Insight into adenosine receptor function using antisense and gene-knockout approaches

Jonathan W. Nyce

The extensive role of adenosine in discriminating input from the extracellular environment is effected through a series of cell membrane-spanning proteins – the adenosine A_1, A_{2A}, A_{2B} and A_3 receptors. New genetic and epigenetic tools have emerged that facilitate the elucidation of the function of these receptors with greater specificity than is generally possible with traditional antagonist drugs. These tools include antisense oligonucleotides (epigenetic) and gene 'knockin' and 'knockout' mice (genetic) and are discussed in this article by **Jonathan Nyce.**

Each of the known adenosine receptors contains seven transmembrane domains, with an intraceullar carboxy terminus and an extracellular amino terminus[1–5]. These receptors interface with the G proteins G_i (A_1, A_3) or G_s (A_{2A}, A_{2B}) and are typically coupled to the adenylate cyclase–cAMP signal-transduction pathway. The A_1 and A_3 receptors are negatively coupled to cAMP and their activation leads to a reduction in cAMP levels. They also signal via phospholipase C (PLC) and Ca^{2+}. These are pertussis toxin-sensitive pathways that are probably mediated by the βγ subunits of $G_{i/o}$ (Ref. 6). The A_{2A} and A_{2B} subtypes are positively linked to cAMP and their activation leads to an increase in intracellular cAMP. The A_{2B} receptor signals through both G_s and G_q. G_q signalling activates PLC and is important, for example, for adenosine-mediated mast-cell stimulation[7].

The A_1 receptor is highly and widely expressed in the brain, particularly in the cortex, cerebellum, thalamus and hippocampus[8]. It is also expressed in the spinal cord, fat cells, testis, kidney, eye, bladder and heart. Human tissues that express high levels of A_1 receptors produce a distinct mRNA transcript containing an exon that is not present in transcripts obtained from tissues that express low levels of A_1. They are also missing an exon (exon 4) which is present in transcripts from tissues that express low levels of A_1 (Ref. 5). Adenosine-mediated effects that occur via the A_1 receptor include depression of neurotransmission, sleep induction, antinociception, ethanol-induced motor incoordination, autonomic control of cardiac function, bronchoconstriction, negative chronotropy, inotropy and dromotropy, anti-β-adrenoceptor action and renal sodium retention (Table 1).

The A_{2A} receptor is abundant in the striatum, nucleus accumbens and olfactory tubercle; smaller amounts are present in the cortex and midbrain and no detectable amounts in the hypothalamus or cerebellum. Antagonistic interactions between A_{2A} receptors and dopamine D2 receptors (as well as between A_1 and D1 receptors) has been reported[9–11]. Stimulation of the A_{2A} receptor leads to a reduction in the affinity of D2 receptors for D2 receptor agonists[9]. A_{2A} receptors are also present in the heart, brain, kidney, lung, liver and platelets[8]. They play an important role in vasodilation, inhibition of platelet aggregation and immune system function.

A_{2B} receptors are ubiquitous in their expression, being highly expressed in the caecum, colon and urinary bladder, with lesser amounts in brain, spinal cord and lung[8]. However, compared with A_{2A} receptors, less is known of their physiological function. Recent evidence suggests that mast-cell degranulation is mediated by the A_{2B} receptor[7], and this is supported by the fact that A_{2B} receptors evoke interleukin 8 secretion in human mast cells by an enprofylline-sensitive mechanism[12].

The A_3 receptor also has a widespread distribution. It is expressed in highest quantities in mature sperm and is also found on mast cells, lung, kidney and heart, while lower levels are detected in brain regions including cortex, striatum and olfactory bulb[8]. It has been shown recently that activation of the adenosine A_3 receptor inactivates eosinophil migration[13,14].

Epigenetic tools to elucidate adenosine receptor function

A variety of traditional small-molecule adenosine receptor agonists and antagonists have provided important insights into the various functions of these receptors. However, none appears to be totally selective for a specific adenosine receptor, which complicates their use as pharmacological tools. From a therapeutic point of view, although adenosine receptors have been targets for a multitude of drug-discovery efforts, such efforts have not been fruitful. Perhaps because of the ubiquitous nature of adenosine receptors, clinical candidates targeting adenosine receptors are generally eliminated from therapeutic consideration because of systemic toxicity.

