>2A</sub>-D<sub>2</sub> versus A<sub>2A</sub>-A<sub>1</sub>) at postsynaptic and presynaptic sites<sup>208,209</sup>. Moreover, it was recently suggested that the presence of the A<sub>2A</sub> receptor may be important for the proper targeting of A<sub>2B</sub> receptors to cell surface membranes, through the formation of A<sub>2A</sub>-A<sub>2B</sub> receptor heterodimers<sup>210</sup>.

Given that adenosine receptors can be co-immuno-precipitated with many different GPCRs even in the same cell and definitely in the same tissue, this represents a major challenge<sup>211-213</sup>. However, these results have not yet been confirmed *in vivo* and there are numerous reports indicating unequivocally that monomeric receptors are sufficient to induce

signalling<sup>214</sup>. Therefore, until it is demonstrated that GPCR heterodimerization occurs in intact animals and that it confers major pharmacological consequences, we suggest that this additional potential complexity should not yet be taken into account for drug development.

**Implications of widespread caffeine use**—The methylxanthine caffeine is undoubtedly the most widely consumed psychoactive substance. It is estimated that the majority of the world's adult population consumes caffeine in sufficient doses to influence behaviour on a daily basis<sup>184</sup>. It was realized many years ago that methylxanthines antagonize adenosine receptors and it is now accepted that many of the actions of caffeine are due to its effect of reducing the number of adenosine receptors to half the normal levels<sup>215</sup>. Nonetheless, the fact that long-term caffeine use is not associated with increased morbidity, and the fact that mice with reduced numbers of receptors fare well, strongly suggests that even the long-term use of drugs that block adenosine receptors can be safe.

Substantial adenosine receptor antagonism has already been achieved in so many people via their daily consumption of caffeine-containing beverages; this complicates the interpretation of drug trials studying adenosine receptor-targeted agents. A novel adenosine receptor antagonist must therefore be proven to confer benefit over and above that provided by readily available and low-cost caffeine. Caffeine use in humans is limited by effects that are independent of adenosine receptor blockade. Even at the receptor that is most readily blocked by caffeine — the A<sub>2A</sub> receptor — it is difficult to achieve as much as 50% occupancy. It can be calculated that the daily consumption of three to four regular cups of coffee results in approximately 50% A<sub>1</sub> and A<sub>2A</sub> receptor occupancy for several hours<sup>184</sup>. This conclusion is supported by genetic studies in mice: heterozygous *Adora1*- and *Adora2*-knockout mice (that is, mice in which there is a 50% reduction in the expression of A<sub>1</sub> and A<sub>2A</sub> receptors) recapitulate some of the effects associated with long-term caffeine use<sup>215</sup>. A more selective antagonist therefore has the potential of affording more complete blockade and hence — at least in theory — a larger therapeutic benefit.

A clinical trial must very carefully assess the caffeine intake of each person enrolled in the study, as a trial in which the participants do not consume caffeine would not be a representative patient sample, and individuals who have recently refrained from caffeine use would encounter other issues, including relief from some of the (albeit weak) abstinence symptoms.

Caffeine use in the general population provides important clues to the potential therapeutic indications of adenosine receptor antagonists. Recent case-control and prospective studies have linked caffeine consumption with a reduced risk of Parkinson's disease<sup>216,217</sup>, dementia and Alzheimer's disease<sup>218-220</sup>, type 2 diabetes<sup>221,222</sup> and chronic liver cirrhosis<sup>221</sup>. In addition, the common use of caffeine for the treatment of apnoea in premature infants has been associated with reduced retinopathy in 2-year follow-up studies<sup>223,224</sup>. This has prompted the investigation of A<sub>2A</sub> receptor-mediated control of retinal vascularization in the developing eye and the involvement of these receptors in oxygen-induced retinopathy in an animal model of retinopathy of prematurity<sup>11</sup>. Similarly, since 2000, at least five large prospective studies have firmly established a relationship between increased caffeine consumption and a decreased risk of developing Parkinson's disease; the initial study was from the Honolulu Heart Program<sup>217</sup>, which was followed by the Health Professionals' Follow-Up Study and the Nurses' Health Study (involving 47,351 men and 88,565 women)<sup>216</sup>, the Finnish Mobile Clinic Health Examination Survey<sup>225</sup> and the study carried out by the NeuroGenetics Research Consortium<sup>226</sup>. Caffeine, A<sub>2A</sub> receptor antagonists and *Adora2a* knockout have also demonstrated neuroprotective effects in animal models of Parkinson's disease<sup>119</sup>. These findings led to a clinical trial of A<sub>2A</sub> receptor antagonists as a monotherapy for *de novo* early-stage Parkinson's disease<sup>227</sup>.

