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MODULATION DES RÉCEPTEURS DE L'ADÉNOSINE PAR ANTICORPS MONOCLONAUX ET LIGANDS SYNTHÉTIQUES
Application en Physiopathologie Humaine

T H È S E

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1. **By Y**, Durand-Gorde JM, Condo J, Lejeune PJ, Mallet B, Carayon P, Guieu R, Ruf J. Production of an agonist-like monoclonal antibody to the human A_{2A} receptor of adenosine for clinical use. *Molecular Immunology 2009 Jan; 46(3): 400–5*.
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AVANT-PROPOS

Depuis la première description des récepteurs de l'adénosine, il y a 30 ans, les découvertes scientifiques et les applications médicales dans ce domaine se sont remarquablement développées. Le rôle de l'adénosine et de ses récepteurs dans la physiopathologie humaine, en particulier, est devenu un grand chapitre d'actualité aussi bien dans les recherches fondamentales que dans les investigations cliniques. Les récepteurs de l'adénosine sont les cibles majeures de la caféine, la drogue la plus consommée au monde, et ils semblent être impliqués dans une vaste diversité de maladies comprenant les pathologies neurologiques, immunologiques, cardiaques, les désordres du sommeil, l'inflammation ou encore le cancer. Le développement des agonistes et des antagonistes spécifiques des récepteurs de l'adénosine est également en expansion. Notre équipe a été un des premiers laboratoires en Europe, à mettre au point une technique de dosage reproductible de l'adénosine dans les milieux biologiques et à s'intéresser aux rôles de ses récepteurs en physiopathologie humaine.

Même si les applications thérapeutiques des drogues qui ciblent les récepteurs de l'adénosine, en particulier les récepteurs A_1 ou A_{2A}, semblent prometteuses, l'élucidation des mécanismes de régulation des ces récepteurs et le développement de nouveaux ligands plus stables et plus maniables restent toujours à affiner. Nous nous sommes fixé comme objectif au cours de ce travail, d'une part, de produire un anticorps monoclonal spécifique des récepteurs A_{2A} de l'adénosine qui pourra servir à la fois d'outil de diagnostic pour l'étude de l'expression de ces récepteurs, mais aussi d'agent thérapeutique. En collaboration avec l'Institut des Sciences Moléculaires de Marseille (ISM2) de l'Université Paul Cézanne, nous proposons, d'autre part, de tester de nouveaux agonistes et antagonistes des récepteurs A_1 néo-synthétisés. Ces projets devraient apporter une contribution notoire dans le domaine des sciences de la vie et de la santé.

TABLE DES MATIÈRES

CHAPITRE TROISIEME

ABRÉVIATIONS

A	
A_1R	Récepteur A_1 de l'adénosine
A_2R	Récepteurs A_2 de l'adénosine
$A_{2A}R$	Récepteur A_{2A} de l'adénosine
$A_{2B}R$	Récepteur A_{2B} de l'adénosine
A_3R	Récepteur A_3 de l'adénosine
ADA	Adénosine désaminase
ADP	Adénosine diphosphate
APL	Adenosine plasma level
AMP	Adénosine 5'-monophosphate
AMPc	Adénosine 5'-monophosphate cyclique
ANR	Agence nationale de la recherche
ATP	Adénosine triphosphate
C	
CDP-glucose	Cytidine diphosphate glucose
CDR	Complementary determining region
CGRP	Calcitonin gene-related peptide
CNT	Concentrative nucleoside transporter
E	
EC_{50}	Half maximal effective concentration
ENT	Equilibrative nucleoside transporter
F	
FAD	Flavine adénine dinucléotide
FMN	Flavine mononucléotide
G	
GMPc	Guanosine monophosphate cyclique
GPCRs	G protein coupled receptors
GRK	G-receptor kinase
H	
HGPRT	Hypoxanthine guanine phosphoribosyl transférase
HIV-1	Human immunodeficiency virus type 1
HUT	Head-up tilt
I	
IASP	International Association for the Study of Pain
IC_{50}	Half maximal inhibitory concentration
i.c.v.	Intra-cérébro-ventriculaire
i.p.	Intra-péritonéal

K

K_d	Constante de dissociation
K_i	Constante d'inhibition
K_m	Constante de Michaelis

L

LD_{50}	Median lethal dose

M

mAb	Monoclonal antibody
MAP-kinase	Mitogen-activated protein kinases
MOR	μ opiate receptor

N

NAD	Nicotinamide adenine dinucléotide
NADP	Nicotinamide adenine dinucléotide phosphate
NMDA	Acide N-methyl-D-aspartique
NMS	Neurally mediated syncope

P

PAG	Periaqueductal grey
PBMC	Peripheral blood mononuclear cell
PKA	Protéine kinase A
PKC	Protéine kinase C
PLC	Phospholypase C

S

SAH	S-adénosyl homocystéine
SIDA	Syndrome d'immunodéficience acquise
SIRS	Systemic inflammatory response syndrome
SKCs	Calcium-activated potassium channel type SK
SNP	Single nucleotide polymorphism

T

TCR	T-cell receptor

U

UDP-glucose	Uridine diphosphate glucose

RÉSUMÉ

L'adénosine est un nucléoside ubiquitaire qui exerce un contrôle puissant sur les systèmes nerveux, immunitaire et cardiovasculaire par l'intermédiaire de quatre récepteurs membranaires : A_1R, $A_{2A}R$, $A_{2B}R$ et A_3R. L'étude des récepteurs de l'adénosine est nécessaire à la compréhension de physio-pathologies humaines non encore élucidées. Pour étudier l'expression des $A_{2A}R$, nous avons, dans une première étude, produit un anticorps monoclonal, appelé Adonis, d'isotype IgM, κ. Adonis reconnait un épitope linéaire de sept acides aminés sur la partie C-terminale de la seconde boucle extra-cellulaire de l'$A_{2A}R$ humain. Adonis révèle, par Western blotting sur lysats cellulaires, une bande de 45 KDa, correspondant à l'$A_{2A}R$. Adonis se comporte comme un « agonist-like » en augmentant la production d'AMPc et en inhibant la prolifération cellulaire via la stimulation des $A_{2A}R$. Dans une deuxième étude, nous avons utilisé Adonis pour montrer que l'expression des $A_{2A}R$ de cellules mononucléées, qui mime celle des tissus cardiaques, permet de différencier certains patients souffrant de syncope neurocardiogénique. Nous avons monté dans une troisième étude, qu'Adonis induit une « down-régulation » de l'expression des co-récepteurs CXCR4 et CCR5 des cellules T via la stimulation des $A_{2A}R$, et qu'à ce titre il pouvait être un outil thérapeutique dans les infections par HIV. Dans une quatrième étude, nous avons évalué les effets anti-nociceptifs d'Adonis qui, administré par voie intra-cérébro-ventriculaire, augmente de manière dose-dépendante les latences obtenues avec le test du Hot-plate et du Tail-flick chez la souris. Ces effets sont renversés par deux antagonistes des $A_{2A}R$ mais aussi par un antagoniste des récepteurs aux opioïdes. Ceci suggère que les effets anti-nociceptifs d'Adonis sont médiés par la libération d'opioïdes endogènes. En marge de ses études, nous avons également testé les propriétés biologiques de nouveaux ligands des A_1R dans le cadre d'une collaboration entre chimistes et biologistes. Ainsi, nous montrons, dans une cinquième étude, que parmi la trentaine de molécules synthétisées, quatre sont des antagonistes et deux autres des agonistes avec un EC_{50} de l'ordre du micromolaire pour la production d'AMPc. De tels agonistes des A_1R pourraient être utiles dans le traitement des douleurs neuropathiques, tandis que les antagonistes le seraient dans l'insuffisance cardiaque ou utilisés comme diurétique. Enfin dans une sixième étude, nous avons testé une molécule originale, puisque bivalente, possédant un pôle d'activité pour les récepteurs aux opioïdes μ et un autre pour les A_1R. Cette molécule est un antagoniste pour les deux récepteurs. Elle pourrait avoir des applications cliniques dans certaines pathologies comme le choc hypovolémique ou le sevrage aux opiacés.

Mots-clés : Récepteurs de l'adénosine, Anticorps monoclonal, Agoniste, Antagoniste

ABSTRACT

Adenosine interacts on its cell surface receptors, namely A_1R, $A_{2A}R$, A2BR and A3R, to exert physiological effects on target tissues. Modulation of these adenosine receptors appears to be a current topic of research which may bring more comprehensions on human pathophysiology yet to be elucidated. In order to study $A_{2A}R$ expression, we produced, in study 1, a monoclonal antibody anti-human $A_{2A}R$, called Adonis being of IgM, κ isotype. Adonis recognized a linear epitope of seven amino acids on the C-terminal part of the $A_{2A}R$ second extra-cellular loop. By Western blotting, Adonis reveals a 45 KDa band of $A_{2A}R$ in cell lysates. Adonis behaves as an agonist-like which increases the cAMP production and inhibits cell proliferation through $A_{2A}R$ stimulation. In study 2, we showed that using Adonis, to measure the $A_{2A}R$ expression of peripheral blood mononuclear cells which mimic those of the cardiac tissue, was able to differentiate some patients with suspected neurally mediated syncope. We showed, in study 3, that $A_{2A}R$ stimulation by Adonis leads to a down-regulation of CXCR4 and CCR5 expression on T-cells, suggesting that Adonis would be a potential drug to treat HIV infections. In study 4, we showed that intracereboventricular injection of Adonis increased the Hot-plate and Tail-flick test latencies in mice in a dose-dependent manner. Such increases were prevented by two $A_{2A}R$ antagonists and by an opiate receptor antagonist, suggesting that the anti-nociceptive effects of Adonis were mediated, at least in part, by endogenous opioid liberation. The last section focused on biological evaluation of new A_1R ligands in collaborative studies between chemists and biologists. Indeed we showed, in study 5, that among thirty synthesized molecules, four act as A_1R antagonists and two turn out to be A_1R agonists with a micromolar EC_{50} on cAMP production. Those A_1R agonists would be used in neuropathic pains, whereas other antagonists could be used in cardiac failure or as diuretic. Finally, in study 6, we tested an original hybrid molecule which was revealed to be a bivalent antagonist to μ opiate receptors and A_1R. This hybrid compound may have applications in some pathologies such as hypovolemic shock and opiate addiction.

Key-words : Adenosine receptor, Mononclonal antibody, Agonist, Antagonist

Chapitre premier

INTRODUCTION BIBLIOGRAPHIQUE

I. Dérivés puriques

Les dérivés puriques, associés à certains dérivés pyrimidiques, constituent un groupe comprenant les nucléosides, dont le chef de file est l'adénosine, et les nucléotides, dont le principal est l'adénosine triphosphate (ATP). Ces molécules exercent leurs effets cellulaires par l'intermédiaire de leurs récepteurs membranaires spécifiques et induisent de nombreux processus biologiques (Ralevic and Burnstock, 1998).

1. Nucléosides et nucléotides

Un nucléoside est une glycosamine, résultant de la combinaison d'un ose (ribose ou 2-désoxyribose) et d'une base (purique ou pyrimidique), unis par une liaison N-glycosidique. Dans les cellules, les nucléosides peuvent être phosphorylés par des kinases spécifiques pour produire des nucléotides qui sont les constituants moléculaires des acides ribonucléiques (ARN) et désoxyribonucléique (ADN), éléments fondamentaux de toutes les cellules vivantes. Les acides nucléiques comprennent deux bases puriques (adénine et guanine) et trois bases pyrimidiques (cytosine, thymine et uracile), ce qui correspond respectivement à deux nucléosides puriques (adénosine et guanosine) et trois nucléosides pyrimidiques (cytidine, thymidine et uridine). Suivant l'orientation de la liaison N-glycosidique, un nucléoside peut se présenter sous deux formes distinctes (*syn* et *anti*) mais c'est la conformation *anti* qui prédomine dans sa forme naturelle (figure 1).

Les nucléotides sont, non seulement, les unités élémentaires des acides nucléiques, mais leurs dérivés peuvent également avoir des fonctions cellulaires qui leur sont propres : ils interviennent dans de nombreux métabolismes comme transporteur d'énergie (ATP), d'oses (UDP-glucose), de lipides (CDP-choline), d'acides aminés (S-adénosyl-méthionine, homocystéine), de vitamines (B12 cobalamine) ou de groupements phosphate. De plus, les dérivés puriques font partie de la structure de co-enzymes très divers (NAD, NADP, FMN, FAD) et interviennent dans la transduction du signal intra-cellulaire sous formes cycliques (AMPc, GMPc).

2. Origine des dérivés puriques

Les dérivés puriques et pyrimidiques sont synthétisés principalement dans le foie d'où ils sont distribués dans tout l'organisme par les érythrocytes. Ils proviennent, d'une part, de la synthèse de *novo* à partir des éléments intermédiaires du métabolisme énergétique des sucres et des acides aminés et, d'autre part, de la voie de recyclage à partir des produits de dégradations lors du catabolisme des nucléotides ou des nucléosides. Le coût énergétique de la biosynthèse du noyau purique justifie l'activité importante des enzymes de récupération de ces noyaux dans les tissus. L'énergie est fournie par l'ATP synthétisé principalement par phosphorylation oxydative mitochondriale (Schwiebert and Zsembery, 2003). L'alimentation fournit des bases puriques en faible quantité car les enzymes digestives dégradent ces nucléotides en produit final, l'acide urique, insoluble et excrété dans les urines sous forme des cristaux d'urate de sodium. L'ATP est produit à partir de l'adénosine diphosphate (ADP), principalement par phosphorylation oxydative dans la mitochondrie dont il passe la membrane externe par les porines ou les canaux voltage-dépendants et la membrane interne par des antiports ATP/ADP (Schwiebert, 2001; Schwiebert and Zsembery, 2003). La concentration cytosolique de l'ATP se situe entre 3 et 10 mM, ce qui constitue un stock de carburants pour de nombreuses réactions métaboliques et enzymatiques, tandis que son taux extra-cellulaire est environ 10 nM, ce qui traduit un très fort gradient de concentration. Il faut noter que seulement 1% de l'ATP intra-cellulaire libéré, ou encore moins, suffit pour activer au maximum tous les récepteurs purinergiques à la surface cellulaire (Schwiebert and Zsembery, 2003). Ainsi, la signalisation de l'ATP extra-cellulaire peut avoir lieu sans compromettre des métabolismes cellulaires ou d'autres réactions enzymatiques.

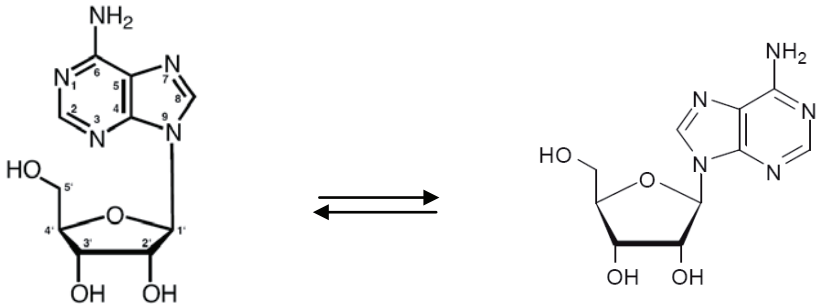

Figure 1. Structure de l'adénosine (*syn* et *anti*)

II. Adénosine

1. Métabolisme

L'adénosine est un nucléoside purique formé d'une base adénine attachée à un noyau ribose via une liaison β-glycosidique (figure 1). Elle est présente dans toutes les cellules de l'organisme. L'adénosine est synthétisée de façon continue aussi bien en milieu intra-cellulaire qu'en milieu extra-cellulaire.

Dans le milieu intra-cellulaire, la formation d'adénosine est assurée, d'une part, par l'enzyme intra-cellulaire 5'nucléotidase, qui déphosphoryle l'adénosine monophosphate (AMP) (Schubert et al., 1979; Zimmermann et al., 1998), et d'autre part, par l'hydrolyse de S-adénosylhomocystéine (SAH) (Broch and Ueland, 1980). Cette adénosine, générée dans la cellule, est transportée dans les espaces extra-cellulaires principalement par des transporteurs spécifiques bi-directionnels via un système de diffusion facilitée qui maintient l'équilibre des concentrations en adénosine dans les deux compartiments cellulaires (Figure 2). Dans certains tissus, par exemple les reins, il y a des transporteurs de nucléosides, dit concentratifs, capables de maintenir des taux élevés d'adénosine contre un gradient de concentration. Ces protéines de transport ont été déjà clonées et nommées ENT1 et ENT2 (pour Equilibrative Nucleoside Transport protein) et CNT1 et CNT2 (pour Concentrative Nucleoside Transport protein) (Anderson et al., 1996; Baldwin et al., 1999; Williams and Jarvis, 1991). En cas de diminution de l'activité des transporteurs, à cause de certaines drogues ou par baisse de la température, le taux d'adénosine extra-cellulaire biologiquement active augmente (Dunwiddie and Diao, 2000). Il faut noter que, pour un tissu donné, certaines cellules sont plutôt productrices d'adénosine et maintiennent donc le taux d'adénosine intra-cellulaire élevé, alors qu'en grande majorité les autres cellules éliminent plutôt les nucléosides.

Dans le milieu extra-cellulaire, la formation d'adénosine est sous la dépendance de CD39, une ecto-apyrase qui transforme l'ATP en AMP et de CD73, une ecto-5'-nucléotidase qui convertit l'AMP en adénosine (Eltzschig et al., 2006). Ces enzymes sont essentielles pour la production d'adénosine à partir de l'ATP neuronale en conditions physiologiques (Dunwiddie

et al., 1997; Zimmermann et al., 1998). Ces réactions s'opèrent en quelques centaines de millisecondes, et l'étape limitante est la déphosphorylation de l'AMP en adénosine par l'ecto-5'-nucléotidase (Dunwiddie et al., 1997). Il existe également une forme soluble de 5'-nucléotidase, de structure inconnue, qui est libérée avec l'ATP par des terminaisons nerveuses sympathiques stimulées et qui participe à l'hydrolyse extra-cellulaire de l'ATP en adénosine (Todorov et al., 1997). Néanmoins, il a été clairement montré que la formation de l'adénosine intra-cellulaire est, quantitativement, au moins aussi importante que celle faite à partir des nucléotides extra-cellulaires (Lloyd and Fredholm, 1995; Lloyd et al., 1993). Cette formation intra-cellulaire peut même être prédominante grâce à l'activité des 5'-nucléotidases intra-cellulaires dont deux formes, cN-I et cN-II, ont été clonées (Sala-Newby et al., 1999). Le cN-I catalyse l'AMP en adénosine, tandis que le cN-II catalyse, respectivement, IMP et GMP en inosine et guanosine (Sala-Newby et al., 2000).

Si la concentration d'adénosine augmente dans le milieu extra-cellulaire, l'adénosine est transportée dans les cellules par l'intermédiaire d'un système de diffusion facilitée (Plagemann, 1986). Ensuite, elle est phosphorylée en AMP par l'adénosine kinase (K_m = 100 nM) (Spychala et al., 1996) ou dégradée en inosine par l'adénosine désaminase (ADA) de moindre d'affinité (K_m entre 20 et 100 µM) (Lloyd and Fredholm, 1995). Dans le cœur et probablement d'autres tissus, l'inhibition de l'ADA, induite par l'hypoxie, favorise la formation d'adénosine à partir de l'AMP et conduit à une augmentation majeure de la concentration en adénosine (Decking et al., 1997). L'adénosine kinase étant quasiment absente du milieu extra-cellulaire, l'adénosine ne peut être rephosphorylée en AMP ou ATP (Lloyd and Fredholm, 1995).

L'adénosine peut être aussi libérée dans le compartiment extra-cellulaire sous l'influence de certains neuro-transmetteurs comme l'acide N-méthyl-D-aspartique (NMDA) et le kaïnate qui augmentent le taux d'adénosine d'une manière dose-dépendante (Carswell et al., 1997; Delaney et al., 1998). L'activation des récepteurs de NMDA semble libérer l'adénosine elle-même plutôt qu'un précurseur (Harvey and Lacey, 1997; Manzoni et al., 1994). Une autre source d'adénosine extra-cellulaire est l'AMPc qui peut être libérée par des neurones et convertie, successivement, par des phosphodiestérases extra-cellulaires, en AMP puis, par une ecto 5'-nucléotidase, en adénosine (Jackson et al., 2007; Jackson and Raghvendra, 2004).

2. Régulation

La production d'adénosine dépend de l'état des cellules. En l'absence de stress oxydant, l'hydrolyse de la SAH produit environ 30% de l'adénosine, le reste est obtenu par déphosphorylation d'AMP (figure 3). Dans ces circonstances, la concentration intra-cellulaire en adénosine est sous contrôle enzymatique et reste faible. En cas de stress cellulaire, la synthèse se fait essentiellement par dégradation d'ATP et la concentration en adénosine extra-cellulaire augmente et peut atteindre jusqu'à 100 fois les valeurs basales (Fredholm et al., 2001; Ralevic and Burnstock, 1998). Dans ces conditions, les cellules en souffrance captent moins bien l'adénosine extra-cellulaire, ce qui contribue à son augmentation locale. En plus, quand la concentration intra-cellulaire en ATP s'effondre, suite à l'utilisation excessive d'énergie, celle en adénosine croît considérablement. Cette adénosine est ensuite transportée dans l'espace extra-cellulaire via des transporteurs mentionnés ci-dessous (Fredholm et al., 1994; Jonzon & Fredholm, 1985). Ainsi en cas d'hypoxie ou d'ischémie, il existe une augmentation des concentrations en adénosine extra-cellulaire qui constitue donc un marqueur très sensible de cet état physiopathologique (Sollevi, 1986). Par ailleurs, la libération directe de l'ATP, qui est ensuite converti dans le compartiment en adénosine, fait qu'il existe une relation entre l'ATP et ses récepteurs et entre l'adénosine et ses récepteurs dans le sens où chacune de ces molécules va agir successivement sur son récepteur spécifique et l'ATP peut alors être considéré comme une pro-drogue de l'adénosine (Matsuoka and Ohkubo, 2004).

La régulation de la concentration extra-cellulaire d'adénosine est un processus très dynamique avec un taux de renouvellement considérable. Les concentrations en adénosine dans le milieu extra-cellulaire varient selon les différents tissus et se situe généralement entre 10^{-8} et 10^{-7} M (Headrick, 1996; Siragy and Linden, 1996). La dégradation et la captation rapide de l'adénosine par les cellules explique que sa demi-vie plasmatique soit très courte, de l'ordre de quelques secondes (Dawicki et al., 1986; Klabunde and Althouse, 1981). Cette demi-vie courte conduit à considérer l'implication de l'adénosine dans le processus autocrine et paracrine mais pas endocrine.

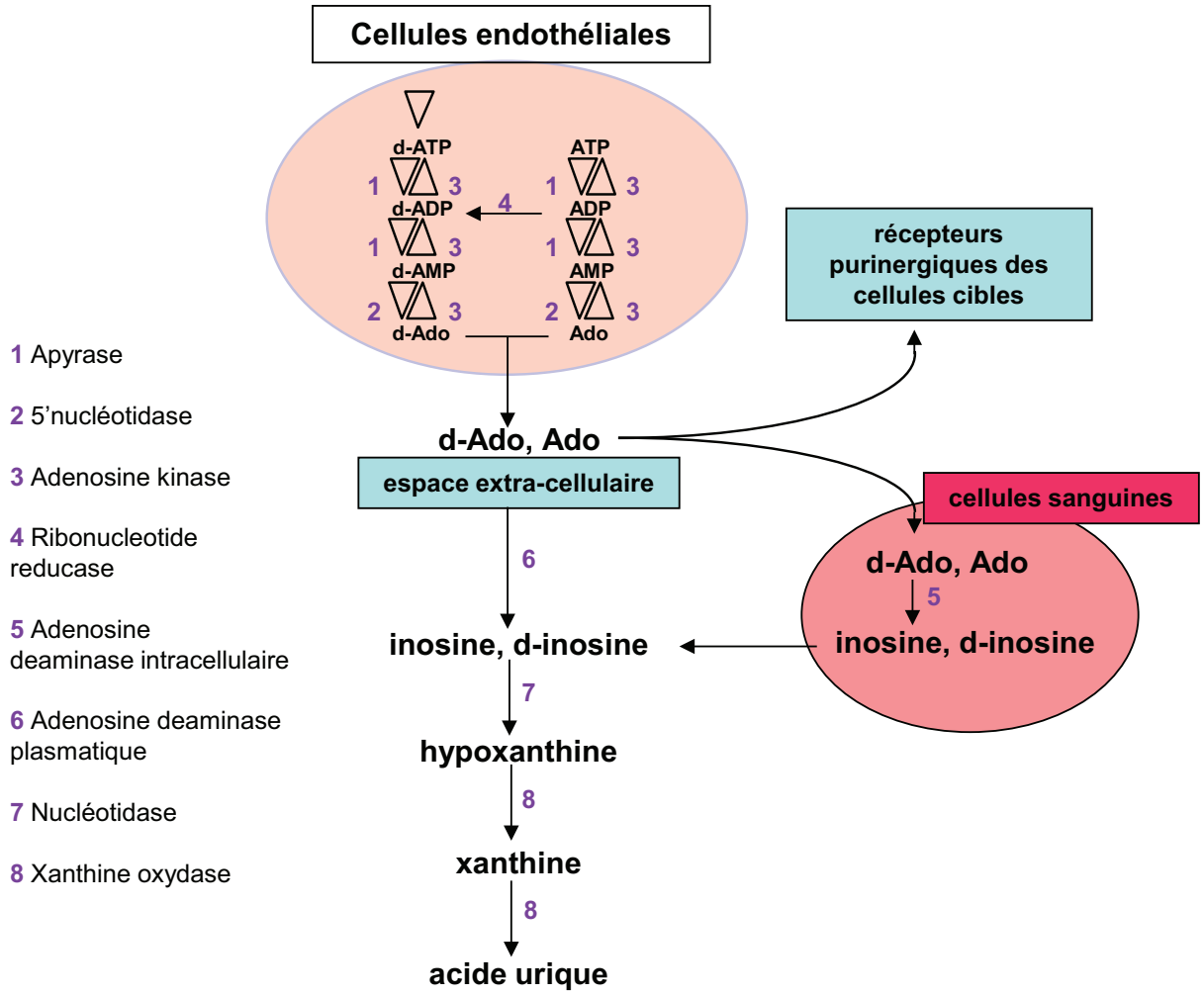

Figure 2. Devenir de l'adénosine extra-cellulaire

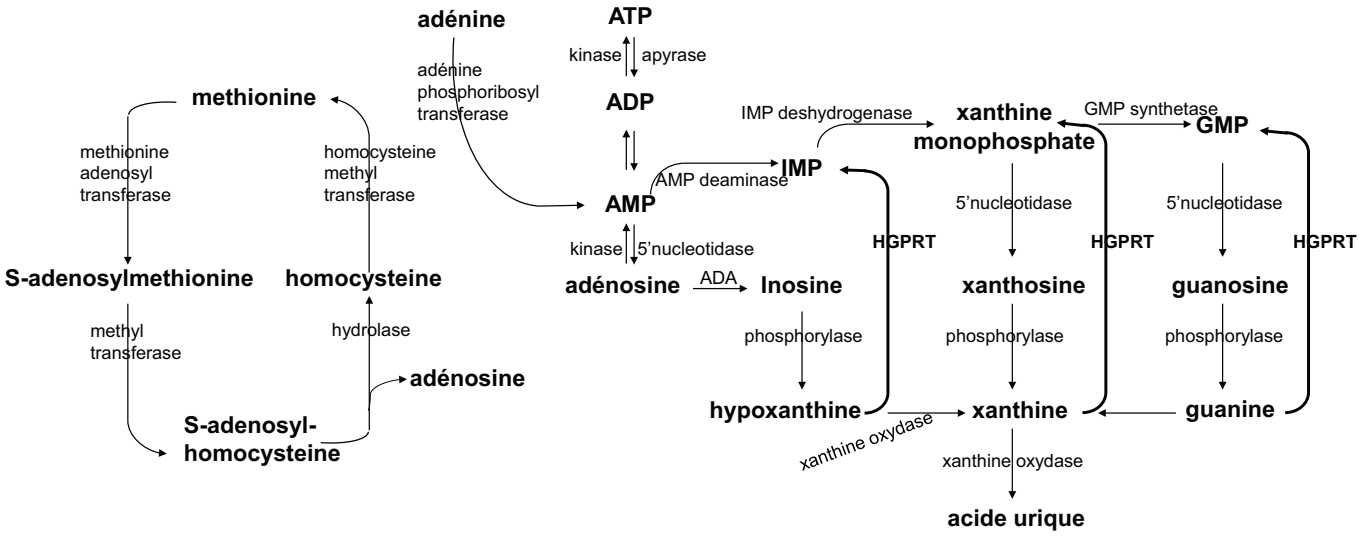

Figure 3. Sources principales de l'adénosine.

ADA : adénosine désaminase, HGPRT : hypoxanthine guanine phosphoribosyl transférase.

III. Récepteurs purinergiques

Les nucléotides puriques ou pyrimidiques exercent des effets physiologiques par l'activation de récepteurs cellulaires dont le clonage et les effets pharmacologiques ont permis de distinguer deux grand groupes de récepteurs : les récepteurs pour l'adénosine et les récepteurs pour les nucléotides puriques ou pyrimidiques. Les récepteurs pour l'adénosine (P1) font tous partie de la superfamille de récepteurs couplés à une protéine G tandis que les récepteurs pour les nucléotides se distinguent en récepteurs canaux (P2X) et en récepteurs couplés à une protéine G (P2Y) (Burnstock, 2007). Seuls les récepteurs de l'adénosine, en particulier les sous-types A_1 et A_{2A}, seront décrits dans ce manuscrit.

IV. Récepteurs de l'adénosine

Les récepteurs de l'adénosine font partie de la superfamille des récepteurs couplés aux protéines G. Ils ont été classés en quatre types, les A_1R, $A_{2A}R$, $A_{2B}R$ et A_3R, en fonction de leur séquence primaire et de leurs propriétés pharmacologiques (Fredholm et al., 2001; Ralevic and Burnstock, 1998; Sebastiao and Ribeiro, 2009). Ces récepteurs ont été clonés à partir d'une grande variété de types cellulaires, dans plusieurs espèces, et ont pu être exprimés dans divers modèles cellulaires. Avant les années 80, on utilisait surtout des tests fonctionnels pour étudier l'effet des ligands sur l'animal ou la genèse de seconds messagers. Actuellement, on utilise des lignées cellulaires transfectées ou des animaux génétiquement modifiés pour l'étude de l'expression ou l'étude fonctionnelle de ces récepteurs (Yaar et al., 2005).

Structure

Les récepteurs de l'adénosine sont caractérisés par une structure formée d'une seule chaine polypeptidique comprenant sept hélices transmembranaires, avec une partie amino(N)-terminale extra-cellulaire et une partie carboxy(C)-terminale cytosolique (Costanzi S et al., 2007). Ces hélices, constituées de 25 à 30 résidus d'acides aminés, sont connectées

par six boucles comprenant trois boucles intra-cellulaires et trois boucles extra-cellulaires. Les régions extra-cellulaires contiennent des sites pour les modifications post-traductionnelles comme la glycosylation. Les A_1R et A_3R contiennent également des sites pour la palmitoylation dans le domaine C-terminal. Les $A_{2A}R$ possèdent un long segment C-terminal de plus de 120 acides aminés qui ne sont pas indispensables pour le couplage à la protéine G mais qui peuvent servir comme sites de fixation pour d'autres protéines accessoires (Zezula and Freissmuth, 2008). Les homologies de séquence pour les récepteurs humains de l'adénosine sont de 49 % entre les A_1R et A_3R, et de 59 % entre les $A_{2A}R$ et $A_{2B}R$. Dans les A_3R, le résidu Histidine dans l'hélice 6 est absent mais un autre résidu Histidine est présent, à un autre emplacement, à l'intérieur de l'hélice 3. L'implication de ces résidus Histidine dans la fonction de reconnaissance et/ou l'activation du récepteur a été démontrée par mutagenèse dirigée (Costanzi S et al., 2007; Kim et al., 2003).