Novel epigenetic and genetic approaches to modulating adenosine receptor function in a more tissue-specific manner have recently been described[15–18]. Epigenetic approaches, which attenuate actual expression of the target receptor, have involved the use of respirable antisense oligonucleotides (RASONs), which can be delivered directly to the target organ, the lung, and direct instillation into specific regions of the brain. Genetic approaches have included the development of transgenic mice in which tissue-specific enhancement of adenosine receptor function has been achieved, and adenosine receptor gene knockout experiments. Interesting approaches toward tissue-specific gene therapy to enhance adenosine receptor expression in a target organ such as the heart have also been described[19].

J. W. Nyce,
Chief Scientific Officer, Department of Molecular Pharmacology and Therapeutics, EpiGenesis Pharmaceuticals, PO Box 7007, Princeton, NJ 08543, USA.

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Table 1. Partial listing of physiological effects mediated by adenosine acting at specific receptors

Receptor	Function
A_1	Depression of neurotransmission Sleep induction Antinociception Maintenance of thalamic spindle rhythms Mediation of various ethanol-induced effects, including motor incoordination Autonomic control of cardiac function Bronchoconstriction Block of atrioventricular nodal function Vasoconstriction Inhibition of Ca^{2+} currents and presynaptic inhibition of GABA Negative chronotropy, dromotropy or inotropy
A_2	Vasodilation Respiration Diverse anti-inflammatory effects
A_{2A}	Facilitation of neurotransmission Adhesion of polymorphonuclear leukocytes to endothelium
*A_{2B}, A_3	Mast cell degranulation

*Species differences exist with respect to A_{2B} and A_3 receptor-mediated effects on mast cells.

Antisense oligonucleotides: background

Antisense oligonucleotides are single-stranded nucleic acids that hybridize to specific target messenger RNAs and thereby impede the template properties of those mRNAs. Because of the avidity and specificity of the Watson–Crick base pairing involved in this hybridization, the specificity and strength of binding of antisense oligonucleotides to their targets can typically surpass that of traditional antagonist drugs to their protein targets. Hybridization of an antisense oligonucleotide appears to simulate RNAase H activity which then degrades the mRNA in the region of oligonucleotide binding. Truncated mRNAs produced in this way might produce a defective protein or no protein (more probable), resulting in ablation of gene function, without effect at the level of the gene itself. Such functional gene ablation can be considered to be a form of gene therapy, i.e. epigene therapy. Because of the ubiquitous presence of nucleases capable of degrading nucleic acids entering the cell, antisense oligonucleotides must be chemically modified to achieve lifetimes sufficient to effect target attenuation. A variety of chemical modifications have been described in the literature[20,21].

Antisense oligonucleotides targeting the A_1 receptor

Traditional antagonists lack specificity

As discussed above, extensive effort has been expended over the years in the development of specific adenosine receptor antagonists. Using a substantial chemical armamentarium resulting from this effort, considerable progress has been made in both the identification of new adenosine receptors (e.g. A_3) that did not fit the developing profile for antagonist sensitivity, as well as elucidation of receptor function. However, although selectivity of these small-molecule antagonists could approach several orders of magnitude in some cases, complete selectivity has proved to be an impossible task. Antisense oligonucleotides offer the advantage that complete receptor specificity is possible if proper design and evaluation criteria are met.

The use of antisense oligonucleotides has been impeded by largely one major factor – how to selectively deliver effective amounts of the agent to the target tissue without inducing toxicity. For adenosine receptor antisense, two avenues of local delivery provide exciting portals into receptor function, potentially solving the delivery problem. These two avenues of local delivery include inhalation of RASONs directly into the target tissue, the lung; and direct injection of antisense oligonucleotides into specific regions of the brain (site-specific functional gene ablation). Figure 1 shows the homology overlapping the A_1 promoter region in several species, and two antisense oligonucleotides. EPI 2010 and EPI 2019 designed for use, respectively, in human/rabbit/monkey and rat.