The main pharmacological targets of caffeine are adenosine receptors, particularly A<sub>2A</sub> receptors, as revealed by mouse knockout studies<sup>20,228</sup>. Therefore, pharmacokinetic and pharmacodynamic (PK/PD) interactions between caffeine and adenosine receptor ligands may contribute to the varying responses to an adenosine receptor-based drug in clinical trials (BOX 2). Indeed, PK/PD analyses show that recent caffeine ingestion (equivalent to two to four cups of coffee) affects A<sub>2A</sub> receptor agonist-induced myocardial perfusion imaging<sup>229</sup> and reduces the efficacy of adenosine in the treatment of paroxysmal supraventricular tachycardia<sup>230</sup>. In this regard, it is somewhat surprising that several current clinical trials of adenosine receptor antagonists have not taken caffeine consumption into consideration in their clinical trial designs. For example, several recent Phase III clinical trials of the A<sub>2A</sub> receptor antagonist istradefylline (KW-6002) and preladenant (SCH420814) in patients with advanced Parkinson's disease apparently did not include data on caffeine consumption<sup>139,227,231-236</sup>. We speculate that if caffeine consumption is taken into consideration in clinical trials, we may see a clearer and better clinical response to adenosine-based drugs in smaller patient populations. Thus, in our view, careful consideration of caffeine pharmacology should be incorporated into most clinical trial programmes of adenosine receptor-based therapies to address this 'elephant in the room' in clinical studies. Genetic studies involving caffeine may offer a unique opportunity for identifying useful pharmacogenetic markers to predict individual responses to caffeine and adenosine drugs in clinical trials (BOX 2).

### Alternative therapeutic approaches

**Indirect receptor targeting**—Adenosine receptors are found on many cells in the body, so exogenous adenosine agonists pose a substantial risk of inducing side effects. As adenosine itself is rapidly degraded and does not readily pass the blood-brain barrier, it can be given acutely — for example, to regulate cardiac rhythm. Adenosine receptor ligands that are metabolically more stable would be able to reach all receptors and, because of their generally high affinity, would provide prolonged stimulation. Alternatively, instead of directly targeting adenosine receptors, one could raise the levels of endogenous adenosine. This approach could provide some degree of tissue specificity, but this has not yet been systematically investigated.

Historically, drugs such as dipyridamole and methotrexate have been found to increase levels of endogenous adenosine. At present, clinical trials are being carried out to increase extracellular adenosine levels in humans. For example, the adenosine uptake inhibitor dipyridamole is used in patients for pharmacological stress echocardiography (as a coronary vasodilator) or as an inhibitor of platelet aggregation. However, dipyridamole could be easily used for enhancing the beneficial effects of adenosine receptors in other biomedical conditions, such as acute kidney injury, myocardial ischaemia or colitis<sup>6</sup>. An FDA-approved adenosine deaminase inhibitor, deoxycoformycin (Nipent; Astex Pharmaceuticals), can increase extracellular adenosine levels and is currently in clinical use for the treatment of haematological malignancies<sup>237</sup>. However, the production of the cytotoxic 2-deoxyadenosine owing to the inhibition of adenosine deaminase is likely to contribute to this therapeutic outcome.

**Allosteric enhancers**—Another possibility is to use so-called allosteric enhancers. This approach was first described for the A<sub>1</sub> receptor<sup>238</sup> when an allosteric enhancer was used to increase the responsiveness of this receptor to endogenous adenosine at sites of its production. At the molecular level, an allosteric enhancer amplifies the action of agonists by stabilizing the ternary complex formed by the agonist, the adenosine receptor and the G protein, thus minimizing the side effects of the drug. At least theoretically, the ability to target the receptor at two sites might increase tissue specificity because the drug would act



in concert with locally increased adenosine under pathological conditions and have little effect on sites where there are low basal adenosine levels. The recent identification of allosteric regulatory sites such as sodium and water control sites by GPCR crystal structure studies<sup>239</sup> has provided a detailed atomic structural framework that can substantially assist the biochemical identification and analysis of allosteric sites on adenosine receptors.

### Multiple target drugs for adenosine signalling

The redundancy of adenosine receptor signalling, as revealed by studies in single *Adora*-knockout mice, indicates that targeting multiple steps and pathways involved in adenosine receptor signalling (such as adenosine generation and metabolism as well as adenosine receptors themselves) may be synergistic and more efficacious than targeting an individual step or pathway. A few multidrug target approaches involving the modulation of adenosine receptor signalling have been postulated and developed. For example, a single drug with dual actions —  $A_{2A}$  receptor antagonism and monoamine oxidase B inhibition — is expected to offer potential synergistic effects on neuroprotection and possible motor stimulation in Parkinson's disease<sup>240</sup>. Similarly, a new drug — T1-11 — that exhibits a dual action composed of adenosine transport (equilibrative nucleoside transporter 1; ENT1) inhibition and  $A_{2A}$  receptor agonism has been developed and tested to show its efficacy in an animal model of Huntington's disease<sup>241</sup>.

Another elegant use of network pharmacology is the development of a novel type of prodrug based on our understanding of the ATP-dependent adenosine signalling cascade<sup>242</sup>. In this approach, 5'-phosphate prodrugs of  $A_{2A}$  receptor agonists are prepared that are preferably cleaved at sites of inflammation where NT5E is highly expressed to release the active  $A_{2A}$  receptor agonists<sup>243</sup>. This prodrug approach not only allows the site-specific action of the  $A_{2A}$  receptor within the tissues where NT5E is enriched but also avoids the potent hypotensive effect of  $A_{2A}$  receptor agonists — a major limiting factor in their development.