Tous les récepteurs humains de l'adénosine ont une structure génomique similaire (Fredholm et al., 2000; Olah and Stiles, 2000). Le gène de l'A_1R a été le plus étudié. Il comporte six exons et cinq introns et il est situé sur le chromosome 1q32.1 (Townsend-Nicholson et al., 1995). Ce gène est à l'origine de deux transcrits qui sont sous la dépendance de deux promoteurs, le promoteur A du côté proximal et B du coté distal. Le transcrit contenant les exons 4, 5 et 6 est présent dans tous les tissus exprimant le récepteur tandis que le transcrit contenant les exons 3, 5 et 6 se trouve dans les tissus à fort niveau d'expression tels que le cerveau, les testicules et les reins. Le gène de l'$A_{2A}R$ humain est localisé sur le chromosome 22q11.2 et possède une structure à deux exons (Fredholm et al., 2000) similaire à celle rapportée chez le rat (Chu et al., 1996).

L'$A_{2A}R$ présente un transcrit hybride dans la plupart des tissus examinés (Maenhaut et al., 1990; Peterfreund et al., 1996; Stehle et al., 1992). L'examen d'ARN isolés de cellules PC12 suggère deux sites différents d'initiation de la transcription (Chu et al., 1996). L'expression de l'$A_{2A}R$ peut être stimulée par les protéines kinases C (PKC) (Peterfreund et al., 1997) et par l'hypoxie (Kobayashi et al., 1998), bien qu'on ne connaisse pas précisément les facteurs de transcription impliqués. Il faut noter que l'$A_{2A}R$ est polymorphe chez l'homme (Fredholm et al., 2000). Il existe un polymorphisme (Single nucleotide polymormphism, SNP) de population, en particulier, le SNP T1083C est plus représenté chez les caucasiens que chez les asiatiques (Deckert et al., 1996; Soma et al., 1998). Ce polymorphisme, bien que muet,

serait associé à certaines pathologies comme les syncopes neurocardiogéniques (Saadjian et al., 2009) ou les attaques de panique (Hamilton et al., 2004).

Le gène de l'A_{2B}R est localisé sur le chromosome 17p11.2 (Jacobson et al., 1995) et présente une structure similaire à celle des gènes des autres sous-types de récepteurs. Chez le rat, l'A_{2B}R présente deux transcrits hybrides de 1.8 et 2.2 kb, ce dernier étant le plus représenté (Stehle et al., 1992). Le gène de l'A_3R humain est situé sur le chromosome 1p13.3. Il présente deux transcrits, dont le plus et le moins représenté sont, respectivement de 2 kb et 5 kb (Atkinson et al., 1997).

SIGNALISATION

De nombreuses évidences supportent l'idée que les récepteurs de l'adénosine régulent la fonction cellulaire par couplage aux protéines G, bien que certains effets indépendants des protéines G aient été également rapportés (Fredholm et al., 2007). L'effecteur principal des quatre sous-types de récepteurs de l'adénosine est l'adényl cyclase dont l'activité est soit stimulée, soit inhibée suivant le sous-type de récepteur présent sur la cellule. Les A_1R et A_2R ont été initialement distingués par leurs propriétés respectives d'inhibition et de stimulation de l'adényl cyclase. Ainsi les A_1R et A_3R sont couplés aux protéines G_i induisant une diminution de la concentration d'AMPc, tandis que les A_{2A}R et A_{2B}R stimulent l'adényl cyclase par activation des protéines G_s avec, par conséquent, une augmentation d'AMPc. Par ailleurs, la signalisation peut être transmise par d'autres protéines G telles que la G_q ou la G_{olf}. Il a été montré que les A_{2A}R pouvaient se coupler aux différentes protéines G dans les différents tissus (Kull et al., 2000). La protéine G_s est associée aux A_{2A}R dans les systèmes périphériques mais pas dans le striatum où la densité des A_{2A}R est plus élevée. Ainsi, la signalisation des A_{2A}R du striatum passe essentiellement via la G_{olf}. D'ailleurs, un récepteur de l'adénosine peut aussi se coupler à plusieurs protéines G après transfection. Par exemple, les A_{2B}R humains transfectés dans les cellules de mastocytes humains (HEK 293) ou de mastocytes canins (HMC-1) sont couplés à la fois aux G_s et G_q (Auchampach et al., 1997; Linden et al., 1999).

Après l'activation des protéines G, la voie de signalisation affecte les enzymes et les canaux ioniques. L'activation des A_1R induit une inhibition de l'adényl cyclase, une activation de quelques types des canaux K^+ (probablement via les sous-unités β,γ de la protéine G), une inactivation des canaux Ca^{2+} de types N, P et Q, une activation de la phospholipase Cβ et une activation des MAP-kinases. Cette voie de signalisation semble être identique pour les A_3R (Englert et al., 2002). Les $A_{2A}R$ et $A_{2B}R$ peuvent aussi induire une augmentation intra-cellulaire de Ca^{2+} selon le type de cellule. La voie de signalisation par les MAP-kinases a été aussi démontrée pour les $A_{2A}R$ et $A_{2B}R$ (Schulte and Fredholm, 2000).

1. Récepteurs A_1 de l'adénosine

L'A_1R a été cloné chez de nombreuses espèces telles que la souris, le rat, le lapin, le chien, les bovins et l'homme. Son polymorphisme présente jusqu'à 10% de variation entre les espèces. Ceci peut expliquer les variations d'affinité (Tucker et al., 1992) et certaines différences dans les processus de régulation (Palmer and Stiles, 1997) ainsi que dans le couplage aux protéines G (Jockers et al., 1994). Il a un poids moléculaire d'environ 36 kDa et une forte affinité pour l'adénosine.

1.1 Ligands

Ligands naturels : L'adénosine est le ligand endogène naturel pour les A_1R. Des études de fixation sur des ovaires de hamster chinois exprimant les A_1R humains ont permis de mettre en évidence des A_1R de forte (0.7 µM) et de faible (150 µM) affinité pour l'adénosine (Cohen et al., 1996). Les antagonistes xanthiques, comme la théophylline (1,3-dimethylxanthine) et la caféine (1,3,7-trimethylxanthine), sont non spécifiques et ont une affinité de l'ordre du micromolaire pour les A_1R, $A_{2A}R$ et $A_{2B}R$. Ces ligands naturels sont évidemment très présents dans l'alimentation courante.

Ligands synthétiques : Les principaux agonistes des A_1R sont obtenus par des substitutions à la position N^6 de l'adénosine (Figure 4) qui donnent des molécules comme le N^6-cyclopentyl adénosine (**CPA**) et le 2-chloro-CPA (**CCPA**). Le CCPA (K_i = 0.83 nM) présente un peu plus d'affinité que le CPA (K_i = 2.3 nM), mais ces deux composés sont plus spécifiques

de l'A_1R murin que de l'A_1R humain. Un autre agoniste, le **S(-)-ENBA**, apparait très affin et spécifique aussi bien chez le rat que chez l'homme (Gao et al., 2003). La modification des xanthines permet d'obtenir des antagonistes hautement spécifiques pour les A_1R tels que le 1,3-dipropyl-8-cyclopentylxanthine (**DPCPX**) avec un K_i de 3.9 nM. Certains antagonistes non xanthiques, dérivés de l'adénine, comme le 8-(*N*-methylisopropyl)amino-N^6-(5'-endohydroxy-endonorbornyl-)9-méthyl adénine (**WRC 0571**) sont aussi spécifiques pour les A_1R avec un K_i de 1.7 nM.

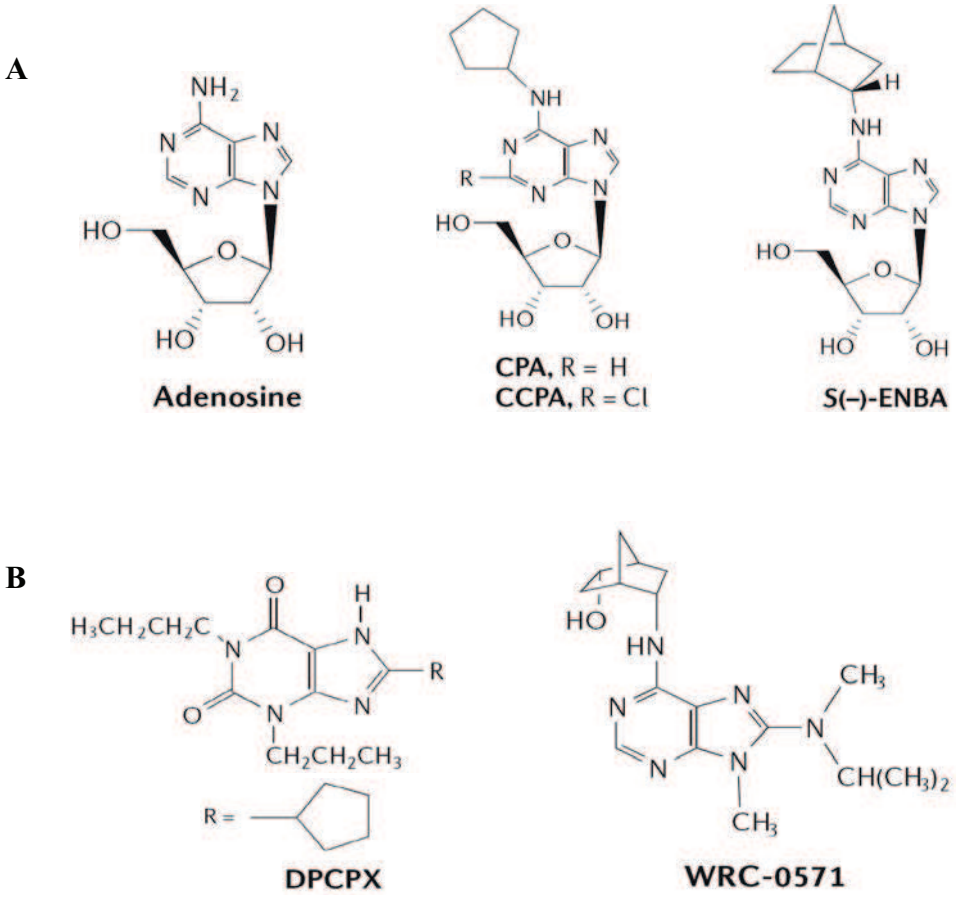

Figure 4 : Agonistes (A) et antagonistes (B) des A_1R

1.2 Distribution

La répartition des A_1R est large et leur transcription est bien corrélée à leurs expressions dans les tissus. On trouve une forte expression dans certaines parties du cerveau (cortex, cervelet, hippocampe, thalamus et tronc cérébral), les cornes postérieures de la moelle, les yeux, les surrénales et le cœur. L'expression des A_1R est moyenne dans le reste du cerveau,

les muscles squelettiques, le foie, les reins, les adipocytes, les glandes salivaires, l'œsophage, l'estomac, le colon et les testicules. L'expression est faible dans les poumons, le pancréas et l'intestin grêle (tableau 1).

Tableau 1 : Expression des sous-types de récepteurs de l'adénosine dans différents tissus

	Expression forte	**Expression intermédiaire**	**Expression faible**
A_1R	Cerveau (cortex, cervelet, hippocampe) cornes dorsales de la moelle, yeux, cœur, surrénales	Autres régions du cerveau, muscle squelettique Foie, rein, tissus adipeux, glandes salivaires, œsophage, colon, testicules	Poumons, pancréas
$A_{2A}R$	Rate, thymus, leucocytes, plaquettes, striatum, bulbes olfactifs	Cœur, poumons, vaisseaux sanguins	Autres régions du cerveau
$A_{2B}R$	Cæcum, colon	Poumon, vaisseaux, yeux, éminence médiane, Mastocytes	Tissu adipeux, surrénale, cerveau, reins, foie, ovaires
A_3R	Testicules Mastocytes	Cervelet, hippocampe Poumon, rate, hypophyse	Thyroïde, cerveau, cœur, intestin, surrénales, foie

1.3 Récepteurs A_1 dans le système nerveux

L'activation des A_1R au niveau pré- et post-synaptique conduit à une réduction de l'activité neuronale par une hyperpolarisation et, par conséquent, à une inhibition de la libération des neurotransmetteurs, pour revue voir (Guieu et al., 1998; Sebastiao and Ribeiro, 2009). En cas d'ischémie tissulaire, l'adénosine libérée donne un effet neuro-protecteur par le biais des A_1R en réduisant la consommation locale d'oxygène et en diminuant les entrées de calcium via l'inhibition des récepteurs au glutamate. Par ailleurs, l'activation des A_1R conduit à des effets sédatifs, anti-convulsivants et anxiolytiques

(Malhotra and Gupta, 1997). L'adénosine exerce des actions multiples sur la transmission de la douleur au niveau périphérique et central (voir figure 5). L'activation des A_1R produit un effet anti-nociceptif, en partie mais pas seulement, par diminution de la libération d'AMPc dépendante de certains neurotransmetteurs dans les terminaisons nerveuses sensorielles (Sawynok, 1998). Les A_1R contrôlent la nociception par trois mécanismes principaux:

1) au niveau pré-synaptique (Figure 5), l'activation des A_1R conduit , via le complexe α_i de la protéine G, à une inhibition de l'activité adényl cyclasique responsable de la diminution de la concentration d'AMPc dans les cordons postérieurs de la moelle et en périphérie ainsi que de la diminution des neurotransmetteurs impliqués dans la genèse de la nociception comme le peptide CGRP, la substance P, l'acétylcholine ou le glutamate (Burnstock and Wood, 1996; Rubino et al., 1993) ;

2) au niveau pré-synaptique, l'activation des A_1R conduit aussi, via le complexe $\beta\gamma$ de la protéine G, à une inhibition des canaux calcium voltage-dépendants de types L, N et P responsable du relâchement des fibres musculaires lisses, de la vasodilatation et de l'inhibition de la libération des neurotransmetteurs impliqués dans la nociception ;

3) au niveau post-synaptique, l'activation des A_1R conduit, également via $G_{\beta\gamma}$, à une activation et une ouverture des canaux potassium (en particulier rectifiant rentrant) engendrant une hyperpolarisation post-synaptique et un blocage de la neurotransmission. Cet effet participe, entre autres, aux effets anti-nociceptifs. À noter que les mécanismes moléculaires conduisant à ces effets anti-nociceptifs sont comparables à ceux obtenus lors de l'activation des récepteurs aux opioïdes dont il existe trois sous-types : mu, delta et kappa.

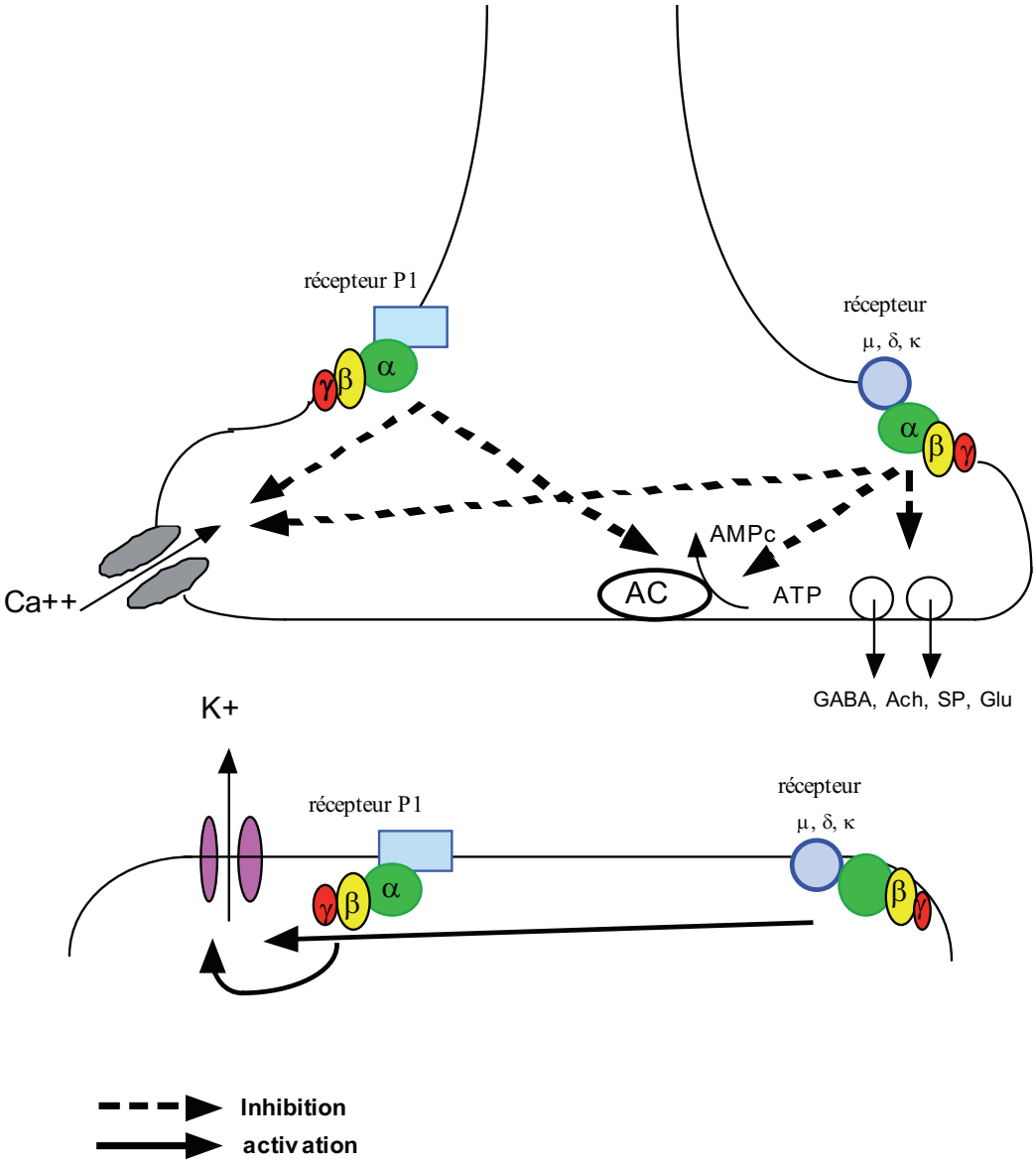

Figure 5 : Effets synaptiques de l'activation des récepteurs (P1) de l'adénosine

1.4 Récepteurs A_1 dans le système cardiovasculaire

L'activation des A_1R induit de nombreux effets dans le système cardiovasculaire tels qu'une diminution du rythme et de la contractilité cardiaque ainsi qu'une atténuation de l'action stimulatrice des catécholamines sur le cœur (Fraser et al., 2003; Zablocki et al., 2004) ; pour revue voir (Shryock and Belardinelli, 1997). Les effets chronotropes (fréquence cardiaque), dromotropes (vitesse de conduction myocardique) et inotropes (contractilité myocardique) négatifs de l'A_1R induits par l'adénosine intraveineuse (Olsson and Pearson, 1990) ont été utilisés dans le traitement des tachycardies supra-ventriculaires. L'activation des A_1R conduit à une modulation indirecte du tonus vasculaire par inhibition de la libération des neurotransmetteurs au niveau pré-jonctionnel dans les fibres sympathiques péri-vasculaires (Goncalves and Queiroz, 1996) et dans les afférences sensitives, sensibles à la capsaïcine (8-méthyle *N*-vanillyle 6-nonénamide) qui est le composé actif du piment *Capsicum* (Rubino et al., 1993). Suivant l'espèce et le tissu étudié, les effets peuvent être opposés. Les A_1R sont largement décrits comme responsables des effets protecteurs de l'adénosine dans les accidents d'ischémie-reperfusion myocardiques (Stambaugh et al., 1997), pulmonaires (Neely and Keith, 1995) et cérébraux (Heurteaux et al., 1995). Les canaux potassium ATP-sensible et les PKC semblent être les voies principalement impliquées dans ces phénomènes.

1.5 Récepteurs A_1 dans le système immunitaire

Les A_1R sont exprimés dans les cellules du système immunitaire telles que les cellules myéloïdes immatures (Panther et al., 2001) et les cellules dendritiques (Schnurr et al., 2004). L'adénosine recrute les cellules dendritiques immatures au niveau des sites inflammatoires via les A_1R et A_3R. L'activation des A_1R résulte des effets pro-inflammatoires dans de nombreux tissus et cellules. À la différence des $A_{2A}R$ qui inhibent l'adhésion des neutrophiles à l'endothélium, les A_1R favorisent le chimiotactisme et l'adhésion des neutrophiles à certaines molécules adhésives sur l'endothélium (Cronstein et al., 1992). Cependant, des études chez les souris génétiquement modifiées suggèrent que les A_1R possèdent également des fonctions anti-inflammatoires dans certains contextes. Par exemple, il a été rapporté que les souris knockout (KO) déficientes pour le gène de l'ADA et pour celui des A_1R développent des inflammations pulmonaires plus graves que les souris KO déficientes

seulement pour le gène de l'ADA. L'A_1R présente donc un rôle protecteur dans ce modèle d'inflammation pulmonaire (Sun et al., 2005).

1.6 Apport des souris transgéniques

Des souris transgéniques, n'exprimant pas l'A_1R, présentent une augmentation d'anxiété, une hyperalgésie ainsi qu'une baisse d'excitabilité neuronale en réponse à l'hypoxie (Johansson et al., 2001). Les effets modulateurs de l'adénosine sur la neurotransmission glutamatergique excitatrice y sont annulés. Ces souris n'ont plus de rétrocontrôle tubulo-glomérulaire et leurs taux de rénine sont élevés (Brown et al., 2001). Par contre, les souris sur-exprimant les A_1R montrent une résistance augmentée au phénomène d'ischémie-reperfusion et au pré-conditionnement myocardique, et cette résistance est directement liée au nombre de A_1R exprimés (Headrick et al., 1998; Lankford et al., 2006; Matherne et al., 1997)

2. Récepteurs A_{2A} de l'adénosine

L'$A_{2A}R$ a été cloné dans de nombreuses espèces. Sa masse moléculaire est d'environ 45 kDa et sa structure cristallographique a été récemment déterminée par rayons X (Jaakola et al., 2008). Par rapport aux A_1R, les $A_{2A}R$ ont une affinité plus faible pour l'adénosine.

2.1 Ligands

Ligands naturels : Les antagonistes xanthiques, comme la théophylline et la caféine, sont non spécifiques et ont une affinité de l'ordre du micromolaire pour les $A_{2A}R$.

Ligands synthétiques : Les agonistes des $A_{2A}R$ sont des dérivés de l'adénosine obtenus généralement par substitution en C2 sur son groupement adénine ou en 5' sur son groupement ribose (Figure 6). La substitution par des groupements amides alkyles en 5' de l'adénosine, comme dans l'agoniste non spécifique **NECA** (5'-*N*-ethylcarboxyamido adénosine), augmente considérablement l'affinité des agonistes sur tous les récepteurs de l'adénosine. Cette approche a été utilisée également pour générer **CGS21680** (2-[p-(2-carbonyl-ethyl)-phénylethylamino]-5'-N-ethylcarboxyadénosine) qui est un agoniste

modérément spécifique pour les $A_{2A}R$ chez le rat avec un K_i de 27 nM (140 fois plus spécifique pour l'$A_{2A}R$ que pour l'A_1R) (Fredholm et al., 2001; Gao et al., 2003). L'agoniste spécifique **ATL146e** (4-[3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl] -cyclohexanecarboxylic acid methyl ester) présente, avec son K_i de 0.5 nM, une affinité 50 fois plus forte pour l'$A_{2A}R$ que CGS21680 qui est l'agoniste de référence (Rieger et al., 2001).

Les principaux antagonistes des $A_{2A}R$ sont des dérivés des bases xanthiques tels que **ZM241385** (4-(2-[7-amino-2-[2-furyl]-[1,2,4]triazolol[2,3-α][1,3,5]triazin-5-yl-amino]ethyl) phénol) et **SCH58261** (5-amino-7-(2-phenylethyl)-2(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine). Ce dernier est le plus spécifique avec une affinité de l'ordre du nanomolaire. ZM241385 fixe également les $A_{2B}R$ mais avec une affinité plus faible (Ongini et al., 1999; Poucher et al., 1995).

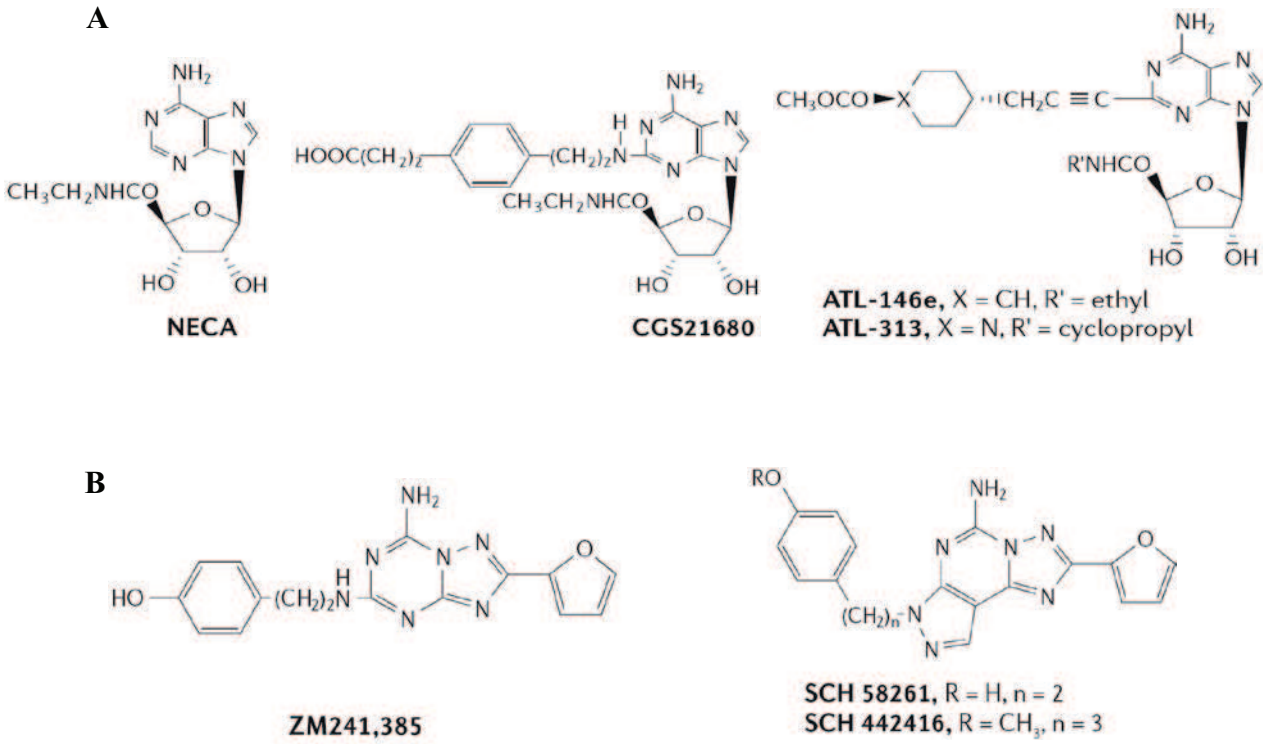

Figure 6 : Agonistes (A) et antagonistes (B) des $A_{2A}R$

2.2 Distribution

Les $A_{2A}R$ sont principalement exprimés dans le cerveau (en particulier les bulbes olfactifs, le noyau accumbens et le striatum), dans les cellules du système immunitaire, dans les plaquettes et dans les fibres musculaires lisses des vaisseaux et de l'endothélium (voir tableau 1). Il semble que les $A_{2A}R$ soient les récepteurs majoritaires de l'adénosine dans les lymphocytes et les plaquettes. Dans les neurones du striatum, les $A_{2A}R$ sont co-localisés avec les récepteurs D_2 de la dopamine surtout dans les neurones GABAergiques striato-pallidaux (Weaver, 1993). D'ailleurs, l'expression des $A_{2A}R$ est faible dans les autres régions du cerveau telles que le cortex et l'hippocampe. L'expression est intermédiaire dans le cœur, les poumons et les vaisseaux.

2.3 Récepteurs A_{2A} dans le système nerveux

Dans le système nerveux, les $A_{2A}R$ facilitent généralement la libération des neurotransmetteurs. Ils sont fortement exprimés au niveau des noyaux gris de la base du crâne, où ils exercent un contrôle puissant sur les voies dopaminergiques via les récepteurs D_2 (Ferre et al., 2007). Cette relation entre $A_{2A}R$ et récepteurs dopaminergiques D_2 permet d'espérer une prochaine utilisation d'antagonistes des $A_{2A}R$ dans le traitement de la maladie de Parkinson pour éviter le phénomène de tolérance (Halldner et al., 2000; Morelli et al., 2009) et dans d'autres désordres du système nerveux central comme la maladie de Hutington (Cunha et al., 2008; Popoli, 2008). Des études pharmacologiques, chez les souris KO déficientes en $A_{2A}R$, montrent que l'inactivation de l'$A_{2A}R$ protège contre la mort des cellules neuronales induite par l'ischémie cérébrale (Chen et al., 1999; Monopoli et al., 1998). Inversement, la reconstitution sélective des $A_{2A}R$ dans les cellules de la moelle épinière, chez ces souris KO, annule l'effet de neuro-protection. Ceci laisse penser que les $A_{2A}R$ pourraient bloquer l'effet protecteur des A_1R et A_3R contre l'hypoxie (Strickler et al., 1996) et ainsi contribuer à aggraver les lésions ischémiques cérébrales (Chen et al., 1999). Des interactions avec les récepteurs D_1 au glutamate et à l'acétylcholine (Sebastiao and Ribeiro, 1996) et les récepteurs aux opioïdes (Halimi et al., 2000; Pham et al., 2003) ont été également décrites. Au niveau comportemental, l'activation des $A_{2A}R$ conduit à une catalepsie (Hauber and Munkle, 1995), tandis que leur blocage par la caféine restaure l'activité motrice (El et al., 2000a).

En ce qui concerne le contrôle de la nociception, le rôle des $A_{2A}R$ reste controversé dans la littérature. L'activation des $A_{2A}R$ a pour résultat des effets pro- ou anti-nociceptifs en fonction du modèle animal utilisé et des conditions expérimentales. Cet aspect sera abordé dans la publication 4.

2.4 Récepteurs A_{2A} dans le système cardiovasculaire

L'activation des $A_{2A}R$ conduit à un relâchement des fibres musculaires des parois vasculaires et à une vasodilatation (Belardinelli et al., 1998; Conti et al., 1993; Iwamoto et al., 1994; Monahan et al., 2000) ; pour revue voir (Shryock and Belardinelli, 1997). Ainsi les souris KO déficientes pour l'$A_{2A}R$ sont spontanément hypertensives (Ledent et al., 1997). Dans les artères coronaires où les $A_{2A}R$ sont prédominants par rapport aux autres sous-types, l'effet de la vasodilatation passe principalement par la voie de l'AMPc (Hussain and Mustafa, 1993; Rekik and Mustafa, 2003). Par ailleurs, ces effets sur la vasodilatation sont, en partie, secondaires à la modulation de canaux ioniques, en particulier à l'inhibition de canaux calcium voltage-dépendant, mais également à la modulation de canaux potassium (rectifiant rentrant, K_{ATP}) (Bryan and Marshall, 1999; Tang et al., 1999). Toujours au niveau coronarien, il a été mentionné l'existence de récepteurs de réserve (spare receptors), qui contrôleraient le flux coronaire (Shryock et al., 1998). L'existence de « spare receptors » est objectivée chaque fois que les effets métaboliques cellulaires sont au maximum (par exemple la production d'AMPc), alors même que tous les récepteurs ne sont pas occupés par le ligand, autrement dit chaque fois que la valeur de l'EC_{50} est inférieure au K_d. L'existence de ces récepteurs de réserve au niveau coronarien permet d'expliquer la vasodilatation coronaire en l'absence d'effets systémiques en particulier sur la pression artérielle (Shryock et al., 1998). Il a été suggéré que l'activation des $A_{2A}R$ au niveau central induisait une tachycardie, tandis que l'activation des $A_{2A}R$ périphériques était responsable d'un effet hypotensif (Schindler et al., 2005). A la frontière entre système cardiovasculaire et système nerveux, il a été montré que les $A_{2A}R$ sont très exprimés dans une région du tronc cérébral qui contrôle la fréquence cardiaque et la pression artérielle (Thomas et al., 2000).