A_1 antisense in the lung

The A_1 receptor is a desirable therapeutic target for diseases such as asthma, acute renal failure and acute respiratory distress syndrome. Evidence suggests that asthma, in particular, could be associated with a disease-associated upregulation of A_1 receptors. Asthmatic individuals have excess amounts of adenosine in their lungs[22], such that the upregulated A_1 receptor would be constitutively stimulated. A_1 receptors are known to be overexpressed in allergic rabbits and rats[23–25]. Inhaled adenosine causes bronchostriction in human asthmatics, and it appears to be a more specific differentiator of asthma than the standard methacholine challenge. Bronchial smooth muscle of human asthmatics contracts in an A_1-dependent manner as judged by response to 2-thio-DPCPX (1,3-dipropyl-8-cyclopentylxanthine)[26]. In addition, it has been reported recently that the A_1 receptor is rapidly upregulated in bronchial smooth-muscle tissue exposed to human asthmatic serum[27]. Recent evidence suggests that the A_1 receptor negatively regulates surfactant secretion[28], and that because surfactant secretion is diminished in asthma and could contribute to bronchial hyperresponsiveness[29–34] this provides further impetus to develop agents capable of attenuating A_1 receptor function. Recent studies suggest that both adult respiratory distress syndrome (ARDS) and acute renal failure (ARF) also involve A_1 receptor activation and could potentially be therapeutically approached via A_1 receptor antagonists[35,36].

The lung as a target for RASONs

Respirable antisense oligonucleotides (RASONs) offer the potential to attenuate A_1 receptor number selectively in the lung, thereby eliminating much or all of the toxicity that might be expected by systemic administration of small molecule A_1 antagonists. In addition, the lung might offer a superb target for local delivery of

RASONs because of its unique surfactant lining[15,37,38] Surfactant is composed primarily of lipids, particularly dipalmitoylphosphatidylcholine, a zwitterionic lipid which, at lung pH acts as a weak cation. It is well known that cationic lipids enhance the uptake of antisense oligonucleotides into cellular interiors, as discussed above. Furthermore, surfactant is recycled rapidly between the air–liquid interface in the lung and the type II pneumocytes, a process which could further facilitate uptake of RASONs. Thus, the lung could offer a particularly receptive target for antisense administration.

EPI 2010 is a RASON designed to target the initiation codon of the human A_1 receptor mRNA. It is being developed as a therapeutic for asthma and other respiratory conditions in which the A_1 receptor is involved. A fortuitous rare homology between the human and the rabbit sequences within the target region of the A_1 receptor mRNA permitted EPI 2010 to be tested in the rabbit model of allergic asthma[15]. This model closely resembles human asthma in many respects including allergen induction of allergen-specific IgE, overexpression of A_1 receptors in bronchial smooth muscle, and hypersensitivity of allergic rabbits to adenosine.

Administration of EPI 2010 to allergic rabbits *via* ultrasonic nebulizer (total dose delivered to the lungs, approximately 100 $\mu g\ kg^{-1}$) produced a dose-dependent attenuation of sensitivity to adenosine (Fig. 2). This effect was highly specific, with no attenuation occurring at either the A_2 receptor or the bradykinin B_2 receptor. Neither of two mismatch control molecules caused any effect at any concentration tested, as judged by receptor-binding studies[15]. EPI 2010 also substantially attenuated sensitivity to a common aeroallergen, house dust mite (*Dermatophagoides farinae*) and histamine. This latter finding provides substantial support for an inflammatory effect mediated by the A_1 receptor, as suggested by previous observations of involvement of the A_1 receptor in the transmigration of neutrophils[39], and supported by further recent cellular data from our laboratory to be reported elsewhere. As suggested by the homology with various monkey species shown in Fig. 1, EPI 2010 is also effective in the *Ascaris*-sensitized primate model of human asthma (Fig. 2). This finding will be reported in detail elsewhere. The duration of effect of EPI 2010 has been shown to be 6.8 days. This durable effect, which could be related to the temporal kinetics of A_1 receptor expression in bronchial hyperresponsiveness, gives EPI 2010 the potential to be the first once-per-week treatment for asthma.