### Signalling pathway-biased drugs

Adenosine receptor activation can trigger alternative signalling pathways via different coupling and by functional interactions with a broad range of other GPCRs and signalling molecules<sup>244</sup>. The functional selectivity of different signalling pathways offers a new opportunity for developing signalling pathway-biased drugs that selectively activate a specific intracellular adenosine receptor signalling pathway that is essential for a particular biological function. By systematically screening compound libraries of GPCRs for this 'biased' form of signalling,  $\beta$ -arrestin-biased  $D_2$  receptor ligands were successfully identified<sup>245</sup> and the discovery of a  $\beta$ -arrestin-biased  $A_1$  receptor ligand was also attempted, albeit with limited success<sup>246</sup>. New knowledge of the diversity and modularity of GPCR structures, the  $A_{2A}$  receptor in particular, from a recent structural biology crystallization study offers opportunities to target receptor structures with different signalling functions<sup>239</sup>. The structure of the  $A_{2A}$  receptor in several functional states was recently described<sup>247-249</sup>, revealing that the  $A_{2A}$  receptor antagonist ZM241385 binds in an extended conformation perpendicular to the plane of the membrane bilayer<sup>249</sup>, whereas  $A_{2A}$  receptor agonists bind in parallel to the plasma membrane, similarly to retinal rhodopsin<sup>248</sup>. Moreover, the allosteric regulatory site (such as sodium and water control sites) was recently identified<sup>239,250</sup>. It is our hope that these exciting results will help in the rational design of novel drugs targeting specific signalling pathways and functions of  $A_{2A}$  receptors as well as other adenosine receptors<sup>251</sup>.

## Conclusions

Over the past 80 years, evidence has accumulated to show that extracellular adenosine is an important modulator of physiological and pathological processes. It has emerged that adenosine receptors can be safely targeted by drugs, and it is possible to generate highly specific agonists and antagonists of adenosine receptors. As a result, increasing numbers of clinical trials testing novel adenosine-based drugs in various indications have been initiated during the past decade. Although adenosine is present in virtually all cells, levels of endogenous adenosine are sufficient to activate adenosine receptors only where they are most abundant. Activation of adenosine receptors has the potential to be beneficial in the treatment of various inflammatory and autoimmune disorders, pain, arrhythmia as well as sleep disorders and some metabolic disorders. However, given the wide-spread distribution of adenosine receptors, agonists of these receptors can have effects in most tissues and produce a variety of responses, which makes them difficult to use. Targeting adenosine receptors indirectly using drugs that enhance endogenous local adenosine production or reduce adenosine degradation may have more general applicability.

Adenosine receptor antagonists will have a larger effect where adenosine receptor activation is enhanced — for example, in various pathological processes, including several instances of local reduction of blood flow or local tissue destruction. However, several large Phase III clinical trials of A<sub>2A</sub> receptor antagonists exhibited insufficient clinical efficacy, despite convincing data from animal models and suggestive evidence from epidemiological studies of caffeine. These Phase III trials did, however, reveal a good safety profile of A<sub>2A</sub> receptor antagonists<sup>138,139</sup>, as also indicated from caffeine use. By contrast, a large Phase III trial with an A<sub>1</sub> receptor antagonist failed because of its toxicity<sup>144,252</sup>, but it is not yet known whether this is a class effect. Activation (or blockade) of adenosine receptors can have very different effects in different cells within the same tissue. Moreover, adenosine receptor-directed pharmacotherapy can have different consequences in different stages of a disease process. A deep understanding of both the disease process being targeted and the complexity of adenosine signalling in different cellular elements and different disease courses is crucial.

There are several factors that need to be explored: examination of adenosine receptor function — globally or locally — in disease models, particularly humans; the careful dissection of the site of action of drugs through their administration in animals with cell-specific receptor deletions; careful examination of adenosine signalling and the effects of the drug over the continuum of specific disease courses (for example, acute and chronic stages of a disease); clinical studies in which individual differences are carefully monitored and related to background factors such as caffeine use; and the examination of the possibility of combining direct adenosine receptor actions with drugs targeting other pathways and/or targets. We anticipate that these considerations will move the field forward and, over the next decade, facilitate the introduction of several additional clinical applications of adenosine receptor-targeting treatments into the clinical setting.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Glossary

<b>Methylxanthines</b>	Purine derivatives with a common xanthine core molecule and methyl group attached in various combinations to nitrogens. The most common methylxanthines include caffeine, theophylline, theobromine and paraxanthine.
<b>G protein-coupled receptor</b>	(GPCR). A cell membrane protein characterized by a seven-transmembrane structure, which is coupled to trimeric G proteins; GPCRs elicit diverse sets of signalling and biological functions.
<b>Ectonucleoside triphosphate diphosphohydrolase 1</b>	(ENTPD1; also known as CD39). A membrane-bound enzyme with enzymatic activity in the extracellular space; ENTPD1 catalyses the conversion of extracellular ATP and/or ADP to AMP — an important step in generating extracellular adenosine.
<b>Ecto-5'-nucleotidase</b>	(NT5E; also known as CD73). A membrane-bound enzyme with enzymatic activity in the extracellular space; NT5E catalyses the conversion of extracellular AMP to adenosine, thereby functioning as a pacemaker enzyme for generating extracellular ATP-derived adenosine.
<b>Acute heart failure</b>	A gradual or rapid change in the signs and symptoms of heart failure. Many pathological factors, including worsening renal function, persistent neurohormonal activation and progressive deterioration in myocardial function, all contribute to the development of acute heart failure.
<b>Parkinson's disease</b>	A neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain and the accumulation of proteinaceous intracellular inclusions (Lewy bodies), leading to decreased dopamine levels in the striatum and cardinal motor symptoms.
<b>Ischaemic preconditioning</b>	A phenomenon in which repeated short periods of exposure to sublethal ischaemia induce tolerance and protection against subsequent lethal ischaemic injury.
<b>Adenosine kinase</b>	An enzyme that converts intracellular adenosine to AMP, which is critically important in setting the basal adenosine level. Hypoxia is associated with transcriptional repression of adenosine kinase, thereby resulting in increased intracellular adenosine levels and enhanced extracellular adenosine signalling.
<b>Hypoxia-inducible factor</b>	(HIF). Key transcription factor for hypoxia-induced responses that are critical in adapting hypoxic or ischaemic tissues to conditions of limited oxygen availability.
<b>Bleomycin-induced lung injury</b>	A widely used antitumour agent causing single- and double-stranded breaks in cellular DNA, leading to genomic instability of damaged cells. Bleomycin induces apoptosis and increases