A coté du rôle de protection contre l'ischémie assuré par les A_1R, il a été démontré chez la souris que l'activation des $A_{2A}R$ avait une action protectrice contre les atteintes secondaires au phénomène d'ischémie-reperfusion, et que cet effet protecteur était dû, en

partie, à une action anti-inflammatoire via les $A_{2A}R$ des lymphocytes (Yang et al., 2005). Dans les plaquettes, l'activation des $A_{2A}R$ induit une inhibition de l'agrégation plaquettaire en augmentant la concentration intra-cellulaire en AMPc (Varani et al., 2000).

2.5 Récepteurs A_{2A} dans le système immunitaire

Les $A_{2A}R$ sont exprimés dans presque toutes les cellules immunitaires comprenant les lymphocytes, les monocytes, les macrophages ainsi que les cellules dendritiques (Sitkovsky et al., 2004). L'activation des $A_{2A}R$ inhibe l'inflammation et les lésions de reperfusion dans différents tissus tels que le foie (Day et al., 2004), les reins (Day et al., 2005), le cœur et l'intestin (Odashima et al., 2005). Via l'activation des $A_{2A}R$, l'adénosine peut inhiber l'activation et la prolifération des cellules T ainsi que la production des cytokines inflammatoires comme IL-2 (Erdmann et al., 2005), en favorisant la production des cytokines anti-inflammatoires. Dans les cellules T $CD4^+$, les agonistes ATL-146^e et ATL-313 des $A_{2A}R$ réduisent la production d'interféron-γ (IFNγ) et modulent les récepteurs des cellules T (TCR), limitant ainsi l'activation des cellules T et des macrophages pendant l'inflammation (Lappas et al., 2005).

2.6 Apport des souris transgéniques

Un nouveau champ de recherche s'est ouvert avec la création des souris KO déficientes pour le gène de l'$A_{2A}R$. Ces animaux présentent une augmentation de l'agressivité, une hypoalgésie, une hypertension artérielle, une tachycardie, une hyperagrégabilité plaquettaire et une réaction inflammatoire majorée (Ledent et al., 1997; Ohta and Sitkovsky, 2001). Ces données constituent un argument pour montrer que l'$A_{2A}R$ exerce un contrôle puissant sur la fréquence cardiaque, la pression artérielle et l'inflammation.

3. Récepteurs A_{2B} de l'adénosine

L'$A_{2B}R$ est le sous-type le moins étudié de la famille des récepteurs de l'adénosine. L'analyse du gène de l'$A_{2B}R$, qui se trouve sur le chromosome humain 17p11.2, montre la présence d'un pseudogène sur le chromosome 1q32 ayant 79% d'homologie avec la séquence codante de l'ADNc de l'$A_{2B}R$ (Jacobson et al., 1995). La transcription de ce

pseudogène dans certains tissus peut conduire à des erreurs d'interprétation des hybridations *in situ* et des Northern blots, si les sondes utilisées sont dans les régions communes.

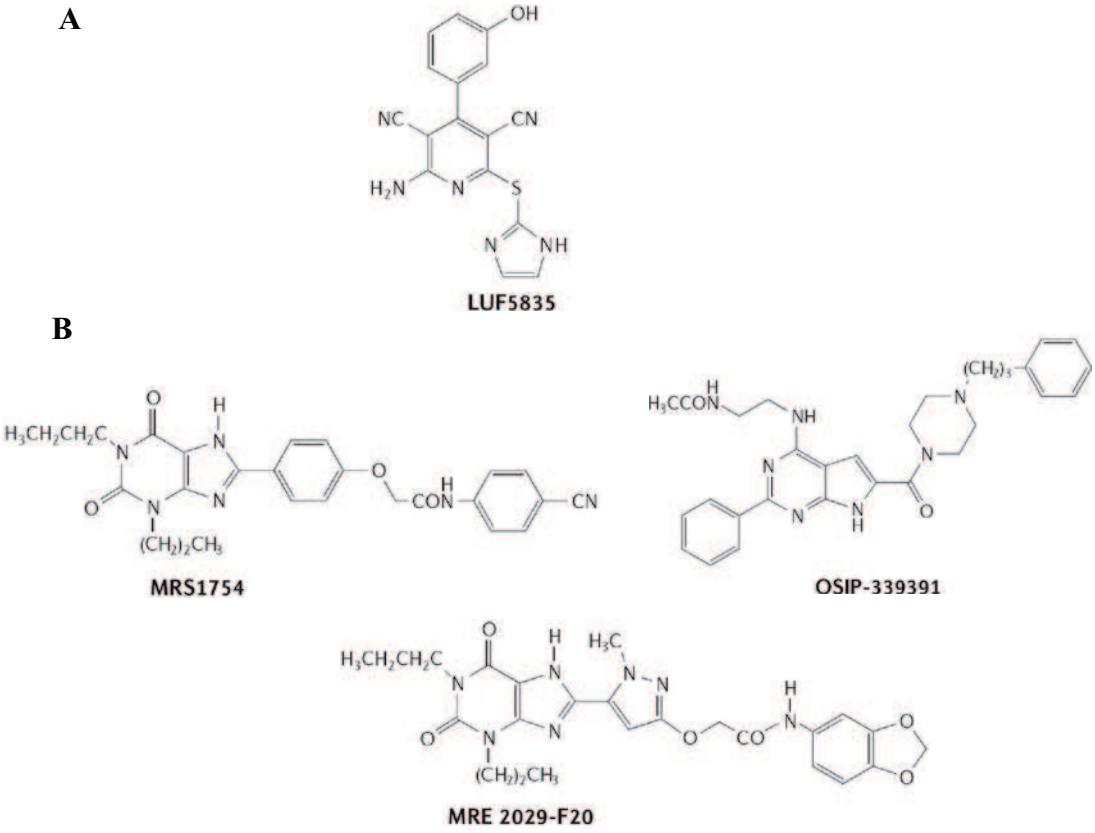

Figure 7 : Agonistes (A) et antagonistes (B) des $A_{2B}R$

3.1 Ligands

Presque tous les agonistes connus de l'$A_{2B}R$ sont des dérivés de l'adénosine de spécificité modeste (voir figure 7). Des composés dérivés de la pyrimidine-3,5-dicarbonitrile ont été développés avec des degrés de spécificité variés, le plus remarquable étant le **LUF5835** avec un EC_{50} de 10 nM (data sur la production d'AMPc) (Beukers et al., 2004). Les xanthines, qui ont été développées comme antagonistes spécifiques de l'$A_{2B}R$, incluent **MRS1754** et **MRE2029-F20** utilisés comme radio-ligands (Baraldi et al., 2004; Ji et al., 2001). Un autre antagoniste spécifique utilisé aussi comme ligand tritié est **[^{3}H]OSIP339391** (Stewart et al., 2004).

3.2 Distribution et rôles biologiques

Les études des ARNm montrent que les $A_{2B}R$ sont présents principalement dans le caecum, le colon et la vessie, ainsi qu'au niveau des noyaux gris de la base du crâne où, à coté des $A_{2A}R$, ils participeraient au contrôle de la motricité. Ils sont exprimés également, quoiqu'à un faible niveau, dans les poumons, les vaisseaux sanguins, les yeux et les mastocytes (voir tableau 1) (Fredholm et al., 2001). L'affinité de ces récepteurs pour l'adénosine reste faible et leur activation nécessite de forte concentration d'adénosine. L'activation des $A_{2B}R$ induit une dégranulation des mastocytes, impliqués dans les réactions allergiques et inflammatoires (Feoktistov and Biaggioni, 1997), et conduit à une vasodilatation comme l'activation des $A_{2A}R$ (Rubino et al., 1995). L'activation des $A_{2B}R$ entraîne aussi une inhibition de la prolifération des fibres musculaires lisses vasculaires (Dubey et al., 1996) favorisant leur apoptose et participant vraisemblablement au processus d'athérosclérose (Peyot et al., 2000). Le rôle de l'$A_{2B}R$ a été mentionné dans le transport des ions chlorures au niveau des cellules épithéliales respiratoires et digestives (Bucheimer and Linden, 2004).

4. Récepteurs A_3 de l'adénosine

L'A_3R, découvert le plus récemment, a été cloné dans diverses espèces et son gène est localisé chez l'homme sur le chromosome 1p13.3 (Monitto et al., 1995). Il est le seul récepteur sur lequel l'inosine a un effet en provoquant la dégranulation des mastocytes (Jin et al., 1997).

4.1 Ligands

Les agonistes prototypiques **IB-MECA** (CF101) et **Cl-IB-MECA** ont été largement utilisés comme agents pharmacologiques pour étudier le rôle physiologique de l'A_3R (Jacobson, 1998). **IB-MECA** est 50 fois plus spécifique *in vitro* pour les A_3R de rat que pour les autres sous-types de récepteurs avec un K_i de 1.8 nM. L'agoniste hautement spécifique des A_3R, **MRS3558** avec un K_i de 0.29 nM, a été récemment rapporté. Un dérivé 4-aminobenzyl peut être radio-iodé pour devenir le **[^{125}I]-I-AB-MECA** qui est très utilisé comme ligand radioactif de haute affinité pour les A_3R (Olah et al., 1994) (figure 8).

Les antagonistes classiques des récepteurs de l'adénosine tels que la caféine et la théophylline présentent une faible affinité pour les A_3R (Zhou et al., 1992). L'affinité de ces antagonistes varie fortement suivant les espèces. Chez l'homme et le mouton, les analogues de la 8-phénylxanthine ont une affinité de l'ordre de 100 nM pour leurs A_3R. Le dérivé de dihydropyridine **MRS1334** et le pyridylquinazoline **VUF5574** (non spécifique chez le rat) sont relativement spécifiques des A_3R humains avec des K_i respectifs de 2.7 et 4.0 nM, tandis que le dérivé de la pyridine **MRS1523** est un antagoniste spécifique des A_3R humains et de rat.

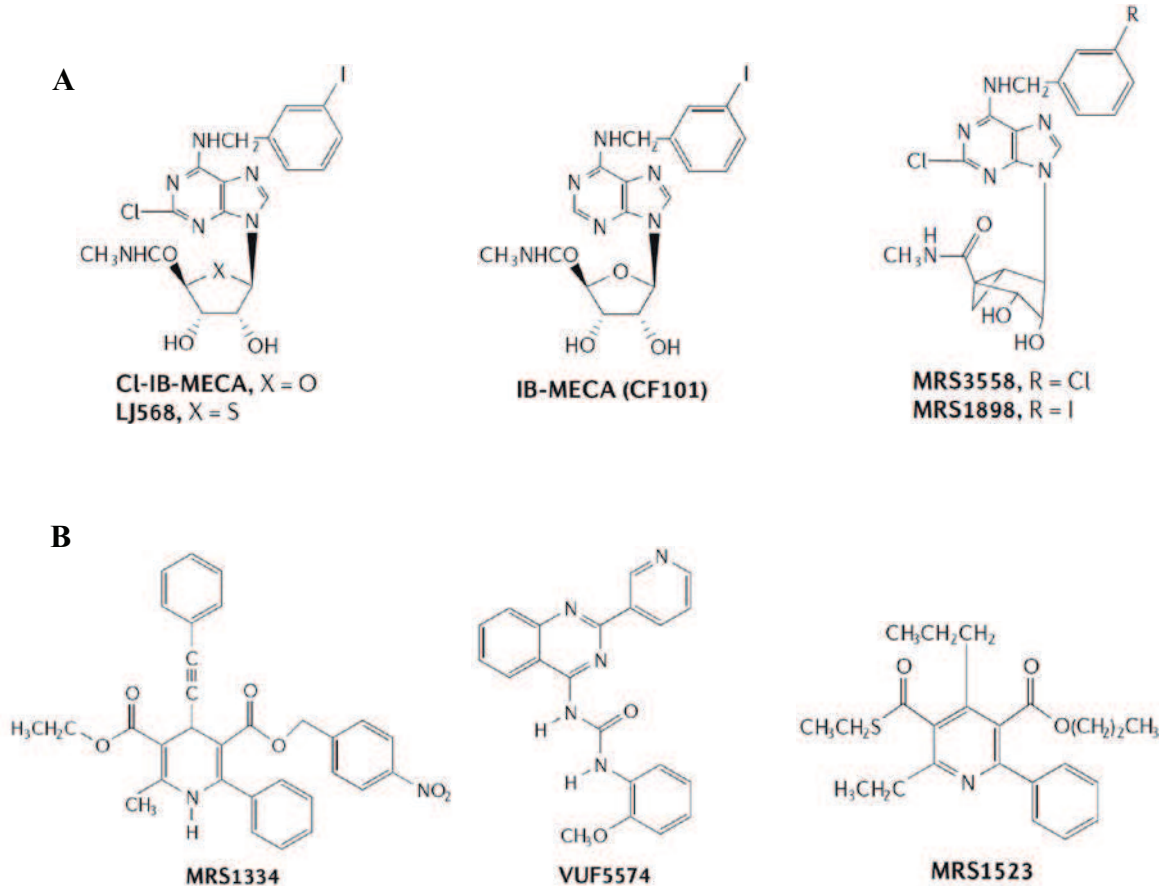

Figure 8 : Agonistes (A) et les antagonistes (B) des A_3R

4.2 Distribution et rôles biologiques

Des études sur les ARNm ont montré un haut niveau d'expression des A_3R dans les testicules et les mastocytes de rat, une expression plus faible dans le cerveau, le cœur, les reins, la rate, le placenta, l'utérus, et l'intestin (Fredholm et al., 2001).

L'activation des A_3R induit une dégranulation des mastocytes, favorisant ainsi l'inflammation (Ramkumar et al., 1993), et conduit à une diminution de l'activité motrice chez la souris (Jacobson et al., 1993). Les A_3R semblent être impliqués dans l'effet cardio-protecteur et neuro-protecteur de l'adénosine comme les A_1R (Tracey et al., 1998; von Lubitz, 1999). Dans les cellules polynucléées, l'endothélium, les cardio-myocytes et les fibres musculaires lisses, l'activation des A_3R stimule le système anti-oxydant par augmentation de l'activité de la superoxyde dismutase, de la catalase et de la glutathion réductase (Maggirwar et al., 1994).

Chapitre second
TRAVAUX ORIGINAUX

PRESENTATION DES TRAVAUX

Nous avons vu la complexité du système adénosinergique qui s'intègre à la physiopathologie humaine via les quatre récepteurs de l'adénosine. Mon projet de thèse concerne plus particulièrement les A_1R et $A_{2A}R$ et peut être récapitulé comme suit :

A. Production et caractérisation d'un anticorps monoclonal anti-$A_{2A}R$ (appelé Adonis) pour l'utilisation diagnostique et thérapeutique :

   i. Production et caractérisation d'Adonis
   ii. Utilisation d'Adonis dans l'étude des niveaux d'expression des $A_{2A}R$ pour la caractérisation des syncopes neurocardiogéniques
   iii. Adonis et désensibilisation des CXCR4 et CCR5 des cellules T
   iv. Adonis et effet anti-nociceptif chez la souris

B. Évaluation biologique des agonistes et antagonistes des A_1R néo-synthétisés :

   v. Évaluation biologique de nouveaux agonistes et antagonistes des A_1R
   vi. Évaluation biologique d'un antagoniste bivalent, spécifiques des récepteurs μ des opioïdes et A_1R.

I. Production et caractérisation d'un anticorps monoclonal anti-A_{2A}R humain

1. Introduction

La compréhension des actions biologiques liées à la modulation des A_{2A}R s'est considérablement élargie pendant ces dernières années aussi bien dans le domaine cardiovasculaire que dans le domaine de l'inflammation, celui de la douleur ou encore du cancer. L'effet anti-inflammatoire de l'adénosine a été attribué aux A_{2A}R, ce qui en fait une cible thérapeutique potentielle pour ses différents ligands (Brown et al., 2008; Sitkovsky et al., 2008). Puisqu'il a été démontré que l'expression des A_{2A}R des cellules mononucléées reflétait celle des A_{2A}R présents dans les tissus du système cardiovasculaire (Varani et al., 2003), l'évaluation des A_{2A}R représente un marqueur très utile dans le suivi de patients atteints de pathologies cardiovasculaires comme l'insuffisance cardiaque ou les syncopes neurocardiogéniques. Nous avons montré récemment, par une méthode isotopique utilisant l'antagoniste radioactif [^{3}H]-ZM241385, qu'il existe une hyper-expression des A_{2A}R chez les patients atteints de syndrome de réponse inflammatoire systémique sévère (SIRS pour Systemic Inflammatory Response Syndrome) après chirurgie cardiaque sous circulation extra-corporelle (CEC) (Kerbaul et al., 2008). Nous avons également montré que la forte expression des A_{2A}R associée à des concentrations élevées en adénosine plasmatique, est un facteur prédictif de mortalité ou de SIRS sévère post-opératoire (voir annexe 1).

Alternativement aux ligands organiques des A_{2A}R, un anticorps monoclonal (mAb) de forte affinité et de haute spécificité constitue un outil précieux dans le domaine de la recherche fondamentale mais aussi de la recherche clinique. Cet anticorps peut être utilisé dans le cadre de plusieurs tests non isotopiques tels que l'ELISA, le Western blotting, l'immunohistochimie ou la cytométrie en flux. Avant notre projet, un seul anticorps monoclonal (mAb 7F6) était rapporté dans la littérature (Rosin et al., 1998). Cet anticorps, accessible dans le commerce, a été obtenu par immunisation de souris avec un A_{2A}R recombinant. Il est dirigé contre un épitope linéaire de 8 acides aminés situé dans la troisième boucle intra-cellulaire de l'A_{2A}R (figure 9), et par conséquence, n'a aucun effet fonctionnel sur les récepteurs à la surface cellulaire. Par ailleurs, le mAb 7F6 nécessite la

rupture préalable des membranes cellulaires pour pouvoir se fixer sur son site et il n'est donc pas utilisable directement sur des cellules intactes comme par exemple en cytométrie de flux.

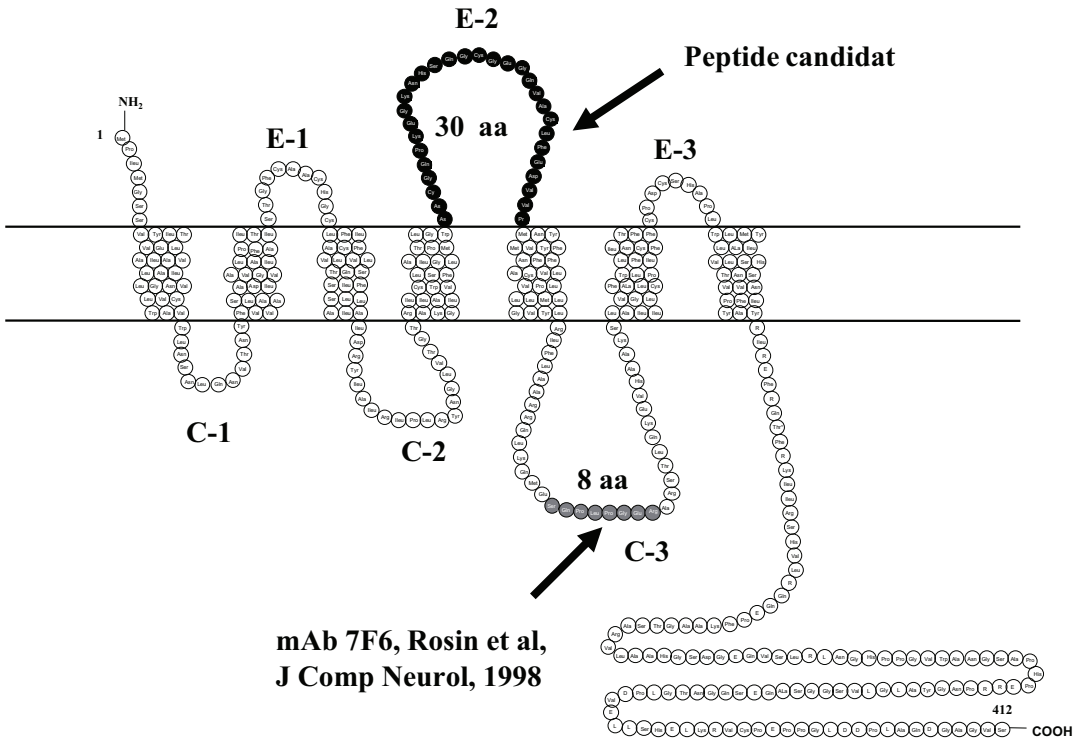

Figure 9 : Schéma de l'$A_{2A}R$ montrant un épitope du mAb 7F6 (C-3, 8aa) et la séquence peptidique immunogène (E-2, 30aa) dans notre étude. E : extracellulaire ; C : cytoplasmique

Quelques anticorps monoclonaux, spécifiques des autres récepteurs couplés à la protéine G, ont été rapportés dans la littérature tels qu'un mAb anti-récepteur M_2 de l'acétylcholine (Elies et al., 1998), un mAb anti-récepteur β_2 adrénergique (Lebesgue et al., 1998), un mAb anti-récepteur β_1 adrénergique (Mobini et al., 2000) et un mAb anti-récepteur 5-hydroxytryptaminergique (Kamel et al., 2005). Tous ces mAbs présentent une activité agonistique et sont dirigés contre un épitope situé dans la seconde boucle extra-cellulaire du récepteur correspondant. Cette boucle est considérée comme une des cibles majeures des auto-anticorps, dans le cadre de diverses maladies auto-immunes comme les maladies de Chagas ou les cardiomyopathies dilatées (Hoebeke, 1996). Nous avons optimisé

la production d'un mAb anti-$A_{2A}R$ par l'immunisation de souris avec un peptide purifié de 30 acides aminés correspondant à la seconde boucle extra-cellulaire de l'$A_{2A}R$ humain (figure 9).

Ce travail est détaillé dans **l'article « Production of an agonist-like monoclonal antibody to the human A_{2A} receptor of adénosine for clinical use »** ci- dessous.

Publication 1

"Production of an agonist-like monoclonal antibody to the human A_{2A} receptor of adenosine for clinical use"

Youlet By, Josée-Martin Durand-Gorde, Jocelyne Condo, Pierre-Jean Leujeune, Bernard Mallet, Pierre Carayon, Régis Guieu and Jean Ruf

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Production of an agonist-like monoclonal antibody to the human A_{2A} receptor of adenosine for clinical use

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ABSTRACT

The second extracellular loop of the A_{2A} receptor ($A_{2A}R$) of adenosine was used to immunize mice for production of Adonis, an IgM monoclonal antibody. Adonis bound to the immunogen peptide and the native receptor in ELISA with K_D values in 6.51–12.35 nM range. It recognized a linear epitope of 7 amino acids (LFEDVVP) at the C-terminal part of the external loop. Adonis revealed a 45-kDa band in lysate of human peripheral blood mononuclear cells in Western blotting in denaturing conditions. This served to monitor the up-regulation of the $A_{2A}R$ expression by caffeine. Adonis stimulated the cAMP production and inhibited the cell proliferation of an $A_{2A}R$ transfected stable cell line. These results confirm the immunogenicity and the functional relevance of the second extracellular loop of the $A_{2A}R$. They suggest that Adonis may be of clinical use in various pathological situations to measure the regulation of the $A_{2A}R$ expression and to act as $A_{2A}R$ agonist drug.

1. Introduction

Adenosine is an endogenous purine nucleoside that mediates a wide variety of physiological functions by interaction with four G-protein-coupled cell-surface receptors termed A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2001). Most of the anti-inflammatory effects of adenosine have been assigned to the A_{2A} receptor subtype ($A_{2A}R$) and growing evidences suggest that both agonists and antagonists for the $A_{2A}R$ may be of therapeutic relevance (Brown et al., 2008; Sitkovsky et al., 2008). $A_{2A}R$ is expressed in many immune and inflammatory cells (Gessi et al., 2000) and is up-regulated by T-helper cell type 1 cytokines (Sitkovsky et al., 2004). Because changes of peripheral $A_{2A}R$ reflect changes that occur at the injured tissue (Varani et al., 2003), $A_{2A}R$ assay appears as a valuable marker for monitoring treatment in patients with inflammatory cells infiltrating the failing organ.

Numerous organic ligands with agonist or antagonist effect on $A_{2A}R$ were synthesized. However they are rarely $A_{2A}R$ subtype specific (Fredholm et al., 2001). Alternatively to organic molecules, monoclonal antibodies (mAb) with high affinity and specificity can be produced for fundamental and clinical research applications. These immune ligands can be conveniently used in various non-isotopic tests such as ELISA, Western blotting, immunohistochemistry or flow cytometry.

To date, only one mAb obtained by immunizing mice with recombinant $A_{2A}R$ was reported (Rosin et al., 1998). This mAb is directed to an 8 residues long linear epitope from the third intracellular loop and consequently have no functional effect on the cell-surface receptor. In addition, the intracellular location of the epitope precludes the mAb binding to intact cells and a cell permeabilisation procedure is needed to use the mAb in flow cytometry (Koshiba et al., 1999).

Several mAb specifics for other G-protein receptors such as the M2 acetylcholine receptor (Elies et al., 1998), the β2-adrenergic receptor (Lebesgue et al., 1998), the β1-adrenergic receptor (Mobini et al., 2000) and the 5-hydroxytryptaminergic (5-HT) receptor (Kamel et al., 2005) were reported. All displayed an agonist activity and were directed to an epitope from the second extracellular loop of the receptor. Interestingly, this part of the receptor was considered as one of the main targets for patient's autoantibodies (Hoebeke, 1996).

Here, to optimize the possibility to obtain $A_{2A}R$ mAb with clinical interest for diagnosis and/or therapy, we immunize mice with a naked purified peptide corresponding to the second extracellular loop of the human $A_{2A}R$. We report on one mAb production and characterization.

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2. Materials and methods

2.1. Immunogen

A 30 residues long peptide (NNCGQPKEGKNHSQGCGEGQ-VACLFEDVVP; residues 144–173) corresponding to the second extracellular loop of the human A_{2A}R (UniProtKB/Swiss-Prot Entry P29274) was synthesized at >95% purity by Genosphere-Biotechnologies (Paris, France). Reverse-phase HPLC gives one eluted peptide peak at 220 nm, which resolves by mass spectrometry (MALDI-TOF) at a molecular weight of 3146.

2.2. Immunization

Six-week-old female BALB/c mice were purchased from Harlan (Gannat, France) and kept for 2 weeks at our animal facility before starting the procedure. Two mice were immunized i.p. twice at 15 days interval with 100 μg peptide in 100 μl saline buffer emulsified (v/v) in complete and then incomplete Freund's adjuvant. Two weeks later mice were i.v. boosted with 20 μg peptide in 100 μl saline in the tail vein. Mice were bled into heparinized tubes before starting the procedure and just before boosting from the retro-orbital sinus.

2.3. Production of the IgM mAb

Immune mice were sacrificed 3 days after the i.v. boost. Spleen cells of mice were hybridized with murine myeloma cells as previously described (Ruf et al., 1989). Positive hybridomas were cloned twice by the limiting dilution technique. Isotypes of the selected mAb were determined using the Serotec kit (Kidlington, UK) according to the manufacturer's instructions. Cells from the selected clone of hybridoma were injected i.p. into pristane-treated BALB/c mice. The resulting ascitic fluid was clarified by filtration through glass fibers and centrifugation and then extensively dialyzed against ultra-pure water at 4 °C. IgM precipitate was centrifuged at 4 °C for 1 h at 20,000 × *g*, washed with ultra-pure water and centrifuged again. Final pellet was dissolved in PBS. The protein concentration of the purified mAb was determined by measuring its absorbance at 280 nm with a molecular extinction coefficient (1%, 1 cm) of 1.25.

2.4. Cell line expressing human $A_{2A}R$

A_{2A}-Chem-3 (Chemicon catalog #HTS048C), a mammalian suspension cell line endogenously expressing the promiscuous G-protein, Gα15, lacking endogenous expression of adenosine receptors and transfected with the full-length human A_{2A}R cDNA (accession number BC013780) was obtained from Millipore (St. Quentin-Yvelines, France) and cultured in RPMI 1640 containing 4.5 g/L glucose, 10% heat inactivated FCS, 0.8 mg/mL Geneticine (G418), 100 IU/mL each penicillin–streptomycin and 1/100 dilution of Chem-3 growth supplement (Millipore).

2.5. Peripheral blood mononuclear cells (PBMC) preparation

Blood sample from a normal individual (28-year-old male) was collected by venepuncture into 8 mL sodium citrate Vacutainer Cell Preparation Tube (CPT, Becton Dickinson, Franklin Lakes, NJ) and PBMC were prepared according to the manufacturer's instructions. This protocol resulted in a >98% viable cell preparation that contained <95% of granulocytes (Sampol et al., 2001).

2.6. Binding test

ELISA was used to detect anti-peptide antibody in immune mice sera, to screen fusion culture supernatants and to test the binding of the purified mAb. Wells of polystyrene microtiter plates (MAXISORP, Nunc, Roskilde, Denmark) were filled with 100 μL PBS containing 1 μg/mL peptide and incubated overnight at 4 °C. Alternatively, 2×10^5 A_{2A}-Chem-3 cells or human PBMC by well were centrifuged at 100 × *g*, for 10 min at 4 °C and fixed with 0.06% glutaraldehyde in PBS for 10 min at 4 °C. Synthetic peptides covalently bound to the wells were used in the epitope mapping described below. After washing and saturation with bovine serum albumin, wells were incubated for 2 h at 37 °C with mice sera, culture supernatants or purified mAb. Antibody binding was revealed using alkaline phosphatase labeled anti-mouse IgG Fab specific antibodies and p-nitrophenyl phosphate substrate by optical density readings at 405 nm. K_D for mAb binding to the immunogen peptide and cell-surface expressed A_{2A}R were estimated by nonlinear regression analysis with the one-site binding equation $Y = B_{max}X/(K_D + X)$ using Prism 5 software (GraphPad Sofware, Inc., La Jolla, CA).

2.7. Epitope mapping

Epitope mapping was performed by ELISA on solid-phase synthetic peptides bound through their C-terminus to a polyethylene 96-well microplate provided by PEPSCAN presto (Lelystad, The Netherlands). 22 nanopeptides overlapping by 7 residues covering the 30 residues long peptide of the A_{2A}R were simultaneously tested in quadruplicate. Because of the surface chemistry, all internal cysteines were replaced by alanines and an extra cysteine was added at the C-termini of the peptides. The mAb was used at optimal dose (30 μg/mL) in the test.

2.8. Western blotting

0.5×10^6 A_{2A}-Chem-3 cells or human PBMC were washed with PBS in Eppendorf tubes. Cell pellets were frozen at −70 °C, thawed at 37 °C and solubilized with 30 μL of 62.5 mM Tris–HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol. Solubilisate was sonicated for 10 min at 47 kHz and loaded onto a 12% acrylamide, 60 mm × 90 mm, 1.5 mm thick minigel. Cell proteins were submitted to standard electrophoresis procedure. Separated proteins in the gel were directly electrotransfered onto a 0.45-μm PVDF membrane. Blotted membrane was saturated with non-fat dried milk and incubated 90 min with 1.25 μg/mL Adonis. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse mAb (Clone GAPDH-71.1, Sigma, Saint Louis, MO) diluted to 0.025 μg/mL was added to Adonis as loading control for normalizing blot results to GAPDH. Blots were visualized by horse-radish peroxidase labeled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescence substrate (Super-Signal West Femto, Pierce Biotechnology, Rockford, IL) using a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY). The staining intensities of the bands were measured densitometrically on a Macintosh computer using the public domain NIH Image software developed at the US National Institute of Health.