These results show the potential of the RASON approach to attenuate selectively lung targets at the level of gene expression – targets that have been difficult to approach using traditional methods for reasons of both insufficient selectivity and unacceptable levels of toxicity. This approach should be amenable to basic science studies designed to elucidate diverse biochemical pathways in the lung, as well as to the development of novel therapeutics targeting mediators of a wide array of respiratory conditions.

a

Human	5′-GTGCCCAGCCTGTG**CCCGCCATGCCGCCCTCCATC**TCAGCTTT-3′
Monkey	5′-GTGCCCAGCCTGTG**CCCGCCATGCCGCCCTCCATC**TCGGCCTT-3′
Rabbit	5′-GTGCCCAGCCTGTG**CCCGCCATGCCGCCCTCCATC**TCGGCCTT-3′
Cow	5′-GTGCCCAGCCTGTG**CCCACCATGCCGCCCTCCATC**TCGGCCTT-3′
Canine	5′-GTGCCCGGCCCGTG**CCTGCCATGCCGCCCGCCATC**TCGGCCTT-3′
Rat	5′-GTGCCCAGCTCCTG**CCCACCATGCCGCCCTACATC**TCGGCCTT-3′
Mouse	5′-GTGCCCAGCTCCTG**CCCACCATGCCGCCGTACATC**TCGGCCTT-3′
Guinea-pig	5′-GTGCCCAGCCTGTG**CCCGCCATGCCGCACTCCGTC**TCGGCCTT-3′

b

Epi 2010	5′-GATGGAGGGCGGCATGGCGGG-3′ [human, monkeys (cynomolgus, rhesus, squirrel), rabbit]
EPI 2019	5′-GATGTAGGGCGGCATGGTGGG-3′ (rat)

Fig. 1. a: Adenosine A_1 receptor gene structure flanking initiation codon. **b:** A_1 receptor antisense oligonucleotide structure. Blue denotes the initiation codon; red denotes bases not identical to the human sequence. Bold and underline denote the target sequence in human, monkeys and rabbit.

Antisense oligonucleotides in the brain: site-specific functional gene ablation

Opportunities exist for the local delivery of antisense oligonucleotides, and for elucidation of physiological pathways mediated by the targeted gene products, that would be much less specifically approached using traditional small-molecule antagonists. Microinjection of antisense oligonucleotides directly into specific regions of the brain achieves a remarkable elevation in the sophistication of classical ablation experiments. Now, instead of ablation of a subregion of the brain, ablation of a single gene within that region is possible. This ability

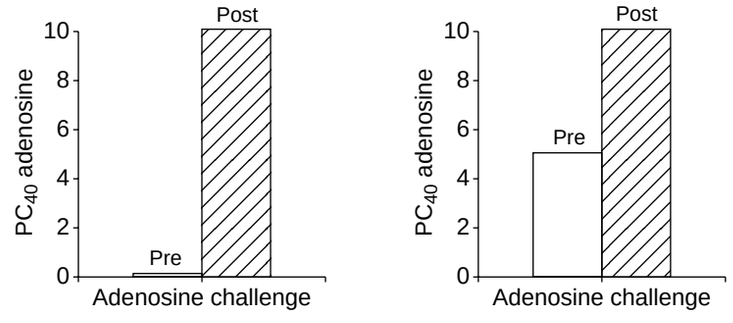

Fig. 2. Adenosine sensitivity of allergic primates and elimination of such sensitivity by aerosolized EPI 2010, a RASON targeting the adenosine A_1 receptor. EPI 2010 was delivered *via* ultrasonic nebulizer once per day for two days, and PC_{40} adenosine values were evaluated 12–16 h later and compared to PC_{40} adenosine values prior to EPI 2010 treatment. PC_{40} adenosine, the Y axis in the above graphs, is defined as that dose of adenosine which reduces dynamic compliance 40% from the baseline. White bars, pre EPI 2010; hatched bars, post EPI 2010. Details of the general experimental protocol are as published elsewhere[15]. Results are shown for two allergic primates.

to achieve site-specific functional gene ablation (SSFGA) presents remarkable opportunities to discern gene function for both well-known and newly discovered genes expressed in the CNS. Adenosine receptors are one such potential target.

Ethanol-induced motor incoordination

One of the many roles that the A_1 receptor appears to play in the brain is as a mediator of the effects of ethanol upon motor coordination[39–41]. Phan *et al.*[42] have recently shown that microinjection of a rat-specific version (EPI 2019) of EPI 2010 into the striatum of rats almost blocks ethanol-induced motor incoordination. Interestingly, rats receiving intrastriatal EPI 2019 are less susceptible to the motor-incoordinating effects of ethanol and can maintain their balance in classical rotorod experiments, while control animals administered a mismatch oligonucleotide or sham injected with saline remain sensitive to the motor incoordinating effects of ethanol. Phan *et al.* demonstrated that these behavioural effects occurred in a dose-dependent manner, that EPI 2019 selectively attenuated A_1 receptor expression, A_1 receptor attenuation occurred in a dose-dependent fashion and a mismatch control oligonucleotide minimally differing from EPI 2019 was without effect upon either ethanol-induced motor incoordination or A_1 receptor attenuation, criteria required to demonstrate an antisense effect of the applied oligonucleotide. These experiments demonstrate conclusively that important effects of ethanol are transduced *via* the A_1 receptor, rather than being directly related to the effects of ethanol upon motoneurones. They also offer clues about the manner in which information is processed in the brain, because SSFGA targeting different regions and performed bilaterally or unilaterally produced similar results.