	the production of reactive oxygen species, resulting in oxidative stress and pulmonary fibrosis.
<b>Adenosine deaminase</b>	An enzyme that converts adenosine to inosine (and deoxyadenosine to deoxyinosine). Lack of adenosine deaminase causes immune deficiency.
<b>Oligomerization</b>	G protein-coupled receptors (GPCRs) can exist in a monomeric state or form dimeric, multimeric or oligomeric structures. Hetero-oligomerization of GPCRs, involving several gene products, can potentially lead to an altered biological response repertoire.
<b>Allosteric enhancers</b>	Allosteric modulators do not have any activity by themselves, but as they bind to the allosteric site (which is distinct from the primary ligand binding orthosteric site) they can alter the receptor confirmation by an orthosteric ligand in such a way that the response to it is increased.
<b>Equilibrative nucleoside transporter1</b>	(ENT1). A channel located in the cell membrane; along with ENT2, ENT1 speeds up the bi-directional transport of adenosine across the cell membrane along its gradient.
<b>Functional selectivity</b>	Also known as biased signalling; an emerging concept of G protein-coupled receptor (GPCR) function. For example, some GPCR ligands preferentially activate signals via $\beta$ -arrestins, others via G proteins.
<b><math>\beta</math>-arrestin</b>	An adaptor protein that was initially recognized as a negative regulator of G protein signalling but is now recognized to be a multifunctional adaptor that can not only mediate G protein-coupled receptor (GPCR) internalization and desensitization but also produce distinct intracellular signalling and hence functional consequences.

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**Box 1****History of adenosine receptor targeting**

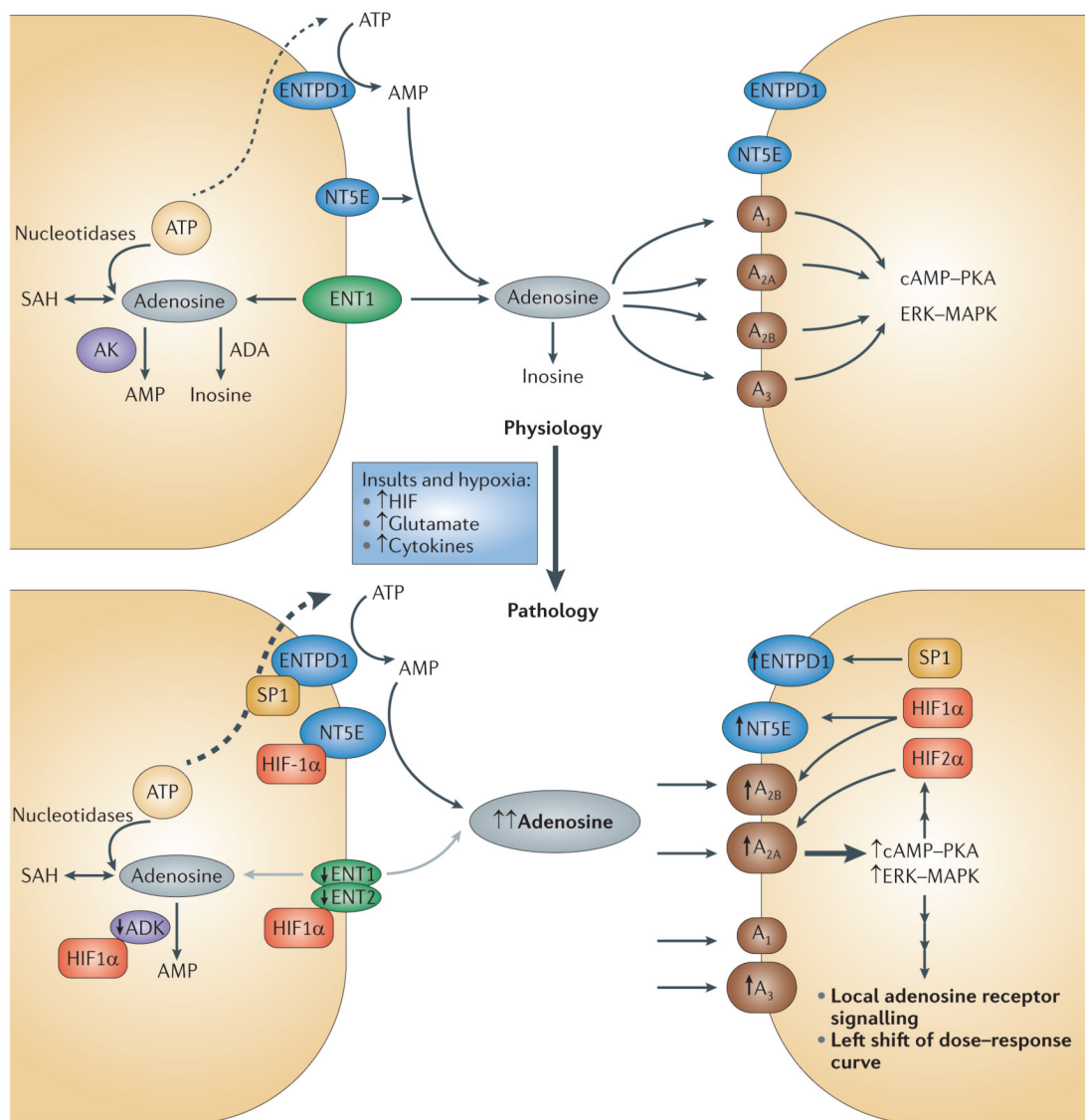
More than 80 years ago, extensive studies on the actions of adenosine reported profound cardiac effects, vasodilation and a marked lowering of body temperature<sup>253,254</sup>. It was proposed that different forms of tissue trauma could release adenosine and/or AMP that could cause vasodilation<sup>253</sup>. Subsequently, the administration of adenosine derivatives was found to have a protective effect<sup>255</sup>. During the Second World War there was interest in the use of such derivatives in the clinic. A relationship was found among the severity of the trauma, the magnitude of the loss of tissue adenine nucleotides and the release of breakdown products. By the mid-1960s it was concluded that the protective effect of adenosine derivatives in the prevention of irreversible trauma may be attributed to vasodilation, energy transfer, anticoagulation or a combination of these mechanisms<sup>256</sup>. The anticoagulatory effect is now attributed to the actions of adenosine on platelet adenosine receptors to reduce their activation<sup>56,257</sup>. The second possibility — that adenine nucleotides enter cells to restore energy charge — is no longer credible. The vasodilatory effect, which was first suggested by Bennet and Drury<sup>253</sup>, was followed up in detail 30 years later<sup>258,259</sup> when it was demonstrated that a lack of energy owing to hypoxia caused the breakdown of myocardial adenine nucleotides, and that the breakdown products were able to cause coronary vasodilation. This led to the very attractive hypothesis that adenosine is a — or the — mediator of hypoxic vasodilation. Indeed, adenosine could be increasing oxygen supply to an energy-depleted tissue, thereby subsequently limiting its own formation — a classical tenet of homeostasis and negative feedback.