To test the caffeine (1,3,7-trimethylxanthine) effect on the A_{2A}R cell-expression, human PBMC were incubated 20 h at 37 °C in the RPMI culture medium under 5% CO_2 with various concentrations of caffeine.

2.9. Cyclic AMP assay

Total cellular cAMP measurement was achieved on A_{2A}-Chem-3 cells with the Amersham Biotrak kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) using the non-acetylation procedure as described by the manufacturer. Various concentrations of Adonis were added to 1×10^6 cells/well in a 96 wells culture plate and incubated for 90 min at 37 °C in the culture medium under 5% CO_2. Various concentrations of the A_{2A}R agonist CGS-21680 (2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine) or the A_{2A}R antagonist caffeine were then added for the next 30 min incubation and the test was stopped by adding the dodecyltrimethylammonium bromide acetate lysis buffer of the kit. For the mix caffeine–Adonis, caffeine was incubated with the cells for 30 min before adding Adonis for the next 90 min. The cAMP content in each well was then determined by a competitive enzyme immunoassay according to the manufacturer's instructions. Tests were in triplicates. Results were given as percent of cAMP produced with respect to the basal level of the A_{2A}-Chem-3 cells.

2.10. Cell proliferation assay

A_{2A}-Chem-3 cells were seeded in 24 wells culture plate (5×10^4 cells/well) in the culture medium (1 mL/well) with or without the presence of various amounts of purified Adonis. During 6 days, the cells within the corresponding wells were day by day suspended and viable cell counts were performed using the trypan blue dye exclusion method and a Malassez haemocytometer.

3. Results

3.1. Production of mAb

As shown in Fig. 1A, mice immunized with the A_{2A}R peptide produced specific antisera with high titer of anti-peptide antibodies. However, only 6 out of 130 hybridomas were found positive and only 1 out of the 6 selected clones remained positive after subcloning. This clone produced an IgM, κ mouse antibody that we called Adonis. Fig. 1B shows the titration curve displayed by the Adonis culture supernatant.

3.2. Binding specificity of Adonis

Purified Adonis specifically bound to A_{2A}R peptide (Fig. 2A) and to the cell-surface native receptor (Fig. 2B) in ELISA with similar affinities. K_D values were estimated to 6.51, 8.94 and 12.35 nM, for the immunogen peptide, the human PBMC and the A_{2A}-Chem-3 cells, respectively.

The fine specificity of Adonis was assessed by PEPSCAN epitope mapping using 22 overlapping nanopeptides encompassing the whole A_{2A}R second extracellular loop. The minimal score was obtained by peptide #5 (OD: 0.10 ± 0.06) and the maximal scores were obtained for the two last successive peptides #21 (OD: 0.99 ± 0.19) and #22 (OD: 1.47 ± 0.03). Adonis recognition was deduced to be restricted to the C-terminus sequence LFEDVVP (Fig. 3A). A similar sequence exists in the A_{2B}R but not in A_1R and A_3R (Fig. 3B).

Since Adonis epitope was not conformational, we tested it by Western blotting in reducing conditions. Adonis recognized a 45-kDa protein band, corresponding to the expected molecular weight of the human A_{2A}R, both in lysates of A_{2A}-Chem-3 cells (Fig. 4A, lane 1) and normal human PBMC (Fig. 4A, lane 2). Adonis slightly cross-reacted with other bands in A_{2A}-Chem-3 cells but not human PBMC lysates.

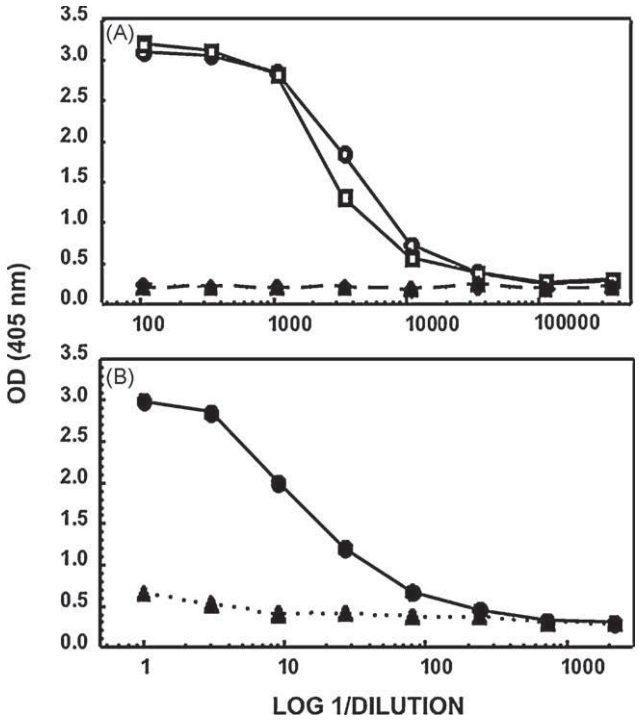

Fig. 1. Titration curves of: (A) the two immune (open symbols) and the corresponding pre-immune (filled symbols) mouse sera used as blanks and (B) the Adonis culture supernatant, the culture medium served as blank. Results are given in optical density read at 405 nm and are the mean values of duplicates.

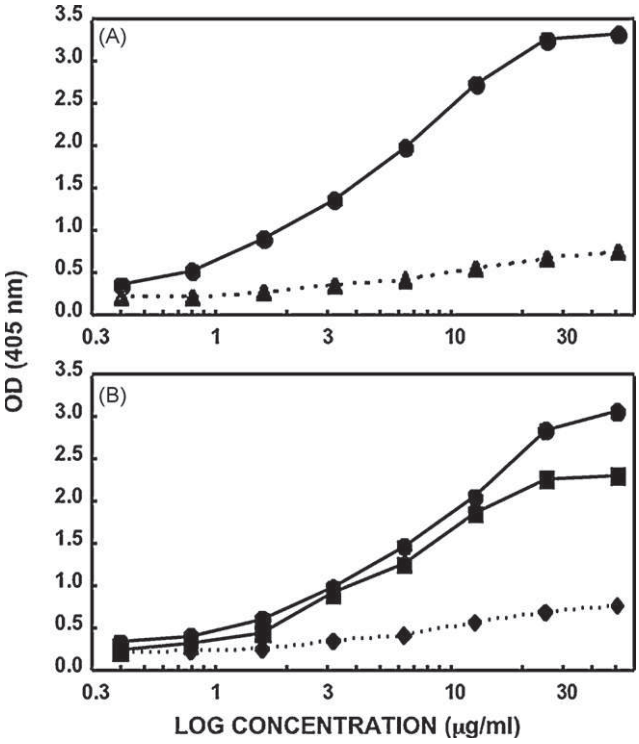

Fig. 2. Dose–response curves of Adonis binding to: (A) the immunogen peptide, uncoated wells served as blanks and (B) glutaraldehyde fixed A_{2A}-Chem-3 cells (circle) and normal human PBMC (square), glutaraldehyde-treated uncoated wells (lozenge) served as blanks. Results are given in optical density read at 405 nm and are the mean values of duplicates.

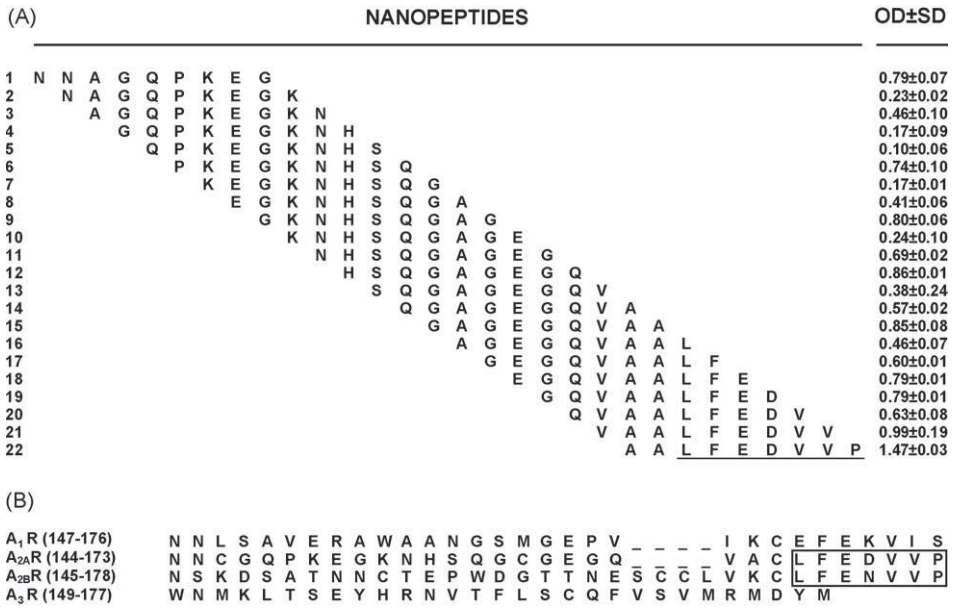

Fig. 3. Epitope mapping of Adonis. (A) Adonis binding was tested in ELISA with a set of 22 nanopeptides covering residues 144–173 corresponding to the second extracellular loop of the human $A_{2A}R$. Results are given in optical density read at 405 nm and are the mean values (±S.D.) of quadruplicates. The binding sequence is underlined. (B) Sequence alignment of the second extracellular loop of the various subtypes of adenosine receptors from UniProtKB/Swiss-Prot (entries P30542, P29274, P29275 and P33765 for human A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, respectively). Homologous binding sequences in $A_{2A}R$ and $A_{2B}R$ are boxed.

To ascertain that the 45-kDa band revealed by Adonis was the $A_{2A}R$, we attempted to regulate the protein expression of the $A_{2A}R$ by incubating normal human PBMC with various concentrations of caffeine, a well-known drug that up-regulates the cell-surface density of the $A_{2A}R$. Fig. 4B shows that in Western blotting performed on PBMC lysates, the 45-kDa band intensity gradually increased with respect to caffeine concentration.

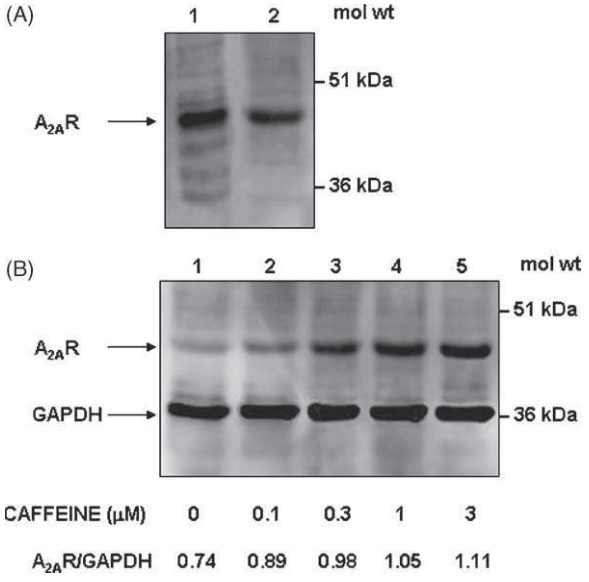

Fig. 4. Western blots of: (A) the Adonis binding on reduced lysate of A_{2A}-Chem-3 cells (lane 1) and human PBMC (lane 2). The molecular weights (kDa) of prestained standards are on the right. The arrow on the left shows the $A_{2A}R$ band at 45 kDa and (B) the effect of caffeine on $A_{2A}R$ cell-expression. Human PBMC cells were previously incubated for 20 h with increasing doses of caffeine (from 0.1 to 3 μM, lanes 2–5; control without caffeine, lane 1). GAPDH mAb served as loading control. The molecular weights (kDa) of prestained standards are on the right. The arrows on the left show the $A_{2A}R$ and GAPDH bands at 45 and 37 kDa, respectively. The blotted bands were analyzed by density reading. Quantitative results are expressed as the $A_{2A}R$/GAPDH ratio of the mean gray values.

3.3. Functional activity of Adonis

Adonis was tested for cAMP production in A_{2A}-Chem-3 cells. Fig. 5 shows that Adonis was able to stimulate the $A_{2A}R$ in a dose-dependent manner as the CGS-21680 agonist. Conversely, caffeine decreased the basal accumulation of cAMP in a dose-dependent manner. Interestingly, the agonist effects of 0.03 μM Adonis and

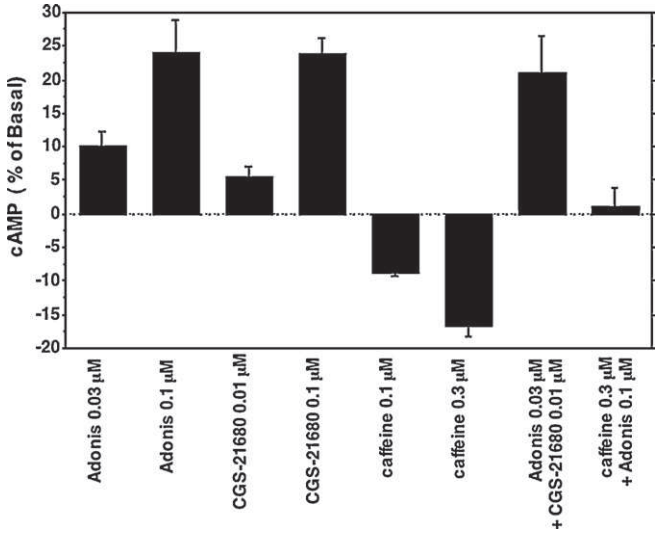

Fig. 5. Effect of Adonis on the cAMP production in A_{2A}-Chem-3 cells. CGS-21680 and caffeine were used as controls. The results are expressed as % of the basal cAMP production and are the mean values (±S.D.) of triplicates. Each bar represents a unique culture condition with the presence of one or two molecules at indicated concentrations. The mean basal level of total cAMP of cells in culture medium is of 462 fmol/well.

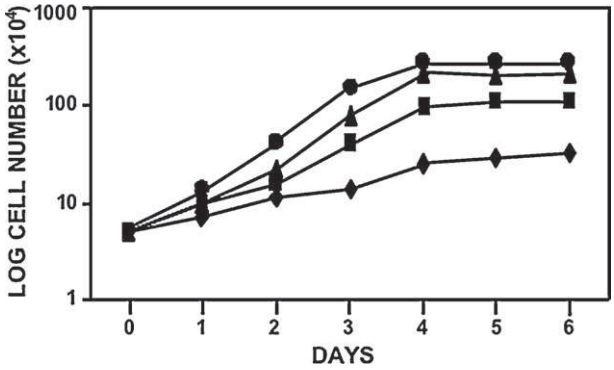

Fig. 6. Growth curves of A_{2A}-Chem-3 cells in the presence of increasing amounts of Adonis in the culture medium. Viable cells counts were reported from days 0 to 6. Standard curve in the absence of Adonis was given as control (circle symbol). The concentrations of Adonis in the medium were successively, 0.06, 0.20 and 0.60 μM (triangle, square and lozenge symbols, respectively). Results are the mean values of duplicates.

0.01 μM CGS-21680 were found to act in synergy. In contrast, a short pretreatment of the cells with 0.3 μM caffeine was able to block the subsequent stimulation of the A_{2A}R by 0.1 μM Adonis.

To assess the stimulating effect of Adonis on cAMP cell production, the A_{2A}-Chem-3 cells were cultured with or without the presence of increasing concentrations of Adonis. Fig. 6 shows that the growth curves of the A_{2A}-Chem-3 cells gradually decreased with the presence of increasing concentrations of Adonis in the culture medium. All the curves reached a plateau value at day 4. Adonis used at 0.60 μM decreased by 10-fold the number of viable cells at day 4.

4. Discussion

The adenosine receptors are made of 7 transmembrane α-helice domains forming a pharmacophore pocket like bacteriorhodopsin (Henderson et al., 1990). This pocket is common to all the subtypes of adenosine receptors that explain the difficulty to obtain ligands with specificity for only one type of receptor. The rationale to choose the second extracellular loop of the A_{2A}R was its length, its low sequence homology with the other receptor subtypes (see Fig. 3B), its structural importance in the function of the receptor and its antigenicity for autoantibodies from patients with cardiovascular diseases (Hoebeke, 1996). Because it is 30 amino acids long, we used the naked peptide to avoid the drawback to couple an irrelevant carrier protein.

The resulting antisera from two immunized mice have a high specific antibody titer but the fusion recovery was poor and only Adonis, an IgM mAb was derived. Purified preparation of Adonis bound to the immunogen peptide and to the native receptor expressed on A_{2A}R-Chem-3 cells and normal human PBMC with similar affinities. This is indicative for the linear nature of Adonis epitope, which is similarly expressed on the whole receptor and a peptide part of it.

PEPSCAN study indicated that the epitope recognized by Adonis is the linear sequence LFEDVVP at position 167–173. For methodological convenience, cysteins in position 146, 159 and 166 were replaced by alanines in the PEPSCAN test. This could have modified the profile of Adonis reactivity. However, the last nanopeptide screened with Adonis was found 15-fold more reactive than the less reactive one of the panel. The immunoreactivity deeply increased with the addition of $Pro1^{73}$. Because $Cyst^{166}$ that was replaced by Ala^{166} in the test is natively involved in a disulfur bridge with Cys^{77} from the first extracellular loop according to Noda et al. (1994), we concluded that the binding sequence was restricted to the last 7 amino acids LFEDVVP.

Adonis would be a valuable reagent to localize and identify A_{2A}R at a molecular level. A_{2A}R and A_{2B}R share a great homology for the binding sequence recognized by Adonis since they only differ by one conserved amino acid (Asp^{170} and Asn^{170}, respectively, see Fig. 3). However, the A_{2A}R sequence is relatively different from the A_1R and at odds from the A_3R sequences. Albeit not tested, this suggests that Adonis would cross-react to the A_{2B}R but not to the A_1R and A_3R.

The specificity of Adonis for the receptor expressed by the A_{2A}-Chem-3 cells would be open to question because several bands in the 30–50 kDa range were revealed on Western blot. However, only one of them was similarly revealed at the expected 45 kDa on the normal human PBMC which contained various immune cells such as T cells, natural killer cells, monocytes and macrophages that express the A_{2A}R (Thiel et al., 2003). This band was proved to be actually the A_{2A}R by successfully enhancing its density using caffeine, a drug that leads to up-regulation of the A_{2A}R (Varani et al., 1999, 2005).

It was reported that PBMC from patients with chronic heart failure produce great amounts of inflammatory cytokines TNF-α and IL-6 (Aukrust et al., 1998) demonstrating that they mirror stimulated immune cells which infiltrate the failing myocardium. Adonis can be conveniently used with PBMC in quantitative Western blotting to test the protein expression of the A_{2A}R in patients from various pathological situations as reported for the congestive heart failure (Carrega et al., 2007a) and neurocardiogenic syncope (Carrega et al., 2007b), but using a radioactive A_{2A}R ligand.

Evidence accumulated that A_{2A}R specific agonists reduce inflammation and tissue damage (Palmer and Trevethick, 2008). Adonis can be planed for use as a pharmacological agent because it is acting on the receptor function. Tested on the A_{2A}-Chem-3 cell line, Adonis behaved as an agonist of adenosine by increasing the level of cAMP. Probably as a consequence of cAMP signaling, Adonis inhibited the cell line proliferation in the micromolar range of concentrations. It was recently reported that stimulated A_{2A}R can inhibit lymphoid cells proliferation by blocking the nuclear translocation of the transcription factors of the NF-κB family (Minguet et al., 2005).

As previously suggested, receptor activation is probably induced by dimerization (Mijares et al., 1996; Hebert et al., 1996). Since IgM is pentameric, Adonis is far more potent than monomeric IgG to couple A_{2A}R at the cell-surface and it would be engineered to have a safer clinical profile for systemic administration in patients.

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2. Conclusion de l'article 1

Adonis est un mAb anti-$A_{2A}R$ d'isotype IgM, κ obtenu par immunisation de souris avec un peptide purifié du récepteur constitué d'une chaine de 30 acides aminés correspondant à la seconde boucle extra-cellulaire de l'$A_{2A}R$. Adonis reconnaît un épitope linéaire de 7 amino-acides (LFEDVVP) situé dans la partie C-terminale de la boucle. Il permet, par Western blotting, de détecter au niveau d'une bande de 45 kDa une « up-régulation » de l'expression de l'$A_{2A}R$ induite par la caféine, dans le lysat cellulaire de cellules mononuclées de sang périphérique humain. Adonis stimule la production d'AMPc et inhibe la prolifération d'une lignée cellulaire transfectée avec l'$A_{2A}R$ humain. Ainsi, Adonis se comporte comme un « agoniste-like ». Ces résultats confirment l'immunogénicité et la fonctionnalité de la seconde boucle extra-cellulaire de l'$A_{2A}R$ et suggèrent qu'Adonis pourrait être très utile pour mesurer le niveau d'expression des $A_{2A}R$ dans diverses situations cliniques où l'implication des $A_{2A}R$ pourrait être envisagée. Adonis pourrait également être utilisé comme agent thérapeutique pour son action agoniste. Puisqu'il s'agit d'une immunoglobuline pentamérique de type IgM, Adonis est beaucoup plus affin qu'une IgG monomérique pour la fixation et le couplage des $A_{2A}R$ à la surface cellulaire et il pourrait être modifié par des technologies de biologie moléculaire pour satisfaire aux caractéristiques d'un agent pharmacologique utilisable chez l'homme.

II. Utilisation d'Adonis dans l'étude des niveaux d'expression des $A_{2A}R$ pour la caractérisation des syncopes neurocardiogéniques

1. Syncope neurocardiogénique

La syncope se définit comme une perte de connaissance temporaire courte et causée par une diminution transitoire du flux sanguin cérébral, généralement due à une hypotension artérielle (Flammang et al., 2005). En dehors des causes neurologiques, les syncopes d'origine cardiovasculaires sont classiquement différenciées en cinq types en fonction de la symptomatologie qui sont : la syncope neurocardiogénique, la syncope posturale orthostatique, les arythmies cardiaques, les pathologies cardio-pulmonaires et les pathologies cérébro-vasculaires.

Les syncopes neurocardiogéniques représentent 1 à 5% des hospitalisations (Goldschlager et al., 2003) et environ 50% des syncopes en général (Brignole, 2005). Ces syncopes sont caractérisées par une vasodilatation artérielle généralisée associée à une bradycardie absolue ou relative entraînant une perte de conscience en rapport avec une défaillance du système nerveux autonome. Deux tests de provocation sont très fréquemment utilisés dans le diagnostic de ce type de syncope :

1. le test d'inclinaison ou head-up tilt test (HUT) (Moya et al., 2009; Sutton and Bloomfield, 1999) qui consiste à allonger le patient sur une table, sous monitorage cardiologique, et à redresser la table en laissant le patient en procubitus. Le test est dit positif lorsqu'on observe à plus ou moins brève échéance une chute de la pression artérielle avec une bradycardie et une perte de connaissance.

2. le test à l'ATP (Mittal et al., 1999; Shen et al., 1996) qui consiste à injecter 20 mg d'ATP en bolus (< 3 secondes) dans une veine brachiale du patient, suivie d'un « flush » de 20 ml de dextrose 5%, le patient étant en décubitus dorsal et sous monitorage cardiologique. Seule la réponse électro-cardiographique est prise en compte et quantifiée pour l'interprétation du résultat du test. Le test ATP est considéré comme positif si, dans l'électro-cardiogramme, apparaît une pause

cardiaque supérieure à six secondes au cours de la phase d'un bloc auriculo-ventriculaire (BVA).

2. Récepteurs A_{2A} de l'adénosine et syncope neurocardiogénique

La symptomatologie des syncopes neurocardiogéniques est complexe mais la contribution de l'adénosine endogène et des $A_{2A}R$ est très vraisemblable. En effet, Il a été rapporté précédemment que les patients souffrant de syncope neurocardiogénique et ayant un tilt test positif, présentaient une augmentation des concentrations plasmatiques en adénosine (APL) (Saadjian et al., 2002) et une surexpression des $A_{2A}R$ (Carrega et al., 2007b). Par ailleurs ces patients possèdent au niveau du deuxième exon codant, un polymorphisme (Single Nucleotide Polymorphism, SNP) particulier dans le gène des $A_{2A}R$ (c.1364 T>C), avec une sur-représentation du variant (Saadjian et al., 2009). Ceci suggère que les $A_{2A}R$ pourraient être impliqués dans la physiopathologie des syncopes neurocardiogéniques dans ces populations (voir annexe 2). Si les tests ATP et HUT permettent d'identifier la syncope comme d'origine neurocardiogénique, ces tests ne semblent pas identifier la même population de patients. En effet, les patients ayant un test HUT positif sont plus jeunes tandis que ceux ayant un test ATP positif sont plutôt des femmes âgées. Par ailleurs, seulement 10 à 20 % des patients, selon les séries, ont les deux tests positifs.

Si le lien entre élévation de l'APL et positivité du test HUT est bien démontré, on ne sait rien des APL, ni de l'expression des $A_{2A}R$ chez les patients présentant un test ATP positif. Ainsi, nous avons mené une étude sur la corrélation entre la concentration plasmatique en adénosine et le niveau d'expression des $A_{2A}R$, chez les patients suspectés de syncope neurocardiogénique, avec les résultats des tests ATP et HUT. La détermination de l'expression des $A_{2A}R$ utilise Adonis, notre mAb anti-$A_{2A}R$, par la méthode du Western blotting.

Ce travail est détaillé dans l'article « **A_{2A} adenosine receptor expression and adenosine plasma level: relationship with laboratory tests in patients with suspected neurally mediated syncope** » ci-dessous.

Publication 2

"A_{2A} adenosine receptor expression and adenosine plasma level: relationship with laboratory tests in patients with suspected mediated syncope"

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Submitted Manuscript

A_{2A} adenosine receptor expression and adenosine plasma level: relationship with laboratory tests in patients with suspected neurally mediated syncope

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Running head: A_{2A} adenosine receptor, adenosine and neurocardiogenic syncope

Abstract

Objective: Endogenous adenosine is thought to act as a mediator in neurally mediated syncope (NMS). This hypothesis is supported by the correlation between high adenosine plasma level (APL), high expression in A_{2A} adenosine receptors ($A_{2A}R$), and positive head-up tilt test (HUT). No study had attempted to correlate APL and $A_{2A}R$ expression with positive ATP test. The purpose of this study was to find possible links between APL, $A_{2A}R$ expression, and laboratory test results in patients with NMS.

Patients and Measures: This prospective study includes a consecutive series of patients referred to our syncope unit for suspected NMS. All patients underwent HUT and ATP testing. Before testing, samples were collected for baseline APL and $A_{2A}R$ expression measurement.

Results: Forty-six patients (25 men and 21 women aged 57 ± 18 years) were enrolled. HUT was positive in 27 patients and ATP in 20. Both tests were positive in 9 and negative in 8. High APL was associated with high probability of positive HUT while low APL was associated with high probability of positive ATP. $A_{2A}R$ expression was lower in patients with positive ATP than with positive HUT but did not differ from controls.

Conclusion: In patients with suspected NMS, low APL was associated with high probability of positive ATP, and high APL was associated with high probability of positive HUT. $A_{2A}R$ expression was lower in patients with positive ATP than with positive HUT. These findings suggest different pathophysiological substrates may be implicated in the genesis of NMS.

Introduction

Neurally mediated syncope (NMS) is characterized by arterial vasodilation, often associated with bradycardia, leading to a transient loss of consciousness [1, 2]. Various humoral factors are suspected to be involved in NMS. Among them, adenosine has been implicated in the genesis of this syndrome [3, 4, 5]. It is well established that the effect of adenosine on blood vessel tone and on sinoatrial and atrioventricular nodes involves activation of four membrane receptor subtypes, designated A_1, A_{2A}, A_{2B}, and A_3 depending on their primary sequence and affinity for ligands [6]. Stimulation of A_1 adenosine receptors (A_1R) is associated with negative chronotropic action [6], stimulation of A_1 or A_3 adenosine receptor is associated with cardioprotection [6,7] and ischemic preconditioning [8], and activation of the A_{2A} adenosine receptors ($A_{2A}R$) or A_{2B} adenosine receptors ($A_{2B}R$) results mainly in vasodilatation [6, 9].

The head-up tilt test (HUT) is a widely accepted diagnostic tool for NMS [10, 11]. Another frequently used test is the adenosine triphosphate (ATP) test, which consists in evaluating susceptibility to syncope after bolus injection of exogenous adenosine or ATP [3, 4, 12].

In previous patient-control studies, patients with positive HUT had high APL [5] and high $A_{2A}R$ expression [13] and displayed particular single nucleotide polymorphism (SNP) in the $A_{2A}R$ gene [14]. These findings indicate that $A_{2A}R$ may be implicated in the pathophysiology of syncope events in this population. However, nothing is known about APL and the $A_{2A}R$ expression in patients with positive ATP test. Thus, the aim of this study was to correlate APL and $A_{2A}R$ expression level in patients with suspected NMS with the results of ATP and HUT tests.

Methods

Patient selection

All consecutive patients referred to our syncope unit for suspected NMS and indicated for laboratory tests from November 2006 to January 2008 were eligible for inclusion in this study. Suspicion of NMS was based on syncope in the absence of (i) severe structural heart disease, significant ECG abnormalities, or rhythm disturbances; (ii) orthostatic hypotension; and (iii) non-syncopal causes of transient loss of consciousness. The criteria for inclusion

were (i) occurrence of one or more episodes of suspected NMS within the preceding year, (ii) absence of drug use for 3 months, and (iii) first-time HUT and ATP testing. Patients with carotid sinus syndrome were excluded. To compare APL in NMS patients and the general population, a matched control group of 14 healthy volunteers was recruited consecutively from the medical and technical staff working at our institution during the study period. None of the controls had a history of syncope.

This study was conducted in compliance with the principles of the Declaration of Helsinki. All patients and controls provided written informed consent to participate in this study approved by the Ethics Committee for Human Research of our university-affiliated hospital.

Study protocol

ATP and HUT tests

ATP and HUT tests were performed during the same session: ATP test then HUT, with a sufficient interval between them to allow complete recovery. The protocols for both tests have been described previously [15]. The ATP test was performed by rapid injection (<3s) of 20 mg of ATP (Striadyne®, Genopharm, Saint Thibault des Vignes, France) through a brachial vein, followed by a 20 ml 5% glucose flush. Positive outcome was defined as complete AV block or sinus pause with a ventricular asystole > 6s [16]. The tilt test was performed after an overnight fast in a quiet room equipped for cardiopulmonary resuscitation. To facilitate blood sampling, an intravenous catheter was inserted into one arm at least 30 min before testing. Patients had to lie on the tilt table for 10 minutes. The table was then tilted to the 60° position for a maximum of 45 minutes. Surface electrocardiogram and finger blood pressure were monitored beat by beat (Task Force Monitor, CNSystems, Graz, Austria). Positive outcome was defined as development of (pre) syncope in association with significant arterial hypotension. To avoid false positive response, no pharmacological challenge was allowed.

Collection of blood samples

Baseline blood samples were collected and treated as described previously [5, 13, 17]. In brief, tubes containing 3 ml of stop solution kept under vacuum were prepared in the laboratory. This method allows whole blood to mix quickly with the cold stop solution so as to prevent adenosine from degradation or uptake by red blood cells [17]. Samples were collected a few minutes before ATP administration. Patients and controls were in supine position during sample collection. After collection, samples were immediately placed on ice until centrifugation.

Adenosine plasma level (APL)

APL was determined as described previously [17]. After deproteinization, adenosine was identified and quantified using HPLC (Hewlett Packard 1100, Palo Alto, CA). The technicians who performed sample treatment and assays were unaware of the result of HUT and ATP tests.