Attenuation of the baroreflex

Evidence suggests that the A_1 receptor plays a role in the baroreflex *via* receptors located in the nucleus tractus solitarius (NTS)[43]. Using EPI 2019 to achieve SSFGA in the NTS, Mao *et al.*[44] demonstrated a role for the adenosine A_1 receptor in baroreceptor-mediated bradycardia in conscious rats[42]. EPI 2019 microinjected into the NTS significantly attenuated baroreflex sensitivity (the slope of the regression line relating decreases in heart rate to phenylephrine evoked increments in mean arterial pressure) by approximately 50%. A mismatch control oligonucleotide was without effect. These results provide evidence that the A_1 receptor in the NTS processes baroreceptor information, suggesting a pivotal role for these receptors in modulating baroreflex control of heart rate.

Genetic tools to elucidate adenosine receptor function

A_{2A} transgenic

Coppee *et al.*[17] and Ledent *et al.*[18] prepared transgenic animals that expressed the A_{2A} receptor under the control of the thyroglobulin gene promoter (Tg-A_{2A}R), thus potently upregulating expression of the receptor specifically in the thyroid gland. This manipulation had the effect of inducing marked hyperthyroidism in the animals. Ledent *et al.*[18] cross-bred Tg-A_{2A}R mice with mice transgenic for the E7 protein of the human papilloma virus, producing Tg-A_{2A}R/Tg-E7 mice. These mice develop a large goitre and severe hyperthryroidism comparable to TgA_{2A}R mice, and the rapid occurrence of malignant thyroid lesions. These malignant thyroid lesions are highly metastatic, generating multiple differentiated and functional metastases in the lung, appearing as early as two months after birth. The frequency of these lesions is reported to reach 75% by three months of age. These studies identify the A_{2A} receptor as a thyroid oncogene, and provide the first genetic animal model of high frequency metastatic thyroid carcinoma.

A_1 knockin gene therapy: manipulation of A_1 gene expression

As adenosine has both cardioprotective and neuroprotective effects, it could have a potential role in reducing the pathological consequences of cardiac and cerebral ischaemia[45]. It might be possible to induce therapeutically the expression of the A_1 mRNA transcript characteristic of tissues that highly express this receptor, thus affording protection from ischaemia to a target tissue such as the heart. It has been shown that A_1 receptor overexpression in transgenic mice increases myocardial resistance to ischaemia[19,46].

A_{2A} knockout mice

As noted above, adenosine is known to possess analgesic, anti-anxiety and vasodilatory actions. Ledent *et al.*[16] created an adenosine A_{2A} receptor knockout mouse and showed that complete loss of this receptor left animals in a viable condition. $A_{2A}R -/-$ mice, however, scored much higher than controls in anxiety tests, showed a delayed response to pain stimuli, and male mice carrying the knockout genotype were much more aggressive towards intruders. Blood pressure, heart rate and platelet aggregation were all increased in $A_{2A}R -/-$ mice, and the A_{2A} receptor agonist CGS21680 lost all biological activity.

Summary

Adenosine is unique among nucleosides in its intrinsic involvement in the physiology of virtually every cell and organ system. Its ubiquitous actions are mediated by at least four receptors that provide important but difficult targets for pharmacological intervention. Because of the ubiquitous nature of adenosine-mediated physiological events, it is important that the ability to modulate selectively individual adenosine receptors within specific target tissues is developed. Such modulation has recently been dramatically improved with the development of a series of epigenetic (RASONs, SSFGA) and genetic (transgenic, gene knockout, and gene therapy) tools. These tools provide the means to elucidate the function

of adenosine receptors in fine detail in experimental systems and to create novel therapeutic approaches to the many important human diseases in which adenosine receptors play an important role.

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