The studies on the effects of adenosine on the heart were also very important in demonstrating that methylxanthines such as theophylline acted as adenosine antagonists<sup>1</sup>. They also established the use of adenosine uptake inhibitors such as dipyridamole as tools in adenosine research<sup>260</sup>. It is now believed that adenosine contributes to basal coronary tone, but has only a minor role in increasing coronary blood flow during exercise<sup>261</sup>, and appears to be a very important factor in pathophysiological conditions including ischaemia<sup>6</sup> and ischaemic preconditioning<sup>30,65,262</sup>. The rapid breakdown of adenosine in the bloodstream was useful in the development of adenosine as a diagnostic tool or a therapeutic agent in supraventricular tachyarrhythmia<sup>17</sup>. Rapid adenosine removal was, however, a disadvantage in other potential applications; efforts to synthesize more stable analogues started in the 1960s, yielding the adenosine analogue 2-chloroadenosine, N6-phenylisopropyl adenosine (R-PIA) and N-ethyl-carboxamido adenosine (NECA). The somewhat different effects of these compounds helped to define the subtypes of adenosine receptors, but all of these compounds exhibited so many effects that their development for therapy proved difficult. In the 1970s it became clear that there are receptors for adenosine, and there was evidence that methylxanthines such as caffeine and theophylline produce many of their actions by acting as antagonists at adenosine receptors. Gradually it also became clear that some drugs with an unknown mechanism of action (for example, dipyridamole and methotrexate) probably acted, at least in part, by increasing the levels of adenosine and the stimulation of adenosine receptors. Towards the end of the twentieth century it became clear that there are subtypes of adenosine receptors, and we now recognize a family of four adenosine receptors that are present in most vertebrates. All of these developments strongly suggest that adenosine receptors are druggable.

**Box 2****Variations in caffeine sensitivity and implications for clinical trial design**

Many studies have examined a possible genetic basis for the known human variation in the response to caffeine<sup>263</sup>. Twin studies have suggested a genetic role of the individual variability in caffeine-related traits, such as withdrawal symptoms. Recent genome-wide association studies (GWAS) have linked genetic polymorphisms of metabolic enzymes (cytochrome P450 enzyme 1A2; *CYP1A2*) and main target receptors (such as the gene encoding adenosine A<sub>2A</sub> receptor; *ADORA2A*) to individual variations in caffeine-induced insomnia<sup>264-266</sup>, anxiety and panic attack<sup>267-269</sup>. Single nucleotide polymorphisms (SNPs) in *ADORA2A* have been found to be associated with the age of onset of Huntington's disease<sup>270</sup> and with a reduced risk of Parkinson's disease<sup>271</sup>. The strongest associations between caffeine and Parkinson's disease were found among slow metabolizers of caffeine who were homozygous carriers of the *CYP1A2* polymorphisms. In 2011, the first GWAS was carried out on coffee consumption in eight Caucasian cohorts comprising >18,000 individuals of Northern European ancestry<sup>272</sup>; the top findings were further replicated in ~8,000 additional independent individuals. Two SNPs located in the 23-kb-long commonly shared 5' flanking regions between the *CYP1A1* and *CYP1A2* genes (rs2470893 and rs2472297) were replicated in the follow-up studies with genome-wide significance. Also, in 2011 the first genome-wide association and interaction study (GWAIS) was performed by the NeuroGenetics Research Consortium to identify genes that influence the inverse association of caffeine consumption with the risk of developing Parkinson's disease<sup>273</sup>, and this study found that the SNP rs4998386 and neighbouring SNPs in *GRIN2A* (which encodes NMDA (*N*-methyl-d-aspartate) glutamate receptor subunit 2A) modulate the risk of developing Parkinson's disease in individuals who drink high amounts of coffee<sup>273</sup>.