Western blot analysis of $A_{2A}R$ expression in peripheral blood mononuclear cells (PBMC)

PBMC from NMS patients were isolated using Vacutainer tubes (Ficoll-based CPT system; Becton Dickinson, Franklin Lakes, NJ). PBMC pellets were frozen at -70°C, quickly thawed at 37°C, and solubilized with a 4% SDS aqueous solution by 30 min sonication at 47 kHz. After protein quantification by microBCA (Pierce Biotechnology, Rockford, IL), 15 µg of PBMC solubilisates in 62.5 mM Tris-HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% mercaptoethanol, were submitted to standard electrophoresis in Mini Protean II system (BioRad, Hercules, CA). Separated proteins in 12% acrylamide minigel were electrotransferred onto a PVDF membrane. The blotted membrane was placed in the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA), saturated with non-fat dried milk, and incubated 20 min with an anti-$A_{2A}R$ mouse monoclonal antibody [18], as primary antibody. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Clone GAPDH-71.1, Sigma) was used as loading control for normalizing blot results. Blots were visualized by horseradish peroxidase-labeled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL) using a Kodak Image Station 440CF (Eastman Kodak

Compagny, Rochester, NY). The staining intensities of the bands were measured densitometrically on a Macintosh computer using the public domain NIH Image software developed at the U.S. National Institutes of Health.

Statistical Analysis

Quantitative variables were described using median and range values. Correlation between APL and quantitative variables was assessed using the Spearman test. Quantitative variables were compared using the non-parametric Mann-Whitney U test or Kruskal Wallis test, depending on the number of groups. The non-parametric Wilcoxon test was used to compare APL between the patient groups defined according to outcome of the ATP and HUT tests. An adjusted P-value was used to control the false positive rate [19]. Probability of a positive ATP test was determined by logistic regression, with adjustment for other variables if any significant association was found. All statistical tests were two-sided and P values less than 0.05 were considered statistically significant. Analyses were performed with R free software (v. 2.8.1).

Results

Clinical characteristics of patients and controls are presented in table 1. No significant differences were found between groups. Women accounted for 45% (21/46) of the patient population and for 60% (12/20) of positive ATP tests (p=0.09). Outcomes of HUT and ATP tests are presented in figure 1. Response to HUT was positive in 27 patients. According to the modified VASIS classification [20], the response was classified as type 1 in 10 patients, type 2A in 5, type 2B in 4, and type 3 in 8. The ATP test was positive in 20 patients. AV block was observed in 18 patients and sinus pause in 2. The duration of the maximum RR interval during positive ATP test was 12.0 ± 5.7 sec.

APL

Figure 2 presents APL measurements for the overall patient population, subgroups obtained according to responses to HUT and ATP tests, and controls. Figure 3 shows the relationships between APL and outcomes of HUT and ATP tests.

As shown in figure 2A, APL was lower in patients with positive ATP than in those with negative ($p < 0.01$) and higher in patients with positive HUT than in those with negative HUT ($p = 0.02$). Compared to controls, patients with positive HUT had significantly higher APL ($p < 0.001$) whereas patients with positive ATP were not statistically different ($p = 0.38$). As shown in figure 2B, APL was lower in patients with positive ATP and negative HUT and higher in patients with positive HUT and negative ATP.

$A_{2A}R$ expression:

$A_{2A}R$ expression was lower in patients with positive ATP and negative HUT than in the other groups of patients, but it did not differ from controls (figure 2C). Patients with positive HUT had higher $A_{2A}R$ expression than the other groups of patients and controls.

APL and outcome of ATP and HUT tests

As shown in figure 3, lower APL was associated with a high probability of positive ATP test, and high APL was associated with high probability of positive HUT test.

Discussion

The main finding of this study is that, in patients with suspected NMS, high and low APL values are associated with opposite responses to the HUT and ATP tests. High APL is associated with a high probability of positive response to HUT test, while low APL is associated with a high probability of positive response to ATP test. Furthermore, patients with positive ATP and negative HUT tests had normal or low expression of $A_{2A}R$, while patients with positive HUT and negative ATP tests had high $A_{2A}R$ expression. Interestingly, patients with both positive or both negative tests had intermediate values in APL. These findings suggest different pathophysiological substrates may be implicated in the genesis of different forms of NMS.

Since the present study shows that baseline APL is more than 4-fold higher in patients with positive HUT and negative ATP than in patients with positive ATP and negative HUT, it can be speculated that the baseline adenosine pattern is able to influence the response to ATP and

HUT tests. In patients with low baseline APL pattern, an exogenous adenosine injection activates high affinity adenosine receptors, mostly A_1R, leading mainly to bradycardia (in general atrio-ventricular block with long asystolic pauses). On the contrary, in patients with high baseline APL pattern, most A_1R could be desensitized and/or down regulated [5]. In this situation, exogenous adenosine injection is ineffective on A_1R because of saturation of high affinity receptors. However, the orthostatic stress induced by HUT is still able to activate low affinity adenosine receptors, mostly $A_{2A}R$, The action on these receptors results mainly in vasodilatation [6, 9] which is well known to be present during positive HUT. These two situations represent the extreme of a wide spectrum in which several intermediate possibilities exist. Data from the literature have shown an overlap between patients with positive HUT and positive ATP tests [22]. This was confirmed by the present study since both tests were positive in 19% of patients. Interestingly, patients in the overlap group had intermediate APL.

A number of clinical findings indicate that the HUT and ATP tests identify different syncope populations. Though some overlap exists, HUT tends to be positive in younger patients of both genders and ATP in older women [21, 22, 23]. Relatively few patients have both positive HUT and ATP [21]. The above findings suggest possible different clinical manifestations, being bradycardia (an asystole) the dominant mechanism of syncope in low-APL (positive ATP) patients and hypotension the dominant mechanism of syncope in high-APL (positive HUT) patients. However, clinical follow-up studies which used prolonged electrocardiographic in order to detect the exact mechanism of spontaneous syncope were unable to confirm such hypothesis [15, 22, 25]. It is likely that syncope due to extrinsic (functional) mechanism which occurs in patients with a normal heart has multiple pathogenesis of which adenosine pathway is only one of the determinants.

We found also that 8 patients had negative HUT in spite of high APL and high expression of $A_{2A}R$. Interestingly, 4 of these 8 patients had xanthine derivatives on their chromatogram (data not shown). It is well known that xanthine derivatives increase both APL 24 and $A_{2A}R$ expression [18]. The antagonist effect of caffeine on adenosine receptor may explain the negative response to HUT.

Study limitations

This study has three limitations. The first is small population size. The population was large enough to reach statistical significance concerning APL and $A_{2A}R$ expression differences, but further study in a larger group of patients will be needed especially to allow detailed analysis of the clinical characteristics of NMS patients with normal or low APL. The second is that, since APL was evaluated only at baseline, we can only assume that APL is a marker of susceptibility for laboratory test response. Further study will be needed to evaluate the implication of different adenosine receptor subtypes in NMS. The third limitation is that the exact mechanisms responsible for development of NMS were not evaluated and thus remain largely unknown [1]. The "adenosinergic" mechanism is probably not the only mechanism involved. In patients with recurrent NMS, central or peripheral baroreceptor reflex abnormalities and alterations in neuro-humoral mechanisms may play a pivotal role [1].

Conclusion

In patients with suspected NMS, high and low APL is associated with opposite responses to ATP and HUT tests. Positive ATP test is associated with low or normal APL and low or normal $A_{2A}R$ expression while positive HUT test is associated with high APL and high expression of $A_{2A}R$. These findings suggest different pathophysiological substrates may be implicated in the genesis of different forms of NMS and in different outcome. Knowing the mechanism is the prerequisite for a successful treatment. How these findings translate into clinical practice is largely unknown. These findings are a useful background for future prospective clinical follow-up studies aimed to correlate the underlying adenosine pattern with the clinical outcome of NMS.

Conflict of interest: none declared

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Table 1: Clinical characteristics of patients: overall population and subgroups as function of laboratory tests.

	Overall population n=46	ATP+ n= 20	ATP- n= 26	HUT+ n= 27	HUT- n=19	Controls n=14
Age (years, mean±SD)	57±18	62±19	53±18	56±17	58±22	54±14
Sex, (male/female)	25/21	8/12	17/9	15/12	10/9	8/7
Heart disease n (%)	1(2)	0 (0)	1(2)	0(0)	1(2)	--
ECG abnormalities n (%)	10(21.7)	5(10.8)	5(10.8)	6(13)	4(8)	--
Total number of syncopal episodes (mean±SD)	7.4±11.8	6.7±11.0	8±12.7	9.4±14.6	4.6±5.6	--
Duration (months, mean±SD)	59±117	36±88.1	77±134	81±147	27±34	--
Triggering factors n (%)	19(41.3)	8(17.3)	11(23.9)	12(26)	7(15.2)	--

Structural heart disease was: stable angina pectoris
ECG abnormalities were: isolated complete right bundle branch block (3 patients), left anterior hemi-block (2 patients) and atrial fibrillation (5 patients) n= number of patients.

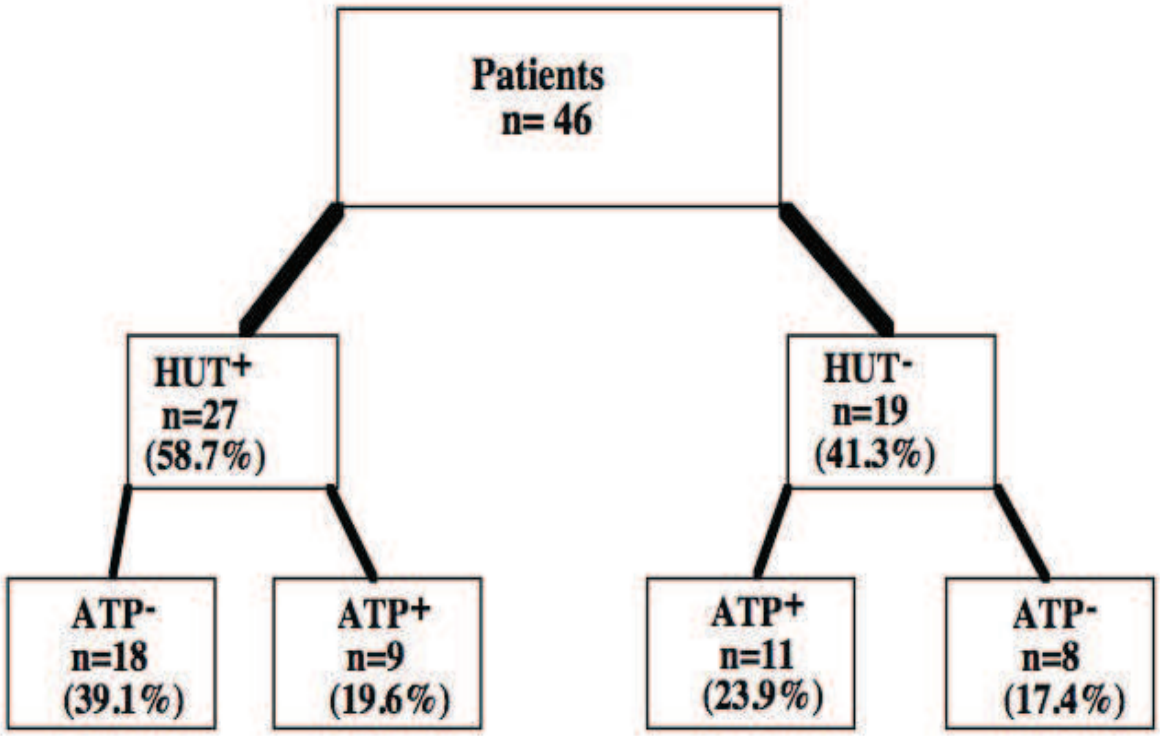


Figure 1: Response to laboratory tests in the 46 patients with suspected neutrally mediated syncope

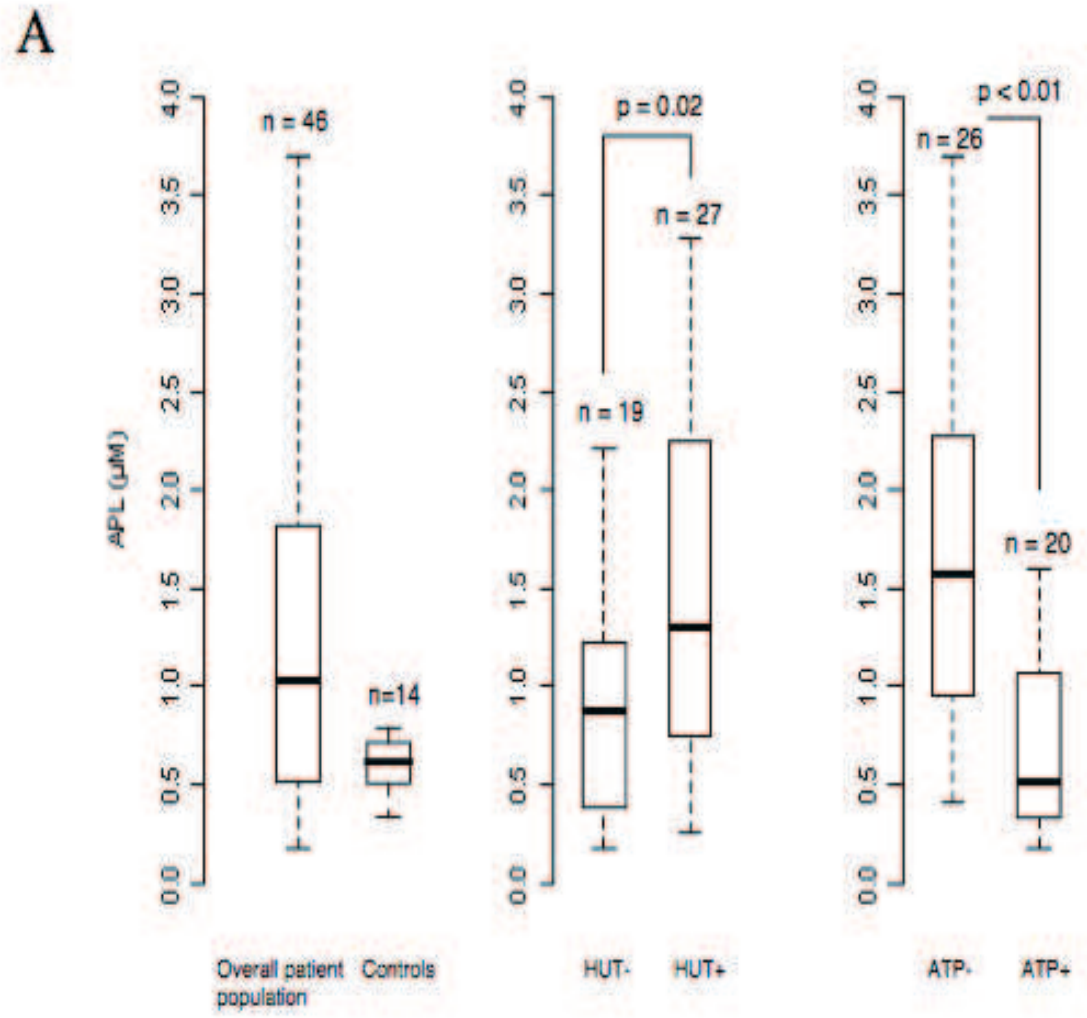


Figure 2A: **Adenosine plasma levels (APL) in patients and controls**
Box plots of baseline APL in 46 patients with unexplained syncope and 14 controls. Box plots show medians, quartiles, and extreme values.

Patients were separated into subgroups, depending on response to ATP or HUT test, considered independently.

B

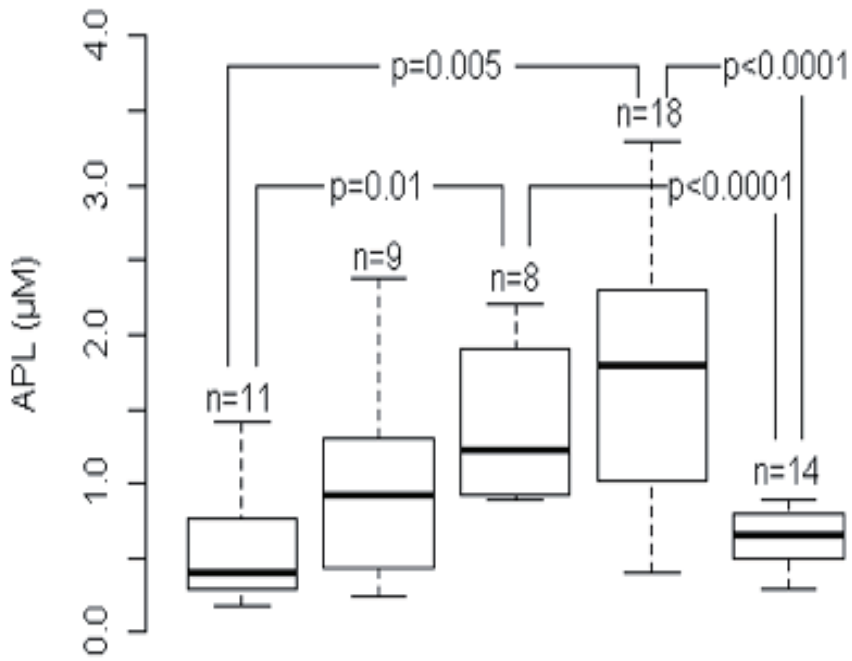
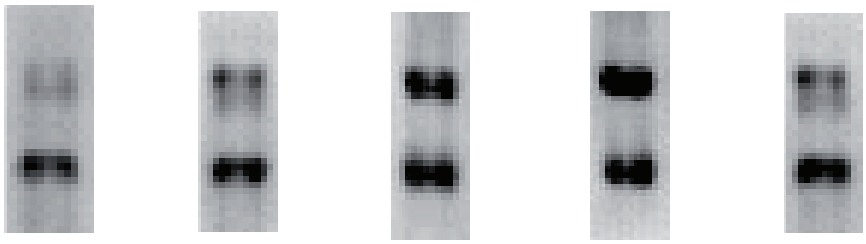


Figure 2B: **Adenosine plasma levels (APL) in patients and controls**
Box plots of baseline APL in 46 patients with unexplained syncope and 14 controls. Box plots show medians, quartiles, and extreme values.

Patients were separated into subgroups, depending on response to both ATP and HUT test.

C



$A_{2A}R$/GAPDH	ATP+/HUT-	ATP+/HUT+	ATP-/HUT-	ATP-/HUT+	Controls
Mean±SD	0.71±0.17*	1.19±0.24	1.3±0.3	1.83±0.47**	0.88±0.12
Median	0.73	1.2	1.2	1.8	0.85
Range	0.45-1.03	0.88-1.5	0.8-1.7	1.2-2.5	0.75-1.1

Figure 2C: Expression level of $A_{2A}R$ in patients and controls

$A_{2A}R$ expression evaluated using Western blot, and homemade $A_{2A}R$ monoclonal antibody. Top of the panel: example of Western blot bands. Quantification of $A_{2A}R$ was done using the $A_{2A}R$/GAPDH ratio. Bottom of the panel: means, SD, median and range of $A_{2A}R$ expression in each group of patients and in controls. *: $p > 0.01$ compared with other groups of patients, and $p > 0.05$ compared with controls. **: $p < 0.05$ compared with other groups of patients or with controls.

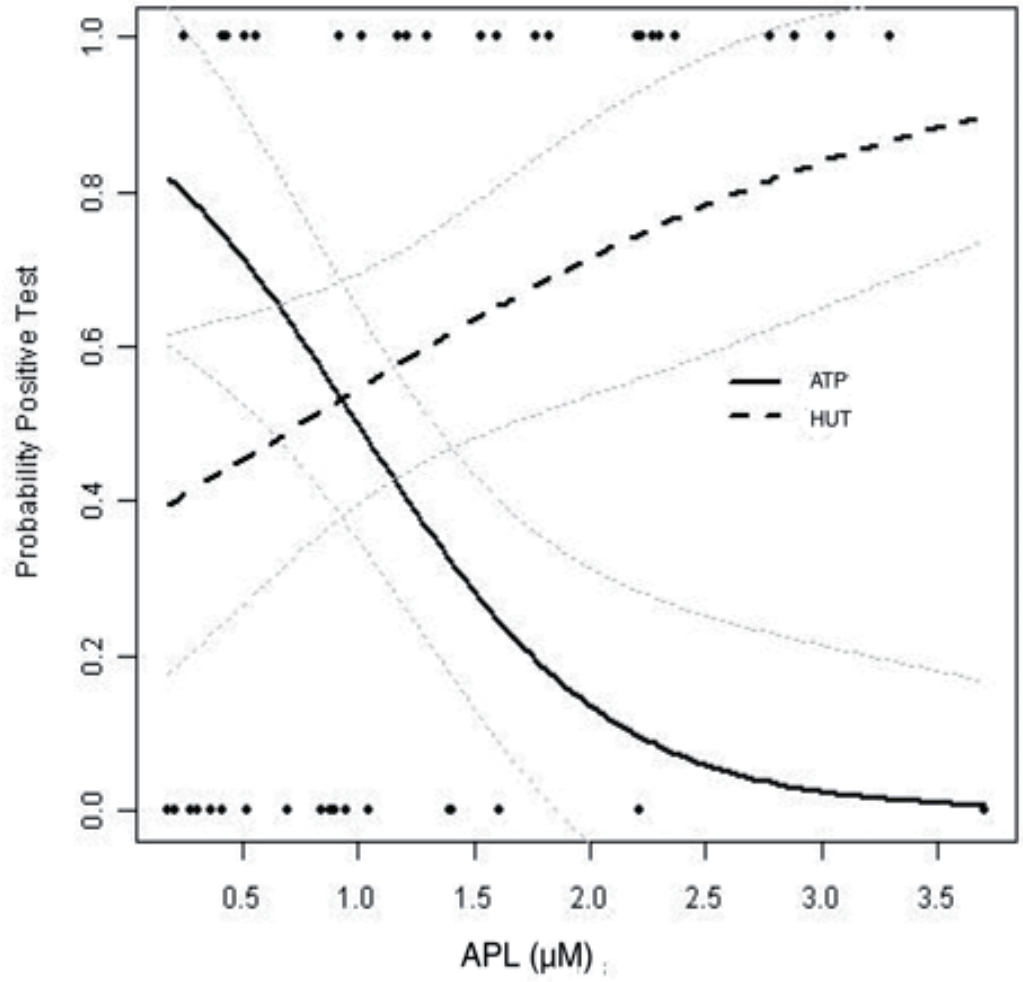


Figure 3: **Probability of positive ATP-test or HUT in function of adenosine plasma levels (APL)**

Probability of positive adenosine triphosphate test (ATP) or head-up tilt test (HUT) (with the 95% confidence intervals; dotted curves) in function of adenosine plasma levels (APL in µM) in 46 patients. Top and bottom dots indicate individual APL values for patients with positive ATP or HUT test (top) and negative ATP or HUT test (bottom)

3. Conclusion de l'article 2

Les principaux résultats de cette étude montrent que, chez les patients souffrant de syncope neurocardiogénique, un APL fort ou faible associé à un niveau fort ou faible d'expression des $A_{2A}R$, se traduit par une réponse positive soit au test HUT, soit au test ATP ; autrement dit :

- le test HUT positif s'associe à un APL élevé ainsi qu'à une surexpression des $A_{2A}R$
- le test ATP positif s'associe à un APL faible ou normal ainsi qu'à une expression faible ou normale des $A_{2A}R$

Nos résultats suggèrent l'existence de différents substrats physiopathologiques et leurs implications probables dans la genèse de différentes formes de syncope. Ainsi, Adonis représente un outil de recherche clinique utile et fiable pour apprécier le niveau d'expression des $A_{2A}R$ en relation avec ces affections cardiovasculaires pas encore totalement élucidées sur plan physiopathologique.

III. Adonis et désensibilisation des CXCR4 et CCR5

1. Récepteurs A_{2A} et inflammation

L'adénosine agit comme un anti-inflammatoire par son action sur les $A_{2A}R$ en inhibant la prolifération des cellules T et la sécrétion des cytokines pro-inflammatoires (Sitkovsky et al., 2004). Les $A_{2A}R$ sont fortement exprimés dans les cellules immunitaires surtout les lymphocytes T qui expriment en plus les récepteurs de chimiokines CCR5 et CXCR4 qui modulent plusieurs fonctions cellulaires dont l'évolution, le déplacement, l'angiogenèse et les réponses immunitaires (Contento et al., 2008). Les récepteurs A_{2A}, CCR5 et CXCR4 font partie de la superfamille des récepteurs couplés aux protéines G, dont la signalisation fait intervenir différentes enzymes cytoplasmiques dans plusieurs voies de communications cellulaires. Ces voies de signalisation sont soumises à des régulations fines qui ont, en retour, un effet sur les récepteurs eux mêmes en induisant une désensibilisation, une redistribution ou une dégradation des récepteurs. Une activation prolongée d'un récepteur entraîne une désensibilisation c'est-à-dire une inactivation de sa propre activité (désensibilisation homologue) ou de l'activité d'un autre récepteur de la même famille (désensibilisation hétérologue) (Ali et al., 1999). La désensibilisation homologue est principalement basée sur l'activation par « feedback » d'une cascade d'inhibiteurs incluant des G-récepteurs de kinases (GRK) et des arrestines. La désensibilisation hétérologue des récepteurs de chimiokines provient essentiellement de la phosphorylation des récepteurs par les protéines kinases C (PKC) suivie parfois de l'internalisation des récepteurs (Lefkowitz, 1998).

Il a récemment été montré que la stimulation des $A_{2A}R$ induit une désensibilisation hétérologue des CCR5 (Zhang et al., 2006). Puisque le CXCR4 et le CCR5 sont recrutés ensemble et peuvent se retrouver en hétéro-oligomère sur les cellules T activées (Contento et al., 2008), nous nous sommes demandés s'il serait possible d'induire une désensibilisation hétérologue et, par la suite, une internalisation des deux co-récepteurs via la stimulation des $A_{2A}R$. Ainsi, nous avons mené une étude sur une lignée de cellules T concernant l'activité fonctionnelle de notre anticorps monoclonal Adonis qui se comporte comme un agoniste spécifique des $A_{2A}R$.

Ce travail est détaillé dans l'article « **Monoclonal antibody-assisted stimulation of adenosine A_{2A} receptors induces simultaneous down-regulation of CXCR4 and CCR5 on $CD4^+$ T-cells** » ci-dessous.

Publication 3

"Monoclonal antibody-assisted stimulation of adenosine A_{2A} receptors induces simultaneous down-regulation of CXCR4 and CCR5 on $CD4^+$ T-cells"

Youlet By, Josée-Martin Durand-Gorde, Jocelyne Condo, Pierre-Jean Leujeune, Emmanuel Fenouillet, Régis Guieu and Jean Ruf

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Brief communication

Monoclonal antibody–assisted stimulation of adenosine A_{2A} receptors induces simultaneous downregulation of CXCR4 and CCR5 on $CD4^+$ T-cells

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ABSTRACT

Immunocompetent cells express various G-protein–coupled receptors that transduce extracellular signals across the plasma membrane. Among them, CXCR4 and CCR5 chemokines receptors and adenosine A_{2A} receptors ($A_{2A}R$) are involved in inflammatory processes. Considering that $A_{2A}R$ activation may have incidence on CXCR4 and CCR5 protein expression through heterologous desensitization process, we tested Adonis, an agonist-like monoclonal antibody to $A_{2A}R$ on $CD4^+$ CEM T-cells. We found that Adonis inhibited the CEM cell growth, upregulated $A_{2A}R$ and downregulated CXCR4 and CCR5 without modifying the CD4 expression. By reducing the expression of CXCR4 and CCR5 chemokines receptors utilized as entry co-receptors by HIV-1 during viral infection of CD4 expressing cells, Adonis stimulation of $A_{2A}R$ appears as a valuable means to treat infected cells.

The A_{2A} receptors ($A_{2A}R$) of adenosine are predominantly expressed on mouse [1] and human [2] T-cells, where they play a critical role in the protection of inflammatory damages and tumors [3,4]. Adenosine blocks the T-cell proliferation and chemokines secretion [5] as a function of the number of $A_{2A}R$ expressed on T-cells [6]. T-cells also express chemokine receptors CCR5 and CXCR4, which mediate several cellular functions such as development, cellular trafficking, angiogenesis and immune response [7]. $A_{2A}R$, CCR5, and CXCR4 are structurally related receptors belonging to the superfamily of the seven-transmembrane G-protein–coupled receptors [8]. Ligand binding to these receptors induces a conformational change of the receptor that links its cytoplasmic part to an intracellular G protein. This signal activates or inhibits intracellular enzymes involved in various pathways of cellular communication. Many of these pathways are dynamically regulated [9]. At the receptor level, regulation can occur via inhibition of receptor/G-protein coupling (desensitization), redistribution of cell surface receptors (trafficking), and receptor degradation (downregulation). Two protein families, G-protein–coupled receptor kinases and arrestins, play a critical role in regulating these processes. Kinases specifically phosphorylate the activated form of the receptor, which in turn promotes arrestin binding. Arrestin binding sterically inhibits coupling of the receptor to its respective G-protein and targets the receptor for internalization via clathrin-coated pits [10]. Receptors then are either recycled back to the plasma membrane or directed to the degradative pathway. $A_{2A}R$ was reported to induce heterologous desensitization of CCR5 [11]. As CXCR4 and CCR5 are co-recruited and may hetero-oligomerize on activated T-cells [12], we hypothesized that it was possible to induce heterologous desensitization and subsequent internalization of both co-receptors through stimulation of the adenosine $A_{2A}R$. Here, we took advantage of production of Adonis, a new agonist-like monoclonal antibody that binds to a linear epitope of the $A_{2A}R$ [13] to downregulate CXCR4 and CCR5 expression on a T-cell line.

CEM, a $CD4^+$ human T-lymphoma cell line endogenously expressing $A_{2A}R$, CXCR4, and CCR5, was obtained from American Type Culture Collection (Rockville, MD) and cultured at 37°C under 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, and 100 IU/ml each penicillin-streptomycin. To establish the growth curve, CEM cells (1×10^5/ml) were seeded into 24-well culture plates, each well containing 1 ml culture medium with or without the presence of various doses of Adonis. During 6 days, the cells within the corresponding wells were day by day suspended and viable cells count was performed using the trypan blue dye exclusion method and a Malassez hemocytometer. Without Adonis, CEM cells exponentially grew until 5 days of culture to reach a plateau (Fig. 1A). Adonis inhibited the CEM cell growth depending on the dose to reach a plateau at day 4 for 0.2 and 0.4 μmol/l and at day 3 for 0.6 μmol/l (Fig. 1A). To further confirm the inhibition of the CEM cell growth by Adonis, we used the CytoX-Violet Cell Proliferation Assay according to the manufacturer's instructions (Epigentek,

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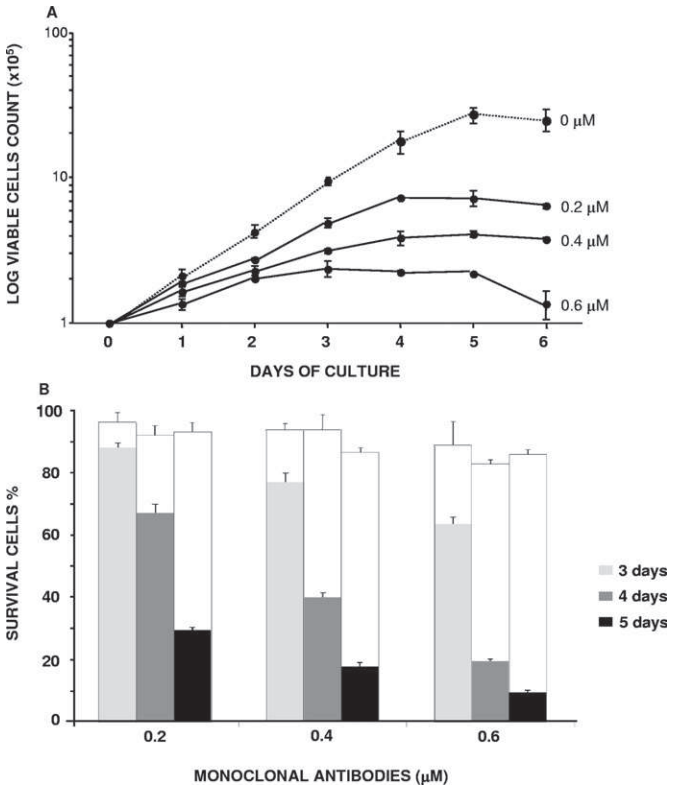

Fig. 1. Adonis effect on CEM cell growth. (A) Viable cells count was reported from day 0 to day 6. Standard curves in the absence of Adonis were given as control (dotted lines). Concentrations of Adonis in the culture medium were successively 0.2, 0.4, and 0.6 μmol/l. Results are mean values ± standard deviation (SD) of duplicates. (B) Percentage of survival cells in presence of Adonis (filled bars) and control (open bars) monoclonal antibodies was reported for 3, 4, and 5 days of culture. Concentrations of monoclonal antibodies in culture medium were successively 0.2, 0.4 and 0.6 μmol/l. Results are the mean ± SD of duplicates.

Brooklyn, NY). This kit is based on the fluorometric change of a special dye composition to measure cell viability that is proportional to the cellular deshydrogenase activity. CEM cells (1×10^5/ml) were mixed with Adonis in 96-microplate wells filled with 200 μl of culture medium. After 3–5 days' incubation at 37°C under 5% CO_2, 20 μl of indicator solution was added to each well. After 4 hours' incubation at 37°C, fluorescence was read at 560/590 nm. The anti-terpolymer (Glu–Ala–Tyr) monoclonal antibody, H56.406.48 [14] with the isotype (IgM, k) as Adonis, was used as control. Results were expressed as percentage of survival cells according to the formula: (fluorescent intensity with monoclonal antibody – blank/ fluorescent intensity without monoclonal antibody – blank) × 100. We found that Adonis gradually decreased in a time- and dose-dependent fashion the CEM cells proliferation, whereas the irrelevant monoclonal antibody remained continuously negative (Fig. 1B). We previously found that Adonis stimulated the cAMP production and inhibited the cell proliferation of an A_{2A}R-expressing cell line [13]. The results obtained on the CEM cell line further agreed with the fact that A_{2A}R downregulate immune response *in vivo* by inducing elevation of intracellular cAMP which in turn inhibits the NF-κB pathway in lymphocytes [15].

To test the A_{2A}R, CXCR4, CCR5 and CD4 expression, CEM cells (1×10^5/ml) were seeded into 25-cm^2 tissue culture flasks containing 10 ml culture medium with or without the presence of various doses of Adonis. Considering the above results, CEM cells were incubated 4 days in the presence of 0.2 and 0.4 μmol/l of Adonis. As control, CEM cells were cultured without Adonis to measure the native expression of the cell receptors. Cells were washed with PBS and frozen at −70°C. Cells were then quickly thawed at 37°C and solubilized with 4% SDS aqueous solution by 30-minute sonication at 47 kHz. After protein quantification by microBCA (Pierce Biotechnology, Rockford, IL), 15 μg of cell solubilisate were diluted in 62.5 mmol/l Tris-HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and subjected to standard electrophoresis procedure in Mini Protean II system (Bio-Rad, Hercules, CA). Separated proteins in 12% acrylamide minigel were electrotransfered onto a PVDF membrane. Blotted membrane was placed into the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA), saturated with nonfat dried milk and incubated 20 minutes with one of the appropriately diluted mouse monoclonal antibody, anti-A_{2A}R (Adonis), anti-CD4 (clone MEM-241, Immunotools, Friesoythe, Germany), anti-CCR5 (clone 12D1, Immunotools) and anti-CXCR4 (clone 1F8, Sigma-Aldrich, Saint Louis, MO). Antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Clone GAPDH-71.1, Sigma-Aldrich) was used as loading control for normalizing blot results. Primary antibody was omitted to specifically reveal Adonis IgM κ light chain in the cell lysates. Blots were visualized by horse-radish peroxidase labeled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL) using a Kodak Image Station 440CF (Eastman Kodak Compagny, Rochester, NY). The staining intensities of the bands were measured densitometrically on a Macintosh computer using the public domain NIH Image software developed at the US National Institutes of Health. Controls were reported for blots of CEM cells incubated with 0.2 μmol/l Adonis and revealed with only the anti-GAPDH monoclonal antibody or the whole set of monoclonal antibodies. In the first control, only the Adonis heavy-chain (65 kDa) and light-chain (25 kDa) bands were revealed in addition to the GAPDH (36 kDa) band and in the second control also appeared the four other bands specific for CD4 (51 kDa), A_{2A}R (45 kDa), CCR5 (41 kDa), and CXCR4 (40 kDa), respectively (Fig. 2A). Using this set of monoclonal antibodies, no crossing bands were evidenced in denaturing conditions and omitting one monoclonal antibody resulted in the loss of the corresponding band in the blot (not shown). We found that Adonis upregulated the A_{2A}R but downregulated the CCR5 and CXCR4 in a dose-dependent manner, whereas CD4 expression remained unchanged (Figs. 2A, 2B). We previously found that caffeine (1,3,7 trimethylxanthine) antagonized the Adonis-induced cAMP production in cells expressing A_{2A}R and was also able to upregulate the A_{2A}R cell-expression in a dose-dependent fashion [13]. Interestingly, Adonis remained linked to the A_{2A}R in a dose-dependent manner as shown by the presence of the 25-kDa IgM κ light chain of Adonis on the blots (Figs. 2A, 2B). The Adonis heavy chain gave faint bands and, consequently, was not chosen for the Adonis binding test. Probably, sustained binding of Adonis to A_{2A}R on the cell surface prevented the complexes made by the multivalent IgM and cross-linked receptors to be endocytosed. In contrast, CCR5 and CXCR4 decreased in net protein term, suggesting a possible internalization of the receptors not recycled back but directed to the degradative pathway [9].

It is likely that the A_{2A}R acts as homodimers at cell surface [16]. Because Adonis is a pentameric IgM antibody, it can stimulate A_{2A}R by stabilizing active homodimers at cell surface. This activating mechanism was already reported for another monoclonal antibody directed to the β_2-adrenergic receptors [17]. It was suggested for a long date that A_{2A}R desensitization is mediated by multiple, temporally distinct, agonist-dependent processes [18]. Agonist short-term desensitization of A_{2A}R was reported in numerous models [19] but, in most instances, long-term desensitization followed by internalization of A_{2A}R was not reported, A_{2A}R being rather considered as fairly resistant to agonist-induced internalization [20]. We found here that A_{2A}R prolonged (chronic) stimulation with Adonis did not downregulate but upregulated A_{2A}R expression. The extent

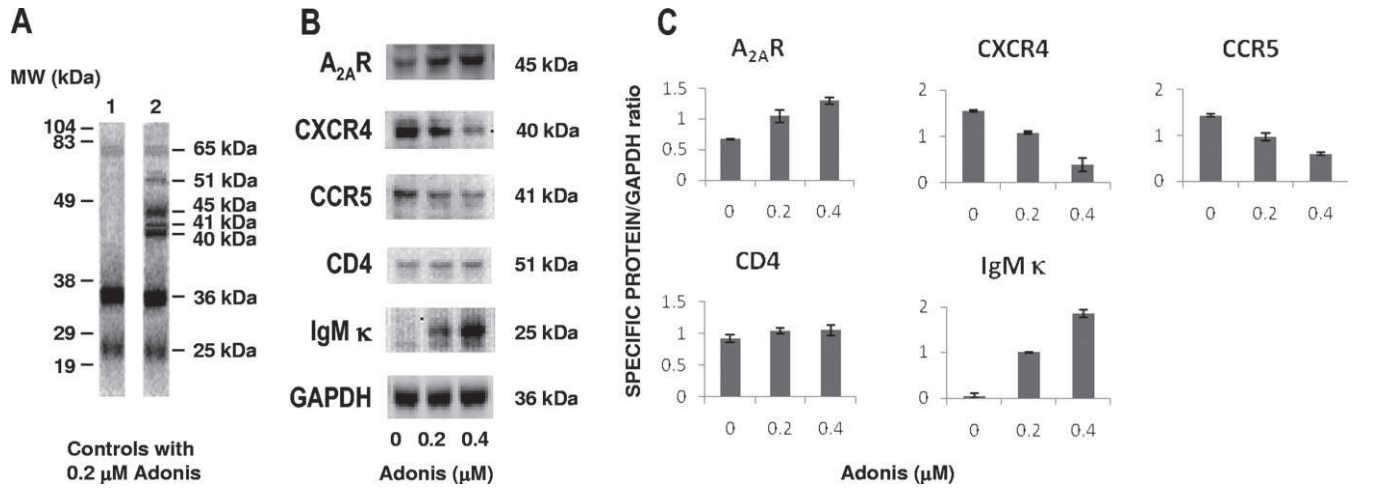

Fig. 2. Adonis effect on protein expression of CEM cell-surface receptors. (A) Controls are blots of CEM cells incubated 4 days with 0.2 μmol/l Adonis and revealed with only the anti-GAPDH monoclonal antibody (lane 1) or the whole set of monoclonal antibodies (lane 2). The molecular weights (kDa) of prestained standards and revealed protein bands are on the left and right, respectively. (B) Representative immunoblots of $A_{2A}R$, CXCR4, CCR5, CD4 expression obtained after 4 days of cell culture with 0.2 and 0.4 μmol/l Adonis. CEM cells cultured without Adonis gave the native expression of the cell receptors. IgM κ light chain was used as Adonis binding control to the $A_{2A}R$ and GAPDH as loading protein control. (C) Results of densitometric analysis of blots are expressed as the specific protein/GAPDH ratio of gray values. Data are mean ± standard deviation of of duplicates.

of $A_{2A}R$ upregulation was measured by Adonis binding on cell lysate electrophoresed under denaturing conditions. It is worth noting that, under these conditions, before being targeted again by Adonis as a primary antibody, $A_{2A}R$ was subjected to the previous binding of Adonis, which resolved on the blot in heavy- and light-chains bands. Adonis might thus have achieved a prolonged binding to $A_{2A}R$, giving a sustained signal vital to support long-term tissue repair, in contrast to classic agonists that only transiently bind into the pharmacophore pocket of $A_{2A}R$. By lowering the cell growth and the chemokine receptor density on the cells, Adonis might better regulate the damaging aspect of inflammation. Our data agree with previous findings suggesting that heterologous chemokine receptors desensitization through $A_{2A}R$ stimulation occurred via cAMP activated PKA without disclosing if PKA directly phosphorylate chemokine receptors [11].

Human immunodeficiency virus type 1 (HIV-1) induces viral-to-cell membrane fusion to gain entry into target cells [21]. Despite the rapid identification of CD4 as the primary cell surface receptor for the HIV-1 binding, it soon appeared that CXCR4 and CCR5 chemokine G-protein receptors act as additional HIV-1 co-receptors that are essential for viral entry into immune cells [22]. Almost all cases of HIV-1 transmission involve R5 viral strains that use CCR5 for entry. X4 viral strains use CXCR4 and usually, but not always, emerge late in disease progression [23]. R5X4 dual strains also exist, as they can use both co-receptors [24]. Molecules that specifically bind to CXCR4 and CCR5 and impede interactions with their ligands may be powerful tools to design effective therapeutic agents to stop HIV-1 entry. Among the various molecules identified to date as inhibitor of CXCR4 and CCR5 are small molecules, peptides, chemokines and their derivatives, and monoclonal antibodies. Some of them (maraviroc and vicriroc) that are antagonists to the CCR5 are in clinical use or in clinical trials [25]. However, there are no molecules that simultaneously block the two receptors. So, by blocking only one receptor, resistance develops and virus enters the cell by switching to the other receptor. By lowering the CCR5 and CXCR4 surface receptors density, we suggest that Adonis can reduce the viral load and therefore the disease progression in HIV-1–infected patients [26].

In conclusion, our results confirm that $A_{2A}R$ stimulation is able to block T-cell proliferation, leading to the possibility of pharmacologically targeting the $A_{2A}R$ to protect tissue in various human diseases. By downregulating the number of CXCR4 and CCR5, $A_{2A}R$ stimulation also seems likely to play a pivotal role in modulating HIV-1 infection. These *in vitro* data on the CEM cell line designate Adonis as a good candidate for further studies with virus-infected cells.

Acknowledgments

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2. Conclusion de l'article 3

Nous avons montré que, par stimulation des $A_{2A}R$, Adonis est capable d'inhiber la prolifération des cellules T, ce qui permet d'envisager ce récepteur comme une cible pharmacologique permettant de protéger les tissus dans diverses pathologies. De plus, cette stimulation induit une « down-régulation » de l'expression protéique des deux co-récepteurs CCR5 et CXCR4 simultanément, reflétant probablement leur internalisation vers une voie de dégradation. Puisque les CCR5 et CXCR4 sont classiquement utilisés par les virus de l'immunodéficience humaine (HIV) pour pénétrer dans leurs cellules cibles $CD4^+$, la diminution de densité de ces récepteurs à la surface des cellules T pourrait être un moyen efficace pour réduire la multiplication virale, donc retarder la progression de la maladie chez les patients atteints d'HIV. Par ailleurs, l'effet simultané sur les deux co-récepteurs d'entrée virale permet d'éviter le phénomène de résistance aux drogues mono-spécifiques, par lequel le virus peut changer son tropisme pour un récepteur d'entrée avec un autre. Ainsi, Adonis pourrait être à la base d'un nouveau médicament susceptible d'intérêt dans le traitement des infections par le HIV. Ces résultats *in vitro* sur une lignée des cellules T nous encouragent à envisager l'étude de l'effet d'Adonis sur des cellules infectées par le virus HIV et ultérieurement sur un modèle *in vivo*.

IV. Adonis et anti-nociception

1. Récepteurs A_{2A} de l'adénosine dans le contrôle de nociception

On distingue schématiquement deux types de douleurs suivant l'IASP (International Association for the Study of Pain) :

i. Les douleurs par excès de nociception ou inflammatoires, qui sont secondaires à l'activation de nocicepteurs spécialisés dans l'acheminement des messages douloureux. On distingue ainsi les nocicepteurs thermiques, chimiques, ou mécaniques en fonction de la nature des stimuli. Certains nocicepteurs sont dits polymodaux car ils répondent à plusieurs types de stimulation.

ii. Les douleurs neuropathiques, qui sont générées par une lésion du système nerveux et, dont la genèse prendrait sa source suite à la perte des contrôles inhibiteurs que le système nerveux met en place (système de contrôle par libération d'opioïdes endogènes, par exemple, ou contrôle inhibiteur médullaire par les fibres afférentes de gros diamètre).

Il est bien établi que l'adénosine et ses analogues exercent un contrôle puissant sur les messages nociceptifs. Ainsi, l'activation des A_1R dans la moelle épinière produit des effets anti-nociceptifs dans les tests de douleur aiguë, inflammatoire ou neuropathique (Sawynok, 2007; Sawynok and Liu, 2003). Chez l'homme, la toute première évidence de l'action anti-nociceptive de l'adénosine a été mise en évidence par l'infusion intra-veineuse d'adénosine qui avait des effets bénéfiques chez des patients atteints de douleurs neuropathiques (Guieu et al., 1994; Sollevi et al., 1995). Quelques années plus tard, il a été montré que l'adénosine pouvait réduire l'hyperalgésie dans un modèle humain de douleur inflammatoire cutanée (Sjolund et al., 1999).

Le mécanisme par lequel l'activation des A_1R conduit à un puissant effet antalgique est bien connu. Il procède au niveau pré-synaptique d'une inhibition de la libération d'AMPc dépendante de neurotransmetteurs algogènes (glutamate, Substance P, voir Figure 5). Au

niveau post-synaptique, l'effet antalgique est dû principalement à la modulation de canaux potassiques engendrant une hyperpolarisation post-synaptique. Ces deux effets sont très voisins de ceux obtenus avec la morphine (voir figure 5).

Si les effets anti-nociceptifs de l'adénosine via l'activation des A_1R sont bien identifiés, ceux engendrés sur le contrôle de la nociception par l'activation des $A_{2A}R$ sont controversés. Certains travaux font état d'une action pro- ou anti-nociceptive des $A_{2A}R$ en fonction du modèle animal utilisé ou de la voie d'administration. Bien que l'activation des $A_{2A}R$ périphériques puisse induire des réponses douloureuses (Sawynok, 1998), ses effets anti-inflammatoires peuvent également contribuer à diminuer la douleur inflammatoire. Ces effets contradictoires pourraient s'expliquer en partie par la spécificité plus ou moins importante des agonistes des $A_{2A}R$ utilisés.

Actuellement, des agonistes des A_1R sont en phase clinique d'évaluation pour la lutte contre la douleur neuropathique, tandis que les agonistes des $A_{2A}R$ sont en phase d'évaluation clinique comme agents anti-inflammatoires (Gao and Jacobson, 2007). Cependant, les études sur les effets des agonistes synthétiques des $A_{2A}R$ (ATL-313, CGS21680) sur la douleur neuropathique ont aussi rapporté des effets anti-nociceptifs au niveau spinal (Loram et al., 2009) et supra-spinal (Regaya et al., 2004). Ces évidences suggèrent que les $A_{2A}R$ pourraient être une cible thérapeutique dans le contrôle des différentes types de douleur et que les agonistes spécifiques de ce récepteur pourraient être des candidats potentiels à la mise au point de nouveaux médicaments.

2. Adonis et tests de nociception chez la souris

A côté de son utilisation comme outil de diagnostic, Adonis peut être envisagé pour une utilisation thérapeutique dans le traitement de pathologies bien définies grâce aux effets fonctionnels très spécifiques de l'$A_{2A}R$. Nous avons vu qu'Adonis se comporte comme un « agoniste-like » en augmentant la concentration en AMPc par stimulation des $A_{2A}R$. Bénéficiant de ce matériel déjà bien caractérisé, nous avons souhaité tester Adonis pour son éventuel effet anti-nociceptif chez la souris en utilisant deux tests classiques qui sont les tests thermiques Hot-plate et Tail-flick.

2.1 Test Hot-plate

La chaleur est souvent utilisée comme un stimulus nocif dans les modèles de douleur aiguë. Pour déterminer si une molécule a une propriété analgésique ou antalgique, il faut comparer le temps de réaction (latence) à l'exposition à une chaleur nocive d'un animal traité avec celui d'un animal contrôle chez lequel on a administré un placebo, par exemple du sérum physiologique. L'augmentation significative du temps de latence est interprétée par une réponse anti-nociceptive ou analgésique. Dans le test Hot-plate (figure 10A), l'animal est placé sur une plaque chauffée à 55°C et le temps de latence de l'animal répondant au stimulus thermique est enregistré au moment où il commence à se lécher les pattes avant ou à sauter.

2.2 Test Tail-flick

Le test Tail-flick permet d'explorer la nociception par l'intermédiaire d'une chaleur radiante nocive. Ce test a été décrit initialement par *D'Amour et Smith* en 1941. L'appareil du test Tail-flick dans notre étude (figure 10B) utilise la chaleur émise par rayonnement laser. La stimulation nocive est appliquée au niveau de la queue et produit une réponse de type réflexe nociceptif simple qui se traduit par un mouvement soudain de la queue qui s'écarte de la source de chaleur. Ce réflexe, au point de départ spinal, est influencé par les voies descendantes supra-spinales, ce qui fait qu'il permet d'étudier toutes les voies de contrôle de la périphérie jusqu'au thalamus, zone d'intégration majeure des stimuli nociceptifs. Par ailleurs, l'avantage du test Tail-flick est que la réponse de l'animal est peu dépendante de l'état des voies motrices par rapport au test Hot-plate.

Pour élucider l'implication des $A_{2A}R$ dans le contrôle de la nociception, nous avons administré Adonis au souris par voie intra-cérébro-ventriculaire et mesuré les temps de latence à l'aide des tests Hot-plate et Tail-flick.

Cette étude est détaillée dans l'article « **Intracerebroventricular injection of an agonist-like monoclonal antibody to adenosine A_{2A} receptor has antinociceptive effect in mice** » ci-dessous.

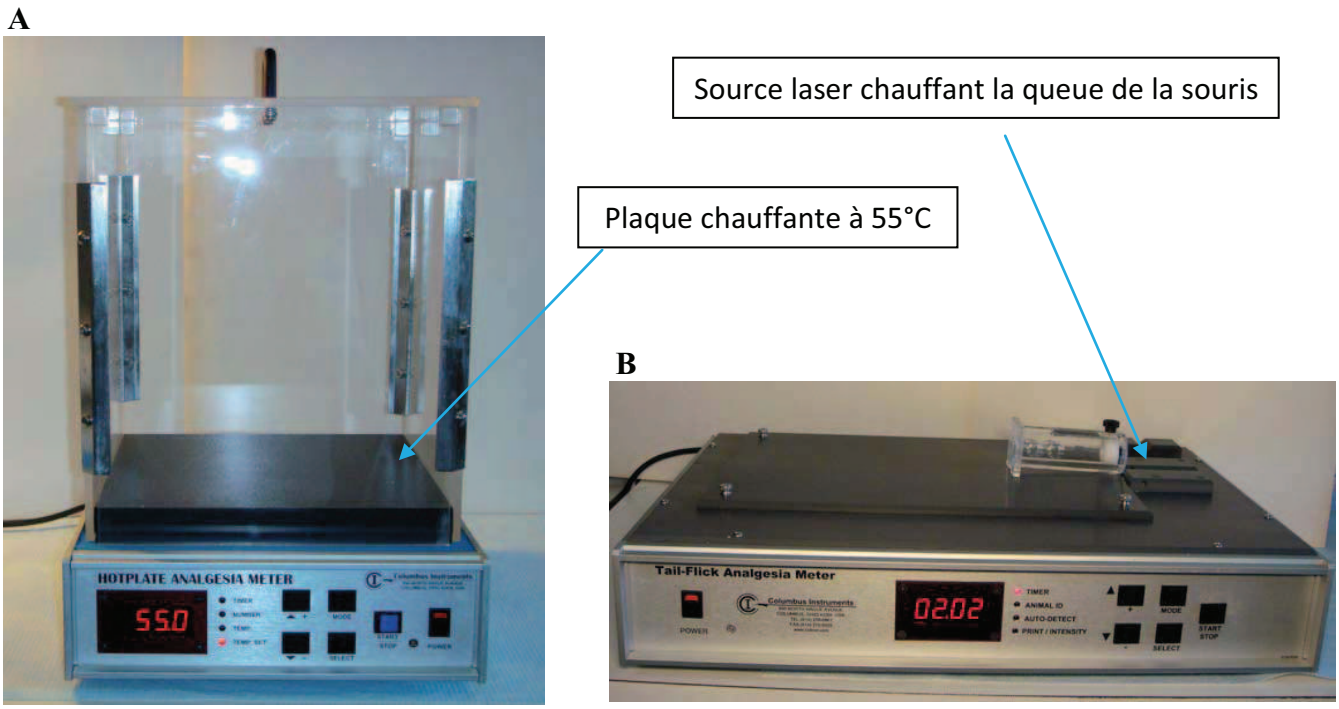

Figure 10 : Appareillage pour les tests Hot-plate (A) et Tail-flick (B) (Colombus Instruments, Columbus, OH)

Publication 4

"Intracerebroventricular injection of an agonist-like monoclonal antibody to adenosine A_{2A} receptor has antinociceptive effect in mice"

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Short communication

Intracerebroventricular injection of an agonist-like monoclonal antibody to adenosine A_{2A} receptor has antinociceptive effects in mice

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ABSTRACT

Adenosine is a modulator of nociceptive pathways, both at the spinal and supraspinal levels. Adenosine A_1 and A_{2A} receptors (A_1R, $A_{2A}R$) are expressed in the basal ganglia where they are the target of caffeine, the most widely use psychoactive drug which acts as an antagonist to both types of receptors. Given the controversial role of $A_{2A}R$ versus A_1R in modulating pain in brain areas, mice received intracerebroventricular injection of Adonis, an agonist-like monoclonal antibody with high specificity for the $A_{2A}R$ and were subjected to behavioral tests investigating nociceptive thresholds. We report that Adonis led to a significant dose-dependent increase in hot-plate and tail-flick latencies in mice and that such increase was prevented by caffeine and ZM 241385, a specific $A_{2A}R$ antagonist. The Adonis antinociceptive effects were also inhibited by naloxone, a non selective antagonist for opioid receptors, suggesting that Adonis acts, at least in part, through the stimulation of the endogenous opioid system. These results confirm the $A_{2A}R$ as a target for pain control and Adonis as a potential drug with therapeutic interest.

1. Introduction

Adenosine is an endogenous nucleoside released from cells by facilitated diffusion and is also produced extracellularly by degradation of ATP. Adenosine receptors are subdivided into four G-coupled protein receptors subtypes (A_1, A_{2A}, A_{2B} and A_3,) depending on their pharmacological properties to exert numerous effects on various tissues including the central nervous system (Ralevic and Burnstock, 1998; Sebastiao and Ribeiro, 2009). Both A_1 and A_{2A} receptors (A_1R, $A_{2A}R$) are involved in the modulation of pain perception at the spinal (Sawynok et al., 1986; Yoon et al., 2005) and supraspinal levels (Pham et al., 2003; Regaya et al., 2004). However, if it is well established that the activation of A_1R leads to antinociceptive effects (Dickenson et al., 2000; Sawynok, 1998), the effects of the activation of $A_{2A}R$ on pain modulation remain controversial. Indeed, antinociceptive (Lee and Yaksh, 1996; Pham et al., 2003; Regaya et al., 2004; Yoon et al., 2005) but also pronociceptive effects (Bastia et al., 2002; Karlsten et al., 1992) have been reported depending on the dose used, the pain model, the animal species and the site of drug administration (for a review see Ferre et al., 2007). Furthermore, $A_{2A}R$ appears to be involved in sleep-inducing mechanisms previously thought to be mediated solely by A_1R (Basheer et al., 2004). In this respect, caffeine, the world's most popular psychoactive drug, binds as a non specific antagonist to A_1R and $A_{2A}R$ and induces insomnia and wakefulness (Fredholm et al., 1999). Deciphering between the involvement of A_1R and $A_{2A}R$ is rather difficult since their opposite effects on the cAMP production are finely tuned: adenosine acting via A_1R leads to a decrease in cAMP whereas activation of $A_{2A}R$ leads to an increase in cAMP (Fredholm et al., 2001). Literature on the effects of agonists and antagonists of the $A_{2A}R$ in pain control is conflicting. For example, studies with $A_{2A}R$ agonists have shown antinociceptive effects in the writhing test, a visceral pain model (Bastia et al., 2002; Pechlivanova and Georgiev, 2002) but the hypoalgesia observed in $A_{2A}R$ knockout mice (Ledent et al., 1997) and the antinociceptive effects of a specific $A_{2A}R$ antagonist in inflammatory models (Hussey et al., 2007), suggest that this is the absence or blockade of the $A_{2A}R$ which have therapeutic potential in pain states. As some differences appear in the involvement of the $A_{2A}R$ depending on the intensity and modality of the stimulus, more detailed studies are needed to determine whether $A_{2A}R$ stimulation might have clinical utility in some pain conditions.

Here, we took advantage of the production of Adonis, an agonist-like monoclonal antibody to the $A_{2A}R$ of adenosine (By et al., 2009) to use it in mouse experimental tests of pain.

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2. Materials and methods

2.1. Drugs

Caffeine (1,3,7-trimethylxanthine), CGS 21680 (2-[*p*-(2-carbonyl-ethyl-)-phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine) and CPA (N^6-cyclopentyladenosine) were purchased from Sigma-Aldrich (Saint Louis, MO). ZM 241385 (4-(2-[7-amino-2-(2-furyl)(1,2,4) triazolo(2,3-a)(1,3,5)triazin-5-ylamino]ethyl)phenol) was from Tocris Bioscience (Bristol, UK). Naloxone hydrochloride (17-allyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one) was from Mylan (Saint-Priest, France). Adonis is an agonist-like monoclonal IgM produced against the extracellular part of the human A_{2A}R. It was shown to stimulate the A_{2A}R in a dose-dependent manner by producing cAMP in A_{2A}R transfected cells as the specific agonist CGS 21680. This effect was prevented by caffeine (By et al., 2009).

2.2. Cell lines

A_1-Chem-3 and A_{2A}-Chem-3 (Chemicon catalog #HTS047C and #HTS048C, respectively), are mammalian suspension cell lines endogenously expressing the promiscuous G protein, Gα15, lacking endogenous expression of adenosine receptors and transfected with the full-length human A_1R and A_{2A}R cDNA, respectively. They were obtained from Millipore (St Quentin-Yvelines, France) and cultured in RPMI 1640 containing 4.5 g/L glucose, 10% heat inactivated FCS, 0.8 mg/mL Geneticine (G418), 100 IU/mL each penicillin-streptomycin and 1/100 dilution of Chem-3 growth supplement (Millipore).

2.3. Animals

Six-week-old male C57/BL6 mice were purchased from Harlan (Gannat, France) and kept for 2 weeks at our animal facility before testing. Animals were housed by group of 9 per cage and had free access to food and water in a quiet environment. The room was maintained at 21–23 °C with a 12 h light/dark cycle. Mice weighed 20–25 g at time of experiment.

2.4. Western blotting

This procedure was used to test Adonis for crossreactivity to mouse A_{2A}R. Cell pellets from the A_{2A}-Chem-3 cell line and brains of 3 male C57/BL6 mice were frozen at -70 °C, quickly thawed at 37 °C and solubilized with 2% SDS aqueous solution by 30 min sonication at 47 kHz. After protein quantification by microBCA (Pierce Biotechnology, Rockford, IL), 10 µg of A_{2A}-Chem-3 cells and murine brain solubilisates were submitted to standard electrophoresis procedure in Mini Protean II system (BioRad, Hercules, CA). Separated proteins in 12% acrylamide minigel were electrotransfered onto a PVDF membrane. Blotted membrane was placed into the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA) and the Western blotting was performed according to the manufacturer's instructions. Blots were visualized by horse-radish peroxidase labeled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescence substrate (Super-Signal West Femto, Pierce) using a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY).

2.5. cAMP measurement

This activity test was used to confirm the agonist-like specificity of Adonis for the A_{2A}R but not the A_1R. Adonis and CGS 21680, an agonist of the A_{2A}R, were tested for cAMP production in the A_{2A}-Chem-3 cell line. Adonis and CPA, an agonist of the A_1R, were also tested for cAMP production in the A_1-Chem-3 cell line. Total cellular cAMP measurement was performed with the Amersham Biotrak kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) using the non-acetylation procedure as described by the manufacturer. Various concentrations of drugs were added to 1×10^6 cells/well in a 96-wells culture plate and incubated for 90 min at 37 °C in the culture medium under 5% CO_2. The test was stopped by adding the dodecyltrimethylammonium bromide acetate lysis buffer of the kit. The cAMP content in each well was then determined by a competitive enzyme immunoassay according to the manufacturer's instructions. Tests were in triplicates. Results were given as percent of cAMP produced with respect to the basal cAMP level of A_{2A}-Chem-3 and A_1-Chem-3 cells.