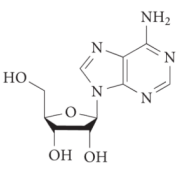
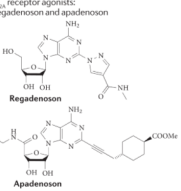
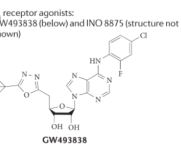
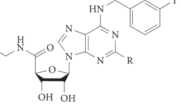
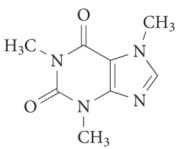
Together, these findings have provided a genetic basis for the individual variation in responses to caffeine and caffeine-related trials. This raises an exciting possibility of predicting the individual responses to caffeine (and possibly other adenosine receptor antagonists) through the identification of genetic polymorphisms of the associated alleles identified by these studies. It was recently demonstrated for the first time that the antiplatelet effect of a P2Y purinergic receptor 12 antagonist is influenced by the *CYP2C19* genotype<sup>274</sup>. Given that caffeine and A<sub>2A</sub> receptor antagonists have a common target and very similar pharmacological effects in the brain, the identification of these SNPs in association with caffeine consumption (in relationship to Parkinson's disease) led us to speculate that clinical trials might yield clearer outcomes if patients are subdivided and analysed by their genotypes for *ADORA2A*, neuronal cell adhesion molecule (*NRCAM*) *GRIN2A*, *CYP1A1* and *CYP1A2* genes. Thus, these studies not only provide proof of concept that taking caffeine consumption into consideration can help identify genes that are missed in GWAS, but they also offer a unique opportunity for personalized medicine — identifying useful pharmacogenetic markers for predicting individual responses to caffeine in Parkinson's disease populations in clinical trials.



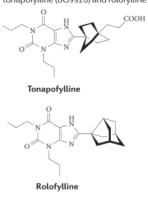
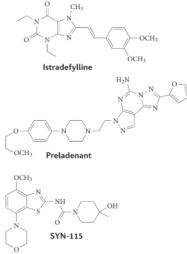
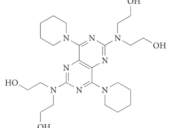
**Figure 1. Local amplification of adenosine signalling in response to insults or hypoxia**  
 Under physiological conditions (upper panel), the extracellular concentration of adenosine is the sum of many biological processes, excluding intracellular adenosine production. Adenosine is transported via equilibrative nucleoside transporter 1 (ENT1) and other transporters. ATP is released via multiple processes. ATP is converted to adenosine by ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1; also known as CD39) and ecto-5'-nucleotidase (NT5E; also known as CD73). Adenosine is metabolized to inosine, AMP or *S*-adenosylhomocysteine (SAH). Many cell types perform all the biological processes displayed in the figure, but some cells show only a limited repertoire. Under pathological conditions (lower panel), local adenosine signalling is markedly amplified in response to insults and hypoxia by a surge in extracellular adenosine concentration from the baseline (20-300 nM) to up to 30 μM in ischaemic or hypoxic tissues. There is also a parallel marked induction of enzymes that are responsible for ATP-dependent adenosine signalling as well as adenosine receptor expression (particularly A<sub>2A</sub> and A<sub>2B</sub> adenosine receptors) and the suppression of enzymes involved in adenosine metabolism, such as adenosine kinase (AK). Adenosine signalling under pathological conditions is controlled by

the following factors: increased extracellular adenosine levels by ATP release; induction of ENTPD1 expression by the transcription factor SP1 and of NT5E expression by hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ); induction of A<sub>2A</sub> receptor expression by HIF2 $\alpha$  and of A<sub>2B</sub> receptor expression by HIF1 $\alpha$ ; repression of AK by HIF1 $\alpha$  and suppression of ENT1 or ENT2 activity by HIF1 $\alpha$ . ADA, adenosine deaminase; cAMP, cyclic AMP; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.

**Table 1**  
**Examples of ongoing or recently completed Phase I–III clinical trials targeting adenosine receptors**

Type of compound	Pharmacology* K <sub>i</sub>	Purpose or name of study	Status	ClinicalTrials.gov identifier	Refs
Adenosine 	A <sub>1</sub> : 77 nM A <sub>2A</sub> : 0.5 nM A <sub>2B</sub> : not determined A <sub>3</sub> : 45 nM	Protection from liver ischaemia following liver surgery	Ongoing	NCT00760708	2,18
		Postconditioning after STEMI	Ongoing	NCT00284323	
		Pretreatment before stenting	Ongoing	NCT00612521	
		Does intradermal adenosine release VEGF and cytokines?	Suspended for re-evaluation	NCT00580905	
A <sub>2A</sub> receptor agonists: regadenoson and apadenoson 	A <sub>1</sub> : >10,000 nM A <sub>2A</sub> : 290 nM A <sub>2B</sub> : >10,000 nM A <sub>3</sub> : >10,000 nM	Is regadenoson superior to adenosine for myocardial perfusion imaging?	Completed	NCT00208312	18,146, 191
		Myocardial perfusion magnetic resonance imaging using regadenoson	Completed	NCT00881218	
		ASPECT study: effectiveness of apadenosin in SPECT imaging compared to adenosine	Completed	NCT01085201	
		Regadenoson approved for treatment of sickle cell anaemia	Recruiting	NCT01566890	
A <sub>2A</sub> receptor agonists: GW493838 (below) and INO 8875 (structure not shown) 	No data available	Analgesic effect of GW493838 in postherpetic neuralgia or peripheral nerve injury	Discontinued	NCT00376454	2,18,275
		Tolerability and safety of INO 8875 in glaucoma and ocular hypertension	Discontinued	NCT01123785	
A <sub>2A</sub> receptor agonists: CF101 (R = H) and CF102 (R = Cl) 	CF101: A <sub>1</sub> : 51 nM A <sub>2A</sub> : 2,900 nM A <sub>2B</sub> : 11,000 nM A <sub>3</sub> : 1.8 nM C3F102: A <sub>1</sub> : 220 nM A <sub>2A</sub> : 5,360 nM A <sub>2B</sub> : >10,000 nM A <sub>3</sub> : 1.4 nM	Safety and efficacy of CF101 in psoriasis	Completed	NCT00428974	2,18,37, 146,147
		Safety and efficacy of CF101 in rheumatoid arthritis	Completed	NCT00556894	
		Safety and efficacy of CF102 in liver cancer	Ongoing	NCT00790218	
Caffeine 	A <sub>1</sub> : 10,700 nM A <sub>2A</sub> : 23,400 nM A <sub>2B</sub> : 33,800 nM A <sub>3</sub> : 13,300 nM	Treatment of apnoea of prematurity and dose study	Currently recruiting participants	NCT01408173	2,18, 223,224
		Treatment of apnoea of prematurity and dose study	Currently recruiting participants	NCT01349205	
		Cognitive long-term effects of caffeine in premature infants	Currently recruiting participants	NCT00809055	
		Caffeine for motor manifestations of Parkinson's disease	Completed	NCT01190735	
		Caffeine for excessive daytime somnolence in Parkinson's disease	Completed	NCT00459420	



Type of compound	Pharmacology* K <sub>i</sub>	Purpose or name of study	Status	ClinicalTrials.gov identifier	Refs
A <sub>1</sub> receptor antagonists: tonapofylline (BG9928) and rolofylline (KW-3902) 	Tonapofylline: A <sub>1</sub> : 7.4 nM A <sub>2A</sub> : 6,410 nM A <sub>2B</sub> : 90 nM A <sub>3</sub> : >10,000 nM Rolofylline: A <sub>1</sub> : 0.72 nM A <sub>2A</sub> : 108 nM A <sub>2B</sub> : 296 nM A <sub>3</sub> : 4390 nM	Safety and tolerability of intravenously administered tonapofylline in individuals with acute decompensated heart failure and renal insufficiency (TRIDENT-1)	Discontinued	NCT00709865	2,18, 21,22
		Effect of rolofylline on heart and renal function in acute heart failure	Discontinued	NCT00328692	
		Effect of rolofylline on heart and renal function in acute heart failure	Discontinued	NCT00354458	
A <sub>1</sub> receptor antagonists: istradefylline (KW-6002), preladenant (SCH-420814) and SYN115 	Istradefylline: A <sub>1</sub> : 841 nM A <sub>2A</sub> : 12 nM A <sub>2B</sub> : >10,000 nM A <sub>3</sub> : 4470 nM Preladenant: A <sub>1</sub> : >1,000 nM A <sub>2A</sub> : 0.9 nM A <sub>2B</sub> : >1,000 nM A <sub>3</sub> : >1,000 nM SYN115: A <sub>1</sub> : 228.4 nM A <sub>m</sub> : 0.38 nM A <sub>2B</sub> : not available A <sub>3</sub> : not available	Efficacy of istradefylline in increasing sleep in patients with advanced Parkinson's disease	Ongoing	NCT00955526	2,18,138, 142,234, 238–243,276
		Effect of preladenant in early Parkinson's disease	Ongoing	NCT01155479	
		Effect of preladenant on 'off-time' in moderate to severe Parkinson's disease	Ongoing	NCT01155466	
		fMRI-aided study of SYN115 on behaviour and brain activity in cocaine addicts	Ongoing	NCT00783276	
		Safety and efficacy study of SYN115 in patients with Parkinson's disease using L-DOPA to treat end of dose wearing off	Completed	NCT01283594	
		Long-term safety study of istradefylline in patients with Parkinson's disease <sup>‡</sup>	Completed	NCT00957203	
		Active-controlled extension study to P04938 and P07037 (P06153 AM3)	Recruiting participants	NCT01215227	
Placebo-controlled study of preladenant in participants with moderate to severe Parkinson's disease (P07037 AM3)	Recruiting participants	NCT01227265			
Adenosine uptake inhibitor: dipyradamole 		Protection after cardiac bypass surgery	Currently recruiting participants	NCT01295567	18,66
		Effects on circulating adenosine levels in relation to genetics	Currently recruiting participants	NCT00760708	
		Supplementation with prednisolone in rheumatoid arthritis	Currently recruiting participants	NCT01369745	

FDA, US Food and Drug Administration; fMRI, functional magnetic resonance imaging; K<sub>i</sub>, inhibition constant; L-DOPA, L-3,4-dihydroxyphenylalanine; SPECT, single-photon emission computed tomography; STEM, ST segment elevation myocardial infarction; VEGF, vascular endothelial growth factor.