2.6. Nociceptive tests

Intracerebroventricular (*i.c.v.*) administration is a conventional route to test drug pharmacological effects in the central nervous system and especially for large molecules such as Adonis (IgM) that does not easily penetrate across the blood-brain barrier. Adonis was diluted in saline (150 mM NaCl) and ZM 241385 was first dissolved in DMSO before to be diluted with saline (0.04% DMSO final). Mice were anesthetised with 1 ml of isofluorane (Forene ®, Abbott, France) on soaked cotton in a closed bottle for 30 s. Mice were *i.c.v.* injected with 5 µl Adonis alone and in association with ZM 241385. Controls were *i.c.v.* injected with 5 µl saline. Mice were or were not pretreated by caffeine and naloxone intraperitoneally (*i.p.*) in 200 µl saline 60 min and 15 min, respectively, before being injected with Adonis. Upon *i.p.* injection and, because of their pharmacokinetic properties, both caffeine and naloxone were rapidly distributed in the spinal and supraspinal components of the central nervous system. Controls were *i.p.* injected with caffeine and naloxone alone without *i.c.v.* post injection of Adonis to test the pre-treatment innocuousness on mouse behavior. Methodology for nociceptive test measurement was previously described (Guieu et al., 2006; Pham et al., 2003). Briefly, in the hot-plate test, mice were placed on a 55 °C hot plate and latencies of forepaw licking were measured 30 and 90 min after *i.c.v.* injection. In the tail-flick test, tails of mice were submitted to laser heating and latencies of tail flicking were measured 30 and 90 min after *i.c.v.* injection. The hot-plate and tail-flick analgesia meters were from Columbus Instruments (Columbus, OH). Statistical analyses were done by the Student's *t*-test (Statview SE+Graphics, Abacus Concepts Software, Berkeley, CA). $p<0.05$ was considered significant.

3. Results and discussion

3.1. Specificity of Adonis for A_{2A}R

First, Adonis was developed against the human A_{2A}R and we had to test it for crossreactivity with mouse A_{2A}R. Previous epitope mapping identified the binding aminoacid sequence (LFEDVVP) at the C-terminal part of the second external loop of the human A_{2A}R (By et al., 2009). Because the mouse A_{2A}R displays the same epitope, we expected that Adonis recognized the mouse receptor in a similar way than the human one. The A_{2A}R of the two species have an almost identical molecular weight i.e. 44707 Da for the human and 44882 Da for the mouse. Effectively, in the Western blot of brain murine lysates, Adonis revealed a band in the same 45 kDa range as the A_{2A}R of the A_{2A}-Chem-3 cell line (Fig. 1).

We found that Adonis stimulates the A_{2A}-Chem-3 cell line for cAMP production with half maximal effective concentration (EC_{50}) of 2×10^{-8} M. The EC_{50} of CGS 21680, a well known A_{2A}R agonist was 5×10^{-7} M (Fig. 2A). To ascertain the specificity of Adonis for the A_{2A}R but not the A_1R, we tested it on the A_1-Chem-3 cell line and we found that Adonis did not display any effect on the basal level of cAMP as compared to the specific A_1R agonist CPA which induced a fall in cAMP production with EC_{50} of 3×10^{-9} M (Fig. 2B). The absence of crossreactivity of Adonis to the human A_1R can be explained by the observation that the seven aminoacids sequence from the C-terminal part of the second external loop (EFEKVIS) of the human A_1R (Swiss-

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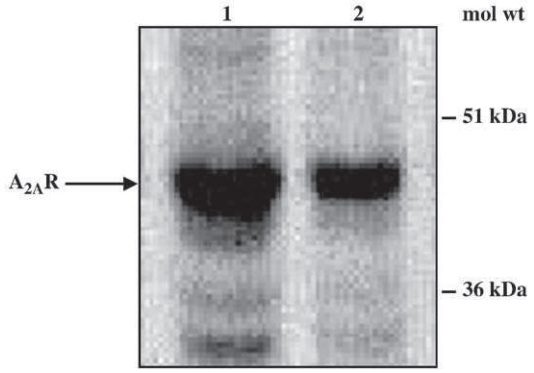

Fig. 1. Cross-reactivity of Adonis for mouse $A_{2A}R$. A_{2A}-Chem-3 cells (lane 1) and murine brain (lane 2) solubilisates were submitted to the Western blotting procedure described in Materials and methods. Results are representative from triplicates. Position of prestained molecular weight standards are on the right. The arrow on the left shows the $A_{2A}R$ at 45 kDa.

Prot: P30542) differs by four aminoacids from the binding sequence (LFEDVVP) of the human $A_{2A}R$ (Swiss-Prot: P29274). Because these different kinds of aminoacid sequences from the A_1R and the $A_{2A}R$ are conserved between the human and mouse species, one would expect that Adonis which crossreacts with the mouse $A_{2A}R$ (Swiss-Prot: Q60613) does not do so with the mouse A_1R (Swiss-Prot: Q60612).

3.2. Effects of Adonis on nociceptive tests

Reactivity to pain was investigated in treated (Adonis) and untreated (saline) mice by using two tests involving thermal stimuli. In the hot-plate test, latencies for licking were significantly increased in a dose- and time-dependent manner in treated mice (Fig. 3A). In the tail-flick test, mean latencies of treated mice were up to two-fold increased with 5 pmol Adonis at 30 min but in a more transient manner than observed in the previous test since the effect slightly decreased at 90 min (Fig. 3B). Interestingly, Adonis acts in the picomoles range whereas CGS21680, the most conventional agonist of the $A_{2A}R$, was reported to act in the nanomoles range in similar tests (Regaya et al., 2004).

In the hot-plate and tail-flick tests, antinociceptive effect of 5 pmol Adonis in mice was significantly prevented with 10 μg caffeine *i.p.* administered 60 min prior *i.c.v.* administration of Adonis (Fig. 3A and B). Evidence was obtained for a predominant role of $A_{2A}R$ in caffeine-induced wakefulness using A_1R and $A_{2A}R$ knockout mice (Huang et al., 2005). Here, caffeine was found to prevent the antinociceptive effects of Adonis without affecting mice sleepiness. This $A_{2A}R$ blockade was further confirmed by the use of a 10-fold mole excess of ZM 241385 (Keddie et al., 1996), a much more selective $A_{2A}R$ antagonist than caffeine, *i.c.v.* co-injected with Adonis in the hot-plate (Fig. 4A) and the tail-flick (Fig. 4B) tests. It can therefore be suggested that the

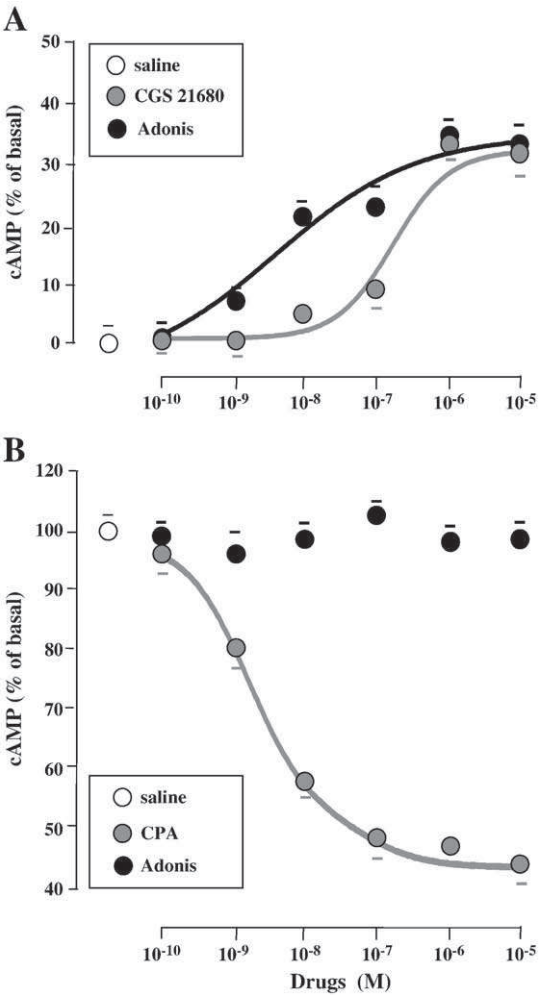

Fig. 2. Agonist specificity of Adonis. Effects of CGS 21680 and Adonis in A_{2A}-Chem-3 cells (A) and effects of CPA and Adonis in A_1-Chem-3 cells (B) were tested on cAMP production. Results are the mean values ± SD of duplicates.

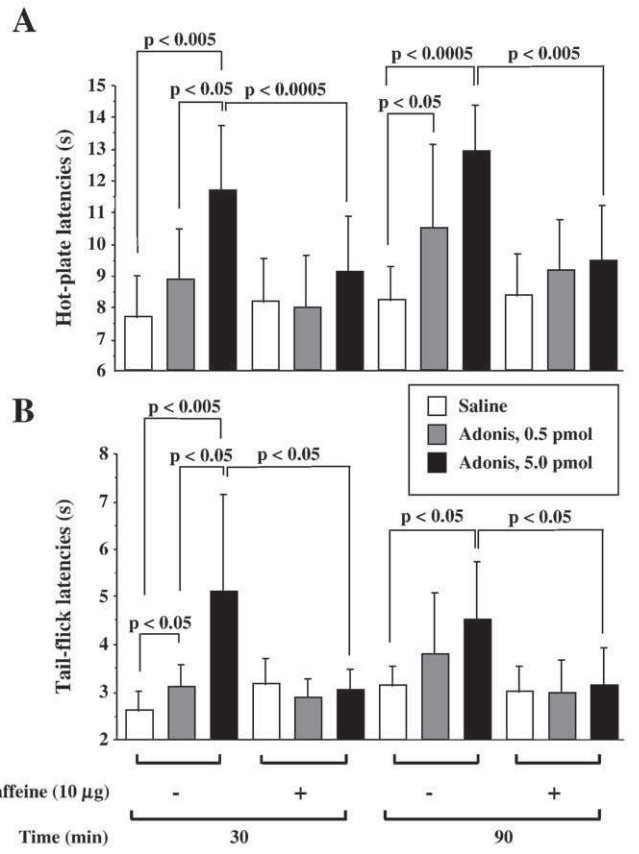

Fig. 3. Antinociceptive effects of Adonis in mice with and without pretreatment with caffeine. Latencies in the hot-plate (A) and the tail-flick (B) tests were performed in mice ($n = 9$ per group) *i.c.v.* injected with 0.5 and 5 pmol Adonis in saline. Results obtained for 30 and 90 min with or without pretreatment of mice with *i.p.* injection of 10 μg caffeine are the mean values ± SD. Statistical differences between groups (at least $p < 0.05$) are indicated.

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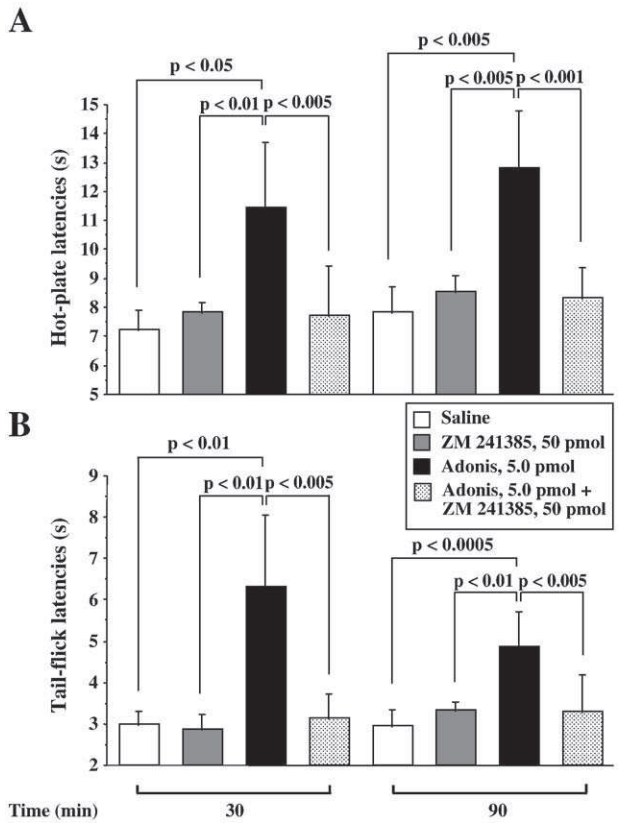

Fig. 4. Blocking effect of ZM 241385 on Adonis-mediated antinociception. Latencies in the hot-plate (A) and the tail-flick (B) tests were performed in mice ($n = 9$ per group) *i.c.v.* injected with 5 pmol Adonis and 50 pmol ZM 241385, alone or together, in saline. Results obtained for 30 and 90 min are the mean values ± SD. Statistical differences between groups (at least $p < 0.05$) are indicated.

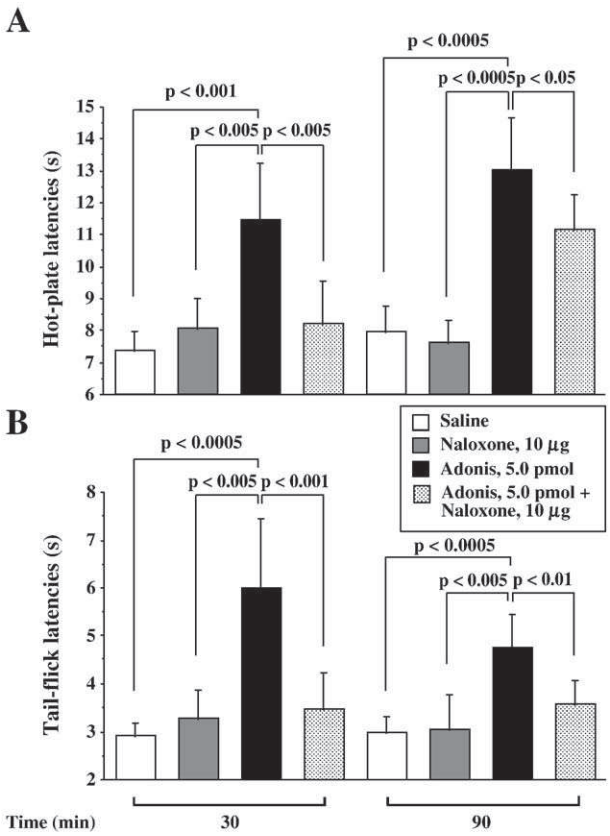

Fig. 5. Blocking effect of Naloxone on Adonis-mediated antinociception. Latencies in the hot-plate (A) and the tail-flick (B) tests were performed in mice ($n = 9$ per group) *i.c.v.* injected with 5 pmol Adonis in saline. Results obtained for 30 and 90 min with or without pretreatment of mice with *i.p.* injection of 10 µg naloxone are the mean values ± SD. Statistical differences between groups (at least $p < 0.05$) are indicated.

effect of caffeine to pain is mediated via blockade of the $A_{2A}R$. Finally, we found that naloxone, a non specific antagonist of opioid receptors, partly prevent Adonis antinociceptive effects in the hot plate (Fig. 5A) and the tail-flick (Fig. 5B) tests, suggesting that endogenous opioids release is involved in the antinociceptive effects of Adonis.

3.3. Possible antinociceptive mechanism of Adonis

$A_{2A}R$ are able to couple to numerous systems. Recent studies strongly suggest a predominant role of $A_{2A}R$ in sleep and locomotor behavior in mice. For example, it was reported that stimulation of $A_{2A}R$ using its agonist CGS 21680 but not CPA (A_1R agonist) infused into the lateral ventricle of mice, induces both non-rapid and rapid eye movement sleep in a dose- and time-dependent manner (Urade et al., 2003). Another report suggested that caffeine stimulates locomotion of mice through $A_{2A}R$ blockade (El Yacoubi et al., 2000). Thus, the question arises if Adonis could act on sleepiness rather than on nociceptive pathways. However, in our experimental protocol and with the picomole doses of Adonis used, we did not observe any sleepiness nor, in contrast, excitability promoting effect in Adonis injected mice which behaved as controls injected with saline.

It is likely that Adonis acts on the motor function through basal ganglia and we cannot exclude that part of Adonis-induced latencies increase may be due to motor disturbances. The literature is quite convincing that $A_{2A}R$ have a prominent role in basal ganglia neurons involved in motor function, and it has even been suggested that $A_{2A}R$ antagonists could be used as a treatment for decreased motor function in Parkinson disease patients (Chen et al., 2007). However, the fact that Adonis effects were prevented by naloxone strongly suggests that Adonis acts, at least in part, on nociceptive function via the opioid system.

High nociceptive threshold was previously observed in patients with Parkinson disease (Guieu et al., 1992). In this neurodegenerative disease with altered motor function, high extracellular adenosine level and high $A_{2A}R$ expression were reported (Varani et al., 2010), suggesting that $A_{2A}R$ are coupled with the dopaminergic system. Interestingly, patients with Parkinson disease have high nociceptive thresholds that are sometimes reversed by naloxone (Guieu et al., 1992), suggesting that $A_{2A}R$ are also linked with an endogenous opioid system.

At the supraspinal level, the periaqueductal gray (PAG) plays a major role in the modulation of nociception through dopamine (Meyer et al., 2009) and opioid receptors (Williams et al., 1995). From our results, and because we used the *i.c.v* route, we cannot exclude that Adonis activates the PAG leading to the release of endogenous opioids, possibly via the cAMP cascade. However, the PAG is not the only area of the brain where endogenous opioids are released and further investigations are needed to specify the Adonis effects on the opioid system. In addition to its putative action on endogenous opioid neurotransmitters, $A_{2A}R$ can also act on cell-membrane channels through its coupled G-subunits independently of the cAMP pathway. As an example, we previously showed that calcium-activated potassium channels (SKCa) but not voltage-gated potassium (Kv) were involved in alleviating pain through $A_{2A}R$ stimulation by CGS 21680 (Regaya et al., 2004). Finally, another possibility is that $A_{2A}R$ couples to different transducing pathways as a function of their heteromerization with different purinergic and dopaminergic receptors (Cunha, 2005; Fredholm et al., 2005).

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3.4. Conclusion

Although one cannot rule out the possibility of Adonis exerting some of its effects on motor function via basal ganglia, it is possible that antinociceptive effects of Adonis would be due, at least in part, to the release of endogenous opioids. Therefore, Adonis strengthens the role of $A_{2A}R$ in pain control and opens an exciting opportunity to manage pain by manipulating the $A_{2A}R$ pathways. For example, it would be valuable to test Adonis under spinal administration in neuropathic animals (Loram et al., 2009). Interestingly, Adonis is a mouse monoclonal antibody and it can be humanized through molecular biology strategies like CDR grafting (Hou et al., 2008) and chain shuffling (Christensen et al., 2009) to fit characteristics of a therapeutic drug.

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3. Conclusion de l'article 4

Nous avons montré qu'Adonis augmente significativement les temps de latence mesurés par les tests Hot-plate et Tail-flick, chez la souris, et que cette augmentation est, au moins en partie, réduite par des antagonistes plus ou moins spécifiques des $A_{2A}R$, la caféine et ZM24385. D'autre part, cet effet anti-nociceptif est également réduit lors de l'administration de naloxone, un antagoniste non spécifique des récepteurs opioïdes, ce qui suggère que Adonis agit, au moins en partie, via la libération d'opioïdes endogènes par le système nerveux central.

Adonis, par l'activation des $A_{2A}R$, exerce un contrôle puissant sur les messages nociceptifs et ouvre une nouvelle opportunité dans le traitement de la douleur. Dans le cadre d'une collaboration, nous proposons également de tester Adonis sur un modèle de rat neuropathique (Loram et al., 2009). Adonis, étant un anticorps monoclonal de souris, pourrait être humanisé pour être compatible avec une utilisation chez l'homme par des stratégies de biologie moléculaire.

V. Évaluation biologique de nouveaux agonistes et antagonistes d'intérêt thérapeutique du récepteur A_1 de l'adénosine

1. Introduction

Les A_1R sont exprimés dans presque tous les tissus de l'organisme comme le cœur, le cerveau, la moelle épinière, les tissus adipeux et les reins. Les effets de la modulation des A_1R pourraient avoir une application thérapeutique surtout dans les domaines cardiovasculaire et neurologique. Des investissements importants aussi bien au niveau privé, de la part de beaucoup de laboratoires pharmaceutiques, qu'au niveau public, pour la recherche dans le domaine de la pharmacologie des A_1R, ont conduit à la découverte de molécules d'intérêt clinique potentiel, qui sont soit des agonistes, soit des antagonistes. Ainsi les agonistes des A_1R pourraient être utilisés dans les douleurs neuropathiques ou certains troubles du rythme cardiaque, tandis que les antagonistes pourraient trouver leur place comme diurétiques, dans l'insuffisance cardiaque ou encore certains troubles du rythme cardiaque.

Ayant acquis des compétences dans l'évaluation des activités biologiques de nouvelles molécules potentiellement intéressantes sur le plan thérapeutique et disposant de cellules transfectées sur-exprimant les récepteurs A_1R, nous avons tout naturellement collaboré avec nos collègues chimistes dans le développement de nouveaux dérivés agonistes ou antagonistes des A_1R dans le cadre d'un projet « NOVOPIOIDES » de l'Agence Nationale de la Recherche (ANR). Dans ce projet, je me suis particulièrement investi dans les tests *in vitro,* sur culture cellulaire, et *in vivo,* sur l'animal, pour déterminer les activités biologiques de ces molécules après leurs synthèses et leurs purifications qui relèvent du domaine d'expertise de nos collaborateurs chimistes. Mon rôle a été, pour l'essentiel, de tester l'affinité des molécules synthétisées pour les A_1R et de mesurer leurs effets cellulaires (production d'AMPc). Chez l'animal j'ai pu tester les propriétés antalgiques de certains de ces dérivés.

La meilleure approche de synthèse des agonistes, rapportée dans la littérature, repose sur la modification en N^6 de l'adénosine ou l'introduction d'une fonction spécifique

en position-2 du noyau d'adénine et/ou en position-3', 4', 5' du groupement ribose (Angus et al., 1971). Par ailleurs, les antagonistes dérivent normalement des xanthines comme la caféine ou la théophylline (Silinsky, 2004). Cependant, l'approche utilisant l'adénosine est limitée par la présence de l'ADA qui convertit de façon irréversible l'adénosine en l'inosine, par désamination sélective en C-6. Pour surmonter ce problème, les chimistes ont choisi de débuter la synthèse à partir d'analogues résistants à l'ADA qui sont des analogues de l'inosine. Dans ce travail, les chimistes ont utilisé la « click chemistry ». Il s'agit d'un concept développé par *K.B Sharpless* qui consiste, par imitation du métabolisme des organismes vivants, à assembler de petites unités chimiques puis à les « clicker » d'un seul coup (Kolb et al., 2001). Ce procédé permet aussi l'assemblage rapide de molécules complexes. C'est la première fois que ce procédé est utilisé pour synthétiser des agonistes ou des antagonistes des A_1R.

Après la synthèse, ces dérivés sont testés, d'une part, pour leur activité fonctionnelle sur le récepteur en terme de production d'AMPc sur les lignées sur-exprimant les A_1R et, d'autre part, pour déterminer leur affinité en compétition avec leurs ligands spécifiques connus (DPCPX, CPA).

Ce travail est détaillé dans l'article « **Expeditious Synthesis and Biological Evaluation of New C-6 1,2,3-Triazole Adenosine Derivatives A_1 Receptor Antagonists or Agonists** » ci-dessous.

Publication 5

"Expeditious synthesis and biological evaluation of new C-6 1,2,3-triazole adenosine derivatives A_1 receptor antagonists or agonists"

Smitha C. Mathew, **Youlet By**, Aurélie Berthault, Marie-Alice Virolleaud, Louis Carrega, Gaëlle Chouraqui, Laurent Commeiras, Jocelyne Condo, Mireille Attolini, Anouk Gaudel-Siri, Jean Ruf, Jean Rodriguez, Jean-Luc Parrain and Régis Guieu

Organic & Biomolecular Chemistry, 2010 Sep 7; 8(17): 3874-81

Expeditious synthesis and biological evaluation of new C-6 1,2,3-triazole adenosine derivatives A1 receptor antagonists or agonists†

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The synthesis of new C-6 1,2,3-triazole adenosine derivatives *via* microwave assisted 1,3-dipolar cycloaddition as key step is described. The binding on membranes of cells that over express A_1 adenosine receptors (A_1AR) was also evaluated. Among them, four compounds increased cAMP production, in a dose-dependent manner acting as antagonists of the A_1AR, while two compounds act as agonists.

Introduction

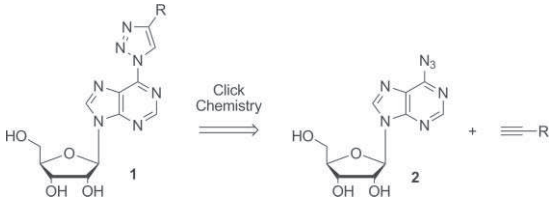
Adenosine, an ubiquitous nucleoside that comes from the dephosphorylation of ATP, acts on G protein-coupled adenosine receptors (AR), named A_1, A_{2A}, A_{2B} and A_3, depending on the pharmacological properties of the receptor subtypes.[1] Each AR has a specific tissue distribution, ligand affinity, and signal transduction mechanism. Because of their presence on nearly every cell type, adenosine receptors have been obvious targets for the development of new drugs for more than three decades.[2] The A_1 adenosine receptors (A_1AR), highly expressed in heart, brain, dorsal spinal cord, adipose tissue and kidney,[3] particularly raised our attention. According to the recent literature the best approach for the discovery of new AR agonists consists of N^6 modifications or introduction of selective functionalization at 2-position of the adenine moiety and at the 3′-, 4′- or 5′-position of the ribose part. Among them N^6-chlorocyclopentyladenosine (CCPA) and N^6-cyclopentyladenosine (CPA) have been reported as being highly selective A_1AR agonists.[4] On the other hand, reported AR antagonists are usually derivatives of xanthines such as caffeine or theophylline.[5] Thus, having drugs that modulate A_1AR at one's disposal could be interesting in different therapeutic areas, including heart failure, diuresis,[6] heart arrhythmia,[7] glucose tolerance,[8] cognitive diseases[9] and contrast media-induced acute renal failure.[10]

Nevertheless, the modified adenosine approach is hampered by the presence of adenosine deaminase (ADA) involved in purines metabolism that irreversibly converts adenosine to the less active inosine nucleoside by selective C-6 deamination. To circumvent this drawback, the design of new analogues resistant to ADA bearing a non metabolisable C-6 amino group is needed. In this context, we propose an expeditious approach based on click-chemistry of specifically functionalized nucleoside analogues starting from commercially available inosine.

New methods for the synthesis of C6 purine derivatives have been widely developed for two decades. Advances in this field employ nucleophilic displacement (S_NAr),[11] and the area of organometallic cross-coupling[12] such as Suzuki–Miyaura,[13] Sonogashira,[14] Stille,[15] and Buchwald–Hartwig[16] coupling reactions. Alternatively, the discovery[17] of the dramatic acceleration of the copper(I) mediated Huisgen 1,3-dipolar cycloaddition of alkyne and azides to yield 1,2,3-triazoles has generated the synthesis of a plethora of new compounds and has accelerated advances in areas ranging from drug discovery to material sciences.[18] Although a number of 1,2,3-triazole nucleoside derivatives has been described,[19] most involve introduction of 1,2,3-triazole at C2,[20] and C8[21] or at the sugar moiety.[22] To the best of our knowledge, when we started the project, this "click reaction" was unexploited for the synthesis of N^6-aminopurine derivatives.[23] As part of our ongoing research towards the synthesis of biologically active adenosine derivatives,[24] we have developed this approach for a rapid access to new N^6 nucleoside derivatives. We describe herein the synthesis, the A_1 binding affinities and the biological evaluation of a novel series of new C-6 1,2,3-triazole substituted purine derivatives **1** *via* microwave assisted 1,3-dipolar cycloaddition.

Results and discussion

As outlined retrosynthetically in Scheme 1, C-6 triazole substituted adenosines **1** could be readily obtained by "click chemistry" between C-6 azido adenosine **2** and different substituted alkynes. Our investigation started with the synthesis of 1,3-dipolar partner **2** (Scheme 2). As described in the literature,[25,26] C-6 azido



Scheme 1 Retrosynthetic strategy for the synthesis of **1**.

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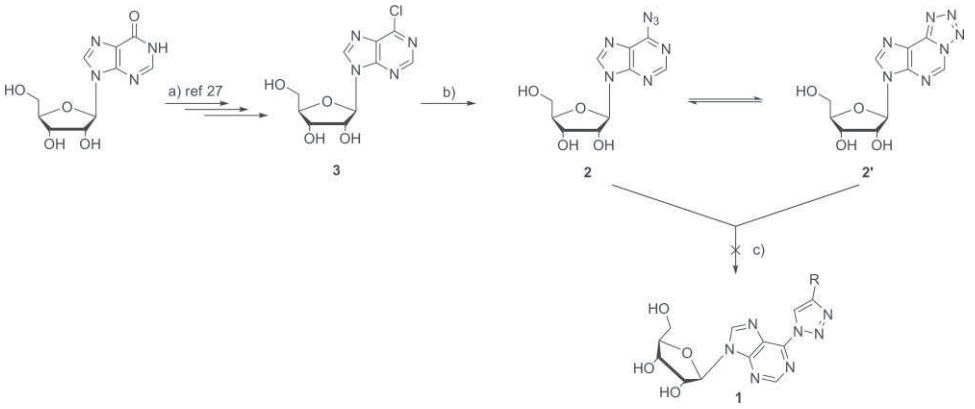
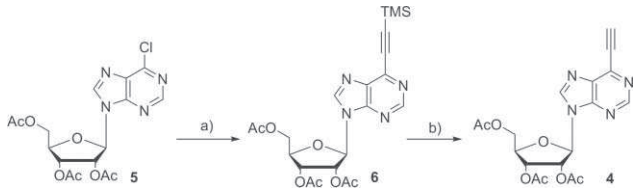


Scheme 2 Preparation of C-6 azido adenosine **2**. *Reagents and conditions*: (a) three steps, 90% overall yield; (b) LiN_3, DMF, rt, 2 days (30%); (c) phenylacetylene, conditions A: $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF, 80 °C, 12 h (45% of adenosine), conditions B: $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF, MW, 100 °C, 30 min (80% of adenosine), conditions C: $CuSO_4{\cdot}5H_2O$, sodium ascorbate, tBuOH : H_2O, rt, 2 h (75% of adenosine) or conditions D: Cu wire, CH_3CN–H_2O, 35 °C, 12 h (65% of adenosine).

adenosine **2** can be synthesized *via* nucleophilic displacement reaction of the electrophilic nucleoside **3** by sodium azide. 6-Chloropurine riboside (**3**) was prepared in three steps (90% overall yield) according to a previously reported procedure from the commercially available inosine.[27]

However, the S_NAr reaction of **3** using the reported conditions did not furnish the corresponding azide **2**. Despite numerous attempts to modify the reaction conditions (lower temperature, microwave irradiation or protection of the alcohol function on the sugar part as an acetate or acetal), **2** was always detected only in trace amount. Finally replacement of sodium azide by lithium azide (DMF, rt, 2 days)[28] led, in 30% yield, to the formation of **2** in equilibrium with its tetrazolo tautomeric form **2′**.[25,28,29]

Even if **2** was obtained in low yield, the copper(I)-mediated Huisgen 1,3-dipolar cycloaddition was then investigated (Scheme 2). Phenylacetylene was chosen as a benchmark substrate and the reaction was initially performed with sodium ascorbate, $CuSO_4{\cdot}5H_2O$ in DMF for 12 h at 80 °C (conditions A). Unfortunately, the desired 1,4-disubstituted 1,2,3-triazole **1** (R=Ph) was never observed. Adenosine, resulting from the reduction of the azido function of **2** into the corresponding amine, was isolated in 45% yield as the main reaction product. This kind of reduction was already described by Frieden *et al.* and can be explain by a thermal decomposition of the azido function to the corresponding nitrene, which evolved into the amine formation.[25] Whatever the conditions used to perform the 1,3-dipolar cycloaddition (Scheme 2, conditions B: $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF, MW, 100 °C, 30 min, conditions C: $CuSO_4{\cdot}5H_2O$, sodium ascorbate, tBuOH : H_2O, rt, 2 h or conditions D: Cu wire, CH_3CN–H_2O, 35 °C, 12 h), adenosine was always the sole product obtained (65–80%). The presence of the C-6 tretrazolyl tautomer could explain the lack of reactivity in the [3+2]-cycloaddition process. This observation drove us to envision a new strategy. Instead of installing the azide function on the C-6 position of the adenosine moiety, it was decided to prepare the corresponding C-6 acetylene **4**. The readily available protected chloroinosine **5** (Scheme 3) underwent a smooth Sonogashira coupling with TMS-acetylene. The installation of the triple bond at the C-6 position of the adenosine derivative was accomplished in 69% yield.[30] Deprotection of the TMS group was performed



Scheme 3 Synthesis of **4**. *Reagents and conditions*: (a) $PdCl_2(PPh_3)_2$, CuI, NEt_3, DMF, 60 °C, 3 h (69%); (b) TBAF, THF, 0 °C, 20 min (43%).

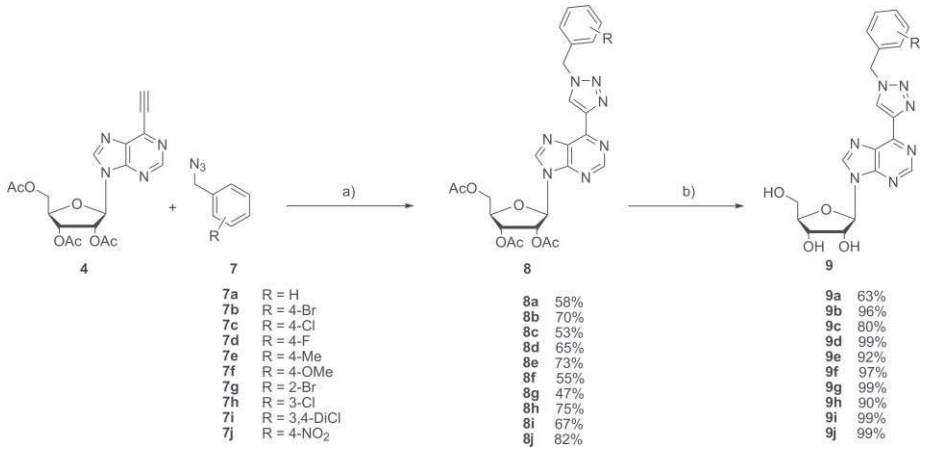
with tetrabutylammonium fluoride[31] to yield the terminal alkyne **4** in 43% yield.

The Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction was next studied. In an initial experiment (Scheme 4), the alkynyl derivative **4** was treated with benzyl azide **7a** in dry DMF at 90 °C in the presence of copper(II) and sodium ascorbate under microwave conditions for 30 min. We were pleased to find that these conditions afforded the triazole derivative **8a** in 58% yield thus validating this new approach (Scheme 4). In order to examine the scope of this approach, the [3+2]-cycloaddition was then performed with a range of different azides **7b–j** (Scheme 4). The latter were prepared according to a safe literature procedure developed by Varma *et al.*[32] based on a nucleophilic substitution reaction of alkyl halides by sodium azide in water using microwave-assisted heating. The 1,3-dipolar cycloaddition has then allowed the formation of several new C-6 1,2,3-triazole adenosine derivatives **8b–j** in good yields (up to 82%) and with a total regioselectivity.

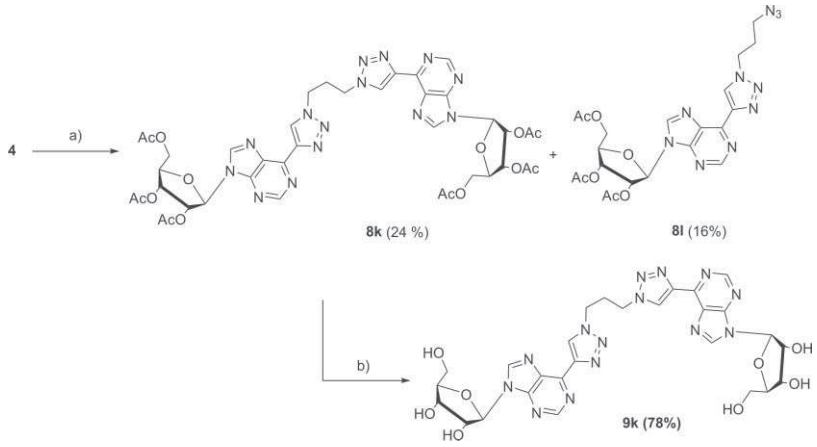
Interestingly, when the reaction was performed with 1,3-diazidopropane **7k**, a separable mixture of bis-C6-1,2,3-triazole bis-adenosine **8k** and mono cycloadduct derivative **8l** were obtained in moderate yield (respectively 24 and 16%, Scheme 5). At last, deprotection of the acetate functions of the sugar part under basic condition (NH_3, MeOH) led to the formation of the corresponding compounds **9a–k** in excellent yields (up to 99%, Scheme 4 and 5).

Biological evaluation

These new C-6 1,2,3-triazole adenosine derivatives **9a–k** were then submitted to biological evaluation. The ability of these



Scheme 4 Preparation of C-6 1,2,3-triazole substituted adenosines **9**. *Reagents and conditions*: (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF, μW, 90 °C, 30 min; (b) NH_3, MeOH, rt, 12 h.



Scheme 5 Reactivity of **4** with 1,3-diazidopropane. (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF, μW, 90 °C, 30 min, 1,3-diazodopropane **7k**; (b) NH_3, MeOH, rt, 12 h.

unprecedented molecules to modulate cAMP production in mammalian Chem-3 cells that over expressed A_1ARs was first tested. Indeed, agonists of A_1AR are believed to decrease the cAMP production whereas antagonists display the opposite effect.

As a matter of fact **9e**, **9f**, **9h** and **9i** increased cAMP production in cell culture, in a dose-dependent manner acting as antagonists of the A_1AR (Table 1), while **9j** and **9k** decreased cAMP production acting as agonists (Table 1). The IC_{50} of compounds was also evaluated, using binding assay on cell membranes. IC_{50} of antagonist compounds range between 0.29 μM and 1.5 μM and between 11 μM and 42 μM for agonist compounds (Table 1). Even if these molecules have weaker affinity when compared to the well known A_1AR antagonist DPCPX, their biological effects occur at reasonable concentrations.

Compounds **9a**, **9b**, **9c**, **9d** and **9g** had an IC_{50} for A1AR binding >100 μM and all compounds tested had an IC_{50} for A_{2A} adenosine receptor binding >50 μM (data not shown). While nitroaromatic compounds have been reported to have A_1AR antagonist properties,[33] interestingly, **9j** exhibits agonist properties. It is also interesting to note that nitroaromatic compounds were known to act *via* the inhibition of NO synthase and cGMP modulation.[34]

Conclusions

In summary we have reported the synthesis of new C-6 1,2,3-triazole adenosine derivatives *via* microwave assisted 1,3-dipolar cycloaddition as key step. Among them, four compounds expressed antagonist properties, while two expressed agonist properties, basing on cAMP production on A_1AR. Their biological effects were obtained at reasonable concentrations.

Experimental

All reactions sensitive to oxygen and moisture were carried out in oven-dried glassware under a slight positive pressure of argon unless otherwise noted. Reagents and solvents were commercial grades and were used as supplied. *N*,*N*-dimethylformamide was distilled from calcium hydride and stored over calcium hydride. THF was obtained from a Solvent Purification System. Triethylamine was distilled from KOH prior to use. Analytical thin layer chromatography (TLC) was performed on Merck precoated analytical plates, 0.25 mm thick, silica gel 60 F254. Flash column chromatography was performed on Merck Kieselgel 40–63 mm. ^{1}H NMR and ^{13}C NMR spectra were recorded on an AC300 using the deuterated solvent as internal deuterium lock. Chemical

Table 1 Effects of compounds on cAMP production and on [^{3}H] DPCPX displacement or [^{3}H] CPA displacement on Chem 3 cells that overexpress the A1 AR. IC_{50} for cAMP inhibition was defined as the concentration of compound that induces half of the maximal cAMP inhibition. EC_{50} for cAMP production was defined as the concentration of compound that induces half of the maximal cAMP production. IC_{50} for DPCPX or CPA displacement was defined as the concentration of competing ligand which displaces 50% of [^{3}H] DPCPX or [^{3}H] CPA used as specific radioligand

	cAMP production		[^{3}H]DPCPX displacement	[^{3}H]CPA displacement	
Compound	EC_{50}/M	IC_{50}/M	IC_{50}/M	IC_{50}/M	Properties
9a			$>10^{-5}$		—
9b			$>10^{-5}$		—
9c			$>10^{-5}$		—
9d			$>10^{-5}$		—
9e	$5.5 \pm 0.9\ 10^{-7}$		$2.9 \pm 0.8\ 10^{-7}$		Antagonist
9f	$7 \pm 2\ 10^{-7}$		$15 \pm 4\ 10^{-7}$		Antagonist
9g			$>10^{-5}$		—
9h	$6 \pm 2\ 10^{-6}$		$6 \pm 1.8\ 10^{-7}$		Antagonist
9i	$7 \pm 2\ 10^{-7}$		$4 \pm 2.9\ 10^{-7}$		Antagonist
9j		$1 \pm 0.6\ 10^{-6}$		$42 \pm 11\ 10^{-6}$	Agonist
9k		$0.5 \pm 0.3\ 10^{-6}$		$11 \pm 4\ 10^{-6}$	Agonist
DPCPX	$3.8 \pm 0.5\ 10^{-9}$		$4 \pm 0.8\ 10^{-9}$		Antagonist

shift data are given in units δ relative to residual protic solvent where δ(chloroform) = 7.26 ppm, δ(DMSO) = 2.50 ppm and δ(methanol) = 3.31 ppm The multiplicity of a signal is indicated as: br – broad, s – singlet, d – doublet, t – triplet, q – quartet, m – multiplet, dd – doublet of doublets, *etc.* Coupling constants (J) are quoted in Hz and recorded to the nearest 0.1 Hz. ^{13}C NMR spectra were recorded on an AC300 spectrometer using the deuterated solvent as internal deuterium lock. Chemical shift data are given in units δ relative to residual protic solvent where δ(chloroform) = 77.16 ppm, δ(DMSO) = 39.52 ppm and δ(methanol) = 49.00 ppm. High resolution mass spectra (HRMS) have been performed using a mass spectrometer equipped with a pneumatically assisted atmospheric pressure ionization. The sample was ionized in positive mode electrospray.

(2*R*,3*R*,4*S*,5*R*)-2-(6-azido-9*H*-purin-9-yl)-5-(hydroxymethyl)-tetrahydrofuran-3,4-diol 2

To a stirred solution of chloroinosine **3** (400 mg, 1.38 mmol) in dry DMF was added lithium azide (750 mg, 15.2 mmol). The reaction was stirred at room temperature for 60 h then the solvent was removed under vacuum. The crude product was recrystallized from methanol to give **2** (120 mg, 30%). δ_H(300 MHz, DMSO) 10.14 (s, 1H), 8.93 (s, 1H), 6.15 (d, J = 5.1 Hz, 1H), 5.66 (d, J = 5.9 Hz, 1H), 5.33 (d, J = 5.3 Hz, 1H), 5.14 (appt, J = 5.3 and 5.5 Hz, 1H), 4.57 (q, J = 5.3 Hz, 1H), 4.21 (q, J = 4.7 Hz, 1H), 4.02 (q, J = 4.0 Hz, 1H), 3.76–3.69 (m, 1H), 3.65–3.57 (m, 1H); δ_C(75 MHz, DMSO) 145.4 (C), 142.6 (CH), 142.0 (C), 136.1 (CH), 120.3 (C), 88.4 (CH), 85.8 (CH), 74.6 (CH), 70.1 (CH), 61 (CH_2).

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-((trimethylsilyl)ethynyl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 6

To a purged argon solution of chloroinosine 3 (1.1 g, 2.6 mmol), trimethylsilyl acetylene (0.6 mL, 3.1 mmol), $PdCl_2(PPh_3)_2$ (54 mg, 0.078 mmol) and copper iodide (30 mg, 0.156 mmol) in DMF was added triethylamine (1 mL, 8.4 mmol). The mixture was then heated at 60 °C for 3 h and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (1/1 pet. ether–ethyl acetate) to afford **6** (800 mg, 69%). δ_H(300 MHz, $CDCl_3$) 8.85 (s, 1H), 8.24 (s, 1H), 6.17 (d, J = 5.3 Hz, 1H), 5.88 (appt, J = 5.5 and 5.3 Hz, 1H), 5.58 (br appt, J = 4.9 and 5.1 Hz, 1H), 4.41–4.26 (m, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H), 0.25 (s, 9H); δ_C(75 MHz, $CDCl_3$) 170.2 (C), 169.5 (C), 169.3 (C), 153.7 (CH), 151.3 (C), 143.9 (CH), 141.7 (C), 134.9 (C), 106.0 (C), 98.2 (C), 86.5 (CH), 80.4 (CH), 73.0 (CH), 70.6 (CH), 62.9 (CH_2), 20.7 (CH_3), 20.5 (CH_3), 20.3 (CH_3), –0.5 (3 × CH_3); HRMS (ESI) calcd for $C_{21}H_{27}N_4O_7Si$ $[M+H]^+$: 475.1644 found 475.1642

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-ethynyl-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 4

To a stirred solution of 6 (1.5 g, 3.1 mmol) in THF (20 mL) at 0 °C was added dropwise TBAF (3.4 mL, 3.4 mmol, 1M in THF) and stirred for 20 min. The reaction mixture was quenched with saturated aqueous ammonium chloride solution and the solvent was removed under vacuum. The residue was extracted with dichloromethane, dried over sodium sulfate then concentrated. The resulting mixture was purified by column chromatography on silica gel (94/6 dichloromethane–methanol) to give **4** (550 mg, 43%). δ_H(300 MHz, $CDCl_3$) 8.78 (s, 1H), 8.25 (s, 1H), 6.12 (d, J = 5.1 Hz, 1H), 5.82 (br appt, J = 5.5 and 5.3 Hz, 1H), 5.51 (br appt, J = 5.1 and 5.3 Hz, 1H), 4.33–4.17 (m, 3H), 3.73 (s, 1H), 1.97 (s, 3H), 1.92 (s, 3H), 1.90 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.0 (C), 169.3 (C), 169.1 (C), 152.4 (CH), 151.0 (C), 144.2 (CH), 140.7 (C), 135.2 (C), 86.6 (C), 86.3 (CH), 80.1 (CH), 77.6 (CH), 72.8 (CH), 70.2 (CH), 62.7 (CH_2), 20.4 (CH_3), 20.2 (CH_3), 20.1 (CH_3); HRMS (ESI) calcd for $C_{18}H_{19}N_4O_7$ $[M+H]^+$: 403.1248 found 403.1241

General procedure for the synthesis of compounds 8a–l.

A mixture of acetylene **4** (70 mg, 0.17 mmol), benzyl azide (0.24 mmol), sodium ascorbate (10 mg, 0.051 mmol) and copper sulfate pentahydrate (2 mg) in dry DMF (2 mL) was subjected to microwave irradiation for 30 min at a temperature of 90 °C. The reaction mixture was then concentrated, extracted with dichloromethane and the residue was purified by column chromatography using dichloromethane–methanol as eluent to afford the triazole derivatives **8a–l**.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-benzyl-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8a. δ_H(300 MHz, $CDCl_3$) 9.00 (s, 1H), 8.64 (s, 1H), 8.24 (s, 1H), 7.40–7.31 (m, 5H), 6.25 (d, $J = 5.3$ Hz, 1H), 5.95 (t, $J = 5.5$ Hz, 1H), 5.68–5.65 (m, 3H), 4.47–4.32 (m, 3H), 2.13 (s, 3H), 2.09 (s, 3H), 2.04 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.4 (C), 153.2 (CH), 151.8 (C), 148.0 (C), 143.9 (C), 143.2 (CH), 134.4 (C), 130.3 (C), 129.3 (2 × CH), 129.0 (CH), 128.2 (2 × CH), 126.9 (CH), 86.5 (CH), 80.5 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 54.5 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.4 (CH_3); HRMS (ESI) calcd for $C_{25}H_{26}N_7O_7$ $[M+H]^+$: 536.1888 found 536.1878.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(4-bromobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8b. δ_H(300 MHz, $CDCl_3$) 8.99 (s, 1H), 8.65 (s, 1H), 8.24 (s, 1H), 7.48 (d, $J = 8.5$ Hz, 2H), 7.18 (d, $J = 8.5$ Hz, 1H), 6.24 (d, $J = 5.1$ Hz, 1H), 5.95 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.66 (br dd, $J = 5.3$ and 4.5 Hz, 1H), 5.60 (s, 2H), 4.47–4.28 (m, 3H), 2.12 (s, 3H), 2.08 (s, 3 H), 2.04 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 170.3 (C), 169.6 (C), 169.4 (C), 153.2 (CH), 151.8 (C), 147.8 (C), 144.0 (C), 143.3 (CH), 133.4 (C), 132.4 (2 × CH), 130.3 (C), 129.8 (2 × CH), 126.9 (CH), 123.1 (C), 86.5 (CH), 80.5 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 53.7 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.4 (CH_3); HRMS (ESI) calcd for $C_{25}H_{25}BrN_7O_7$ $[M+H]^+$: 614.0993 found 614.0994.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(4-chlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8c. δ_H(300 MHz, $CDCl_3$) 9.02 (s, 1H), 8.65 (s, 1H), 8.25 (s, 1H), 7.35 (d, $J = 8.7$ Hz, 2H), 7.26 (d, $J = 8.7$ Hz, 1H), 6.26 (d, $J = 5.3$ Hz, 2H), 5.96 (t, $J = 5.5$ Hz, 1H), 5.68 (br dd, $J = 5.5$ and 4.5 Hz, 1H), 5.64 (s, 2H), 4.49–4.34 (m, 3H), 2.14 (s, 3H), 2.10 (s, 3 H), 2.06 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 170.0 (C), 169.5 (C), 153.3 (CH), 151.8 (C), 147.9 (C), 144.1 (C), 143.3 (CH), 135.1 (C), 132.9 (C), 130.4 (C), 129.6 (2 × CH), 129.5 (2 × CH), 126.8 (CH), 86.6 (CH), 80.6 (CH), 73.3 (CH), 70.7 (CH), 63.1 (CH_2), 53.8 (CH_2), 20.9 (CH_3), 20.6 (CH_3), 20.5 (CH_3); HRMS (ESI) calcd for $C_{25}H_{25}ClN_7O_7$ $[M+H]^+$: 570.1499 found 570.1499.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(4-fluorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8d. δ_H(300 MHz, $CDCl_3$) 9.00 (s, 1H), 8.64 (s, 1H), 8.24 (s, 1H), 7.33–7.29 (m, 2H), 7.07–7.01 (m, 2H), 6.26 (d, $J = 5.3$ Hz, 1H), 5.95 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.67 (br dd, $J = 5.5$ and 4.5 Hz, 1H), 5.62 (s, 2H), 4.48–4.34 (m, 3H), 2.13 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.4 (C), 163.0 (d, $J = 248.7$ Hz, C), 153.2 (CH), 151.8 (C), 147.9 (C), 144.0 (C), 143.3 (CH), 130.3$_4$ (C), 130.3 (d, $J = 3.3$ Hz, C), 130.1 (d, $J = 8.2$ Hz, 2 × CH), 126.8 (CH), 116.3 (d, $J = 22.0$ Hz, 2 × CH), 86.5 (CH), 80.5 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 53.7 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.4 (CH_3); HRMS (ESI) calcd for $C_{25}H_{25}FN_7O_7$ $[M+H]^+$: 554.1794 found 554.1793.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(4-methylbenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8e. δ_H(300 MHz, $CDCl_3$) 9.01 (s, 1H), 8.62 (s, 1H), 8.24 (s, 1H), 7.23 (d, $J = 8.3$ Hz, 2H), 7.17 (d, $J = 8.3$ Hz, 2H), 6.26 (d, $J = 5.3$ Hz, 1H), 5.95 (t, $J = 5.5$ Hz, 1H), 5.68 (br dd, $J = 5.5$ and 4.5 Hz, 1H), 5.62 (s, 2H), 4.48–4.34 (m, 3H), 2.33 (s, 3H), 2.15 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 153.3 (CH), 151.8 (C), 148.1 (C), 144.0 (C), 143.1 (CH), 139.0 (C), 131.4 (C), 130.4 (C), 130.0 (2 × CH), 128.3 (2 × CH), 126.8 (CH), 86.5 (CH), 80.6 (CH), 73.3 (CH), 70.7 (CH), 63.1 (CH_2), 53.4 (CH_2), 21.3 (CH_3), 20.9 (CH_3), 20.6 (CH_3), 20.5 (CH_3); HRMS (ESI) calcd for $C_{26}H_{28}N_7O_7$ $[M+H]^+$: 550.2045 found 550.2044.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(4-methoxybenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8f. δ_H(300 MHz, $CDCl_3$) 9.00 (s, 1H), 8.60 (s, 1H), 8.24 (s, 1H), 7.28 (d, $J = 8.7$ Hz, 2H), 6.89 (d, $J = 8.7$ Hz, 2H), 6.26 (d, $J = 5.3$ Hz, 2H), 5.95 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.67 (br dd, $J = 5.5$ and 4.5 Hz, 1H), 5.59 (s, 2H), 4.48–4.34 (m, 3H), 3.78 (s, 3H), 2.14 (s, 3H), 2.10 (s, 3H), 2.05 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 160.1 (C), 155.6 (C), 153.2$_4$ (CH), 153.1$_9$ (C), 151.8 (C), 143.2 (CH), 129.8 (2 × CH), 126.6 (CH), 126.4 (2 × C), 114.7 (2 × CH), 86.5 (CH), 80.5 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 55.4 (CH_3), 54.1 (CH_2), 20.9 (CH_3), 20.6 (CH_3), 20.5 (CH_3); HRMS (ESI) calcd for $C_{26}H_{28}N_7O_8$ $[M+H]^+$: 566.1994 found 566.1993.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(2-bromobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8g. δ_H(300 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.74 (s, 1H), 8.26 (s, 1H), 7.62 (br d, $J = 7.9$ Hz, 1H), 7.33–7.19 (m, 3H), 6.27 (d, $J = 5.5$ Hz, 1H), 5.96 (t, $J = 5.5$ Hz, 1H), 5.80 (s, 2H), 5.68 (br dd, $J = 5.5$ and 4.4 Hz, 1H), 4.49–4.35 (m, 3H), 2.15 (s, 3H), 2.11 (s, 3 H), 2.06 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 153.3 (CH), 151.9 (C), 148.0 (C), 144.0 (C), 143.3 (CH), 134.0 (C), 133.4 (CH), 130.6 (CH), 130.4 (C and CH), 128.4 (CH), 127.2 (CH), 123.6 (C), 86.5 (CH), 80.6 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 54.2 (CH_2), 20.9 (CH_3), 20.6 (CH_3), 20.5 (CH_3); HRMS (ESI) calcd for $C_{25}H_{25}BrN_7O_7$ $[M+H]^+$: 614.0993 found 614.0992.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(3-chlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8h. δ_H(300 MHz, $CDCl_3$) 9.00 (s, 1H), 8.68 (s, 1H), 8.25 (s, 1H), 7.30–7.28 (m, 3H), 7.19–7.16 (m, 1H), 6.25 (d, $J = 5.3$ Hz, 2H), 5.95 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.67 (br dd, $J = 5.5$ and 4.5 Hz, 1H), 5.63 (s, 2H), 4.47–4.33 (m, 3H), 2.13 (s, 3H), 2.08 (s, 3H), 2.04 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 153.2 (CH), 151.8 (C), 147.8 (C), 144.1 (C), 143.3 (CH), 136.4 (C), 135.1 (C), 130.6 (CH), 130.4 (C), 129.1 (CH), 128.2 (CH), 127.0 (CH), 126.2 (CH), 86.5 (CH), 80.5 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 53.7 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.4 (CH_3); HRMS (ESI) calcd for $C_{25}H_{25}ClN_7O_7$ $[M+H]^+$: 570.1499 found 570.1499.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(3,4-dichlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8i. δ_H(300 MHz, $CDCl_3$) 9.00 (s, 1H), 8.69 (s, 1H), 8.25 (s, 1H), 7.43–7.40 (m, 2H), 7.14 (br dd, $J = 8.3$ and 2.3 Hz, 1H), 6.25 (d, $J = 5.3$ Hz, 1H), 5.95 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.67 (br dd, $J = 5.5$ and 4.7 Hz, 1H), 5.62 (s, 2H), 4.47–4.33 (m, 3H), 2.13 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 153.2 (CH), 151.8 (C), 147.7 (C), 144.2 (C), 143.4 (CH), 134.6 (C), 133.4 (C), 133.2 (C), 131.3 (CH), 130.4 (C), 130.0 (CH), 127.3 (CH), 126.9 (CH), 86.6 (CH), 80.5 (CH), 73.2 (CH), 70.7 (CH), 63.1 (CH_2), 53.1 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.4 (CH_3); HRMS (ESI) calcd for $C_{25}H_{24}N_7O_7Cl_2$ $[M+H]^+$: 604.1109 found 604.1118.

(2*R*,3*R*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(4-nitrobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8j. mp 142.5 °C; δ_H(300 MHz, $CDCl_3$) 9.03 (s, 1H), 8.75 (s, 1H), 8.26 (s, 1H), 8.22 (d, $J = 8.8$ Hz, 2H), 7.48 (d, $J = 8.8$ Hz, 2H), 6.26 (d, $J = 5.1$ Hz, 1H), 5.96 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.79 (s, 2H), 5.68 (br dd, $J = 5.3$ and 4.7 Hz, 1H), 4.49–4.34 (m, 3H), 2.15 (s, 3H), 2.10 (s, 3 H), 2.06 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 153.3 (CH), 151.8 (C), 148.3 (C), 147.6 (C), 144.4 (C), 143.4 (CH), 141.4 (CH), 130.5 (C), 128.8 (2 × CH), 127.2 (CH), 124.5 (2 × CH), 112.9 (C), 86.7 (CH), 80.5 (CH), 73.3 (CH), 70.7 (CH), 63.1 (CH_2), 20.8 (CH_3), 20.6 (CH_3), 20.5 (CH_3); HRMS (ESI) calcd for $C_{25}H_{25}N_8O_9$ $[M+H]^+$: 581.1739 found 581.1743

8k. δ_H(300 MHz, $CDCl_3$) 9.02 (s, 2H), 8.86 (s, 2H), 8.27 (s, 2H), 6.28 (d, $J = 5.3$ Hz, 1H), 5.99 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.70 (br dd, $J = 5.3$ and 4.7 Hz, 1H), 4.62–4.58 (m, 4H), 4.50–4.36 (m, 8H), 2.15 (s, 3H), 2.12 (s, 3 H), 2.08 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 170.5 (2 × C), 169.7 (2 × C), 169.5 (2 × C), 153.3 (2 × CH), 151.8 (2 × C), 147.8 (2 × C), 143.7 (2 × C), 143.4 (2 × CH), 130.5 (2 × C), 127.8 (2 × CH), 86.6 (2 × CH), 80.6 (2 × CH), 73.3 (2 × CH), 70.7 (2 × CH), 61.1 (2 × CH_2), 47.2 (2 × CH_2), 30.7 (CH_2), 20.9 (2 × CH_3), 20.7 (2 × CH_3), 20.5 (2 × CH_3); HRMS (ESI) calcd for $C_{39}H_{43}N_{14}O_{14}$ $[M+H]^+$: 931.3078 found 931.3080

(2*R*,3*S*,4*R*,5*R*)-2-(acetoxymethyl)-5-(6-(1-(3-azidopropyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate 8l. δ_H(300 MHz, $CDCl_3$) 9.04 (s, 1H), 8.77 (s, 1H), 8.28 (s, 1H), 6.28 (d, $J = 5.3$ Hz, 1H), 5.98 (appt, $J = 5.5$ and 5.3 Hz, 1H), 5.68 (br dd, $J = 5.5$ and 4.5 Hz, 1H), 4.60 (t, $J = 6.8$ Hz, 1H), 4.50–4.35 (m, 3H), 3.41 (t, $J = 6.3$ Hz, 2H), 2.31–2.22 (m, 2H), 2.15 (s, 3H), 2.12 (s, 3 H), 2.08 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 170.4 (C), 169.7 (C), 169.5 (C), 153.2 (CH), 151.8 (C), 147.8 (C), 143.5 (C), 143.3 (CH), 130.4 (C), 127.4 (CH), 86.6 (CH), 80.6 (CH), 73.3 (CH), 70.7 (CH), 63.1 (CH_2), 48.1 (CH_2), 47.7 (CH_2), 29.6 (CH_2), 20.9 (CH_3), 20.7 (CH_3), 20.5 (CH_3); HRMS (ESI) calcd for $C_{21}H_{25}N_{10}O_7$ $[M+H]^+$: 529.1902 found 529.1907

General procedure for the synthesis of compounds 9a–9k

A solution of NH_3/MeOH (0.7 mL) was added over triacetate **9** (70 μmol). The mixture was stirred overnight at room temperature and followed by TLC. Once reaction was complete by TLC, the mixture was evaporated under reduced pressure and purified by column chromatography using dichloromethane–methanol (85/15) as eluent to afford the triazole derivatives **9a–9k**.

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-benzyl-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)-5-(hydroxymethyl)-tetrahydrofuran-3,4-diol 9a. mp 76–77 °C; δ_H(300 MHz, DMSO) 9.21 (s, 1H), 8.98 (s, 1H), 8.90 (s, 1H), 7.31–7.50 (m, 5H), 6.07 (d, $J = 5.5$ Hz, 1H), 5.79 (s, 2H), 5.61 (br s, 1H), 5.26 (br s, 1H), 5.15–5.12 (m, 1H), 4.64 (br s, 1H), 4.21 (br s, 1H), 4.99 (br s, 1H), 3.75–3.68 (m, 1H), 3.62–3.56 (m, 1H); δ_C(75 MHz, $CDCl_3$) 151.9 (CH), 150.8 (C), 146.8 (C), 145.3 (CH), 142.8 (C), 134.3 (C), 130.6 (C), 129.3 (2 × CH), 129.1 (CH), 128.2 (2 × CH), 127.4 (CH), 91.5 (CH), 87.7 (CH), 74.0 (CH), 72.6 (CH), 63.2 (CH_2), 54.6 (CH_2); HRMS (ESI) calcd for $C_{19}H_{20}N_7O_4$ $[M+H]^+$ 410.1571 found 410.1570; HPLC purity grade. 254 nm: 98.5%; 300 nm: 100%.

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-(4-bromobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol 9b. mp 126–127 °C; δ_H(300 MHz, DMSO) 9.24 (s, 1H), 8.98 (s, 1H), 8.92 (s, 1H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.38 (d, $J = 8.4$ Hz, 2H), 6.08 (d, $J = 5.5$ Hz, 1H), 5.79 (s, 2H), 5.60 (br s, 1H), 5.29 (br s, 1H), 5.15 (br s, 1H), 4.66 (appt, $J = 5.3$ and 5.1 Hz, 1H), 4.22 (br t, $J = 4.3$ Hz, 1H), 4.00 (br q, $J = 3.8$ Hz, 1H), 3.73 (br dd, $J = 12.1$ and 3.4 Hz, 1H), 3.60 (br dd, $J = 12.1$ and 3.6 Hz, 1H); δ_C(75 MHz, DMSO) 152.2 (CH), 151.6 (C), 146.7 (C), 145.2 (CH), 142.4 (C), 135.3 (C), 131.8 (2 × CH), 130.4 (2 × CH), 129.7 (C), 128.4 (CH), 121.6 (C), 87.8 (CH), 85.7 (CH), 73.7 (CH), 70.2 (CH), 61.2 (CH_2), 52.3 (CH_2); HRMS (ESI) calcd for $C_{19}H_{19}N_7O_4Br$