\* All pharmacology results are cited from REF. 18 and based on human tissue or cells expressing human receptors.

<sup>‡</sup> Istradefylline (KW-6002) is being investigated in a total of 17 Phase I and Phase III clinical trials in patients with advanced Parkinson's disease for its efficacy and safety; see Supplementary information S1 (table) for details.

**Table 2**  
**Physiological and pathological effects of adenosine receptors**

Effects	Physiology and pathophysiology	Adaptation?	Refs
<b>Adenosine A<sub>1</sub> receptors</b>			
Decreased renal blood flow, tubuloglomerular feedback and inhibition of renin release	Physiology	No (or minor)	7,277
Inhibition of lipolysis	Physiology	No (or minor)	5
Vasoconstriction	Pathophysiology?	Not examined	278,279
Bronchoconstriction	Pathophysiology?	Not examined	280
Inhibition of neurotransmitter release	Extreme physiology*	No (or minor)	91
Inhibition of insulin and glucagon release	Physiology	No	281
Reduced heart rate	Physiology	No	282
Osteoclast activation and bone resorption	Physiology?	Not clear	283
Reduced respiration	Extreme physiology*	No	91
Sleep	Physiology	Yes	284,285
Analgesia	Extreme physiology*	No	91,286
Cardiac preconditioning	Pathophysiology	No	262,287
<b>Adenosine A<sub>2A</sub> receptors</b>			
Wakefulness and locomotion	Physiology	No	9,56
Neurodegeneration (including Parkinson's disease, stroke, traumatic brain injury and Alzheimer's disease)	Pathophysiology	No	57,61,117,119,120
Immunosuppression	Extreme physiology* or pathophysiology	Not clear	59,288
Vasodilation and hypotension	Physiology or extreme physiology*	No	56
Blood-brain barrier integrity	Pharmacology	Not clear	289 – 291
Coronary vasodilation	Physiology or extreme physiology*	Yes, but importance not clear	292
Inhibition of platelet aggregation	Extreme physiology*	Not well studied	56
Angiogenesis	Extreme physiology*	Not known	11,293
Sickle cell disease	Pathophysiology	Not known	152,163
Fibrosis	Pathophysiology	No	169
<b>Adenosine A<sub>2B</sub> receptors</b>			
Vascular integrity	Physiology or extreme physiology*	No	64
Cardiac preconditioning	Extreme physiology*	No	65
Sickle cell disease	Pathophysiology	Not known	153
Pro-inflammation (acute injury) and anti-inflammation (some chronic disease states)	Physiology and pathophysiology	Not known	92
Fibrosis	Pathophysiology	Not known	169
<b>Adenosine A<sub>3</sub> receptors</b>			
Increased mast cell activation	Extreme physiology* or pathophysiology	Some, but probably not at	294,295

<b>Effects</b>	<b>Physiology and pathophysiology</b>	<b>Adaptation?</b>	<b>Refs</b>
		receptor level	
Airway contraction	Pathophysiology	Not known	295
Inflammatory pain	Extreme physiology* or pathophysiology	Not known	92
White cell chemotaxis	Extreme physiology* or pathophysiology	Not clear	43
Chronic neuropathic pain relief	Pathophysiology	Not known	149
Anticancer (melanoma)	Pathophysiology	Not known	148

\* Refers to such conditions such as heavy exercise, being at high altitude and unusually high activity in the pathways of the nervous system.

Table 3

## Human adenosine receptors

Receptor name	Human gene	Chromosome	G proteins	Localization	Potency of adenosine <sup>*</sup>
Adenosine A <sub>1</sub> receptor	<i>ADORA1</i>	1q32.1	G <sub>i,o</sub>	Broad distribution: high in nerves, heart, kidney and adipose tissue	10 <sup>-8</sup> to 10 <sup>-7</sup>
Adenosine A <sub>2A</sub> receptor	<i>ADORA2A</i>	22q11.23	G <sub>s/olf</sub>	Broad distribution: very high in basal ganglia; high in nerves, blood vessels and immune cells	10 <sup>-8</sup> to 10 <sup>-7</sup>
Adenosine A <sub>2B</sub> receptor	<i>ADORA2B</i>	17p12-p11.2	G <sub>s</sub> (G <sub>q/11</sub> ; G <sub>12/13</sub> )	Broad distribution, but generally low abundance	3 × 10 <sup>-7</sup> to 10 <sup>-5</sup>
Adenosine A <sub>3</sub> receptor	<i>ADORA3</i>	1p13.2	G <sub>i/o</sub>	Restricted distribution, varying in different species: high in mast cells	10 <sup>-8</sup> to 10 <sup>-7</sup>

\* The potency (in mol per l) is determined by the effect of the agonist in cells expressing approximately 2 × 10<sup>5</sup> receptors per cell; see REF. 1 for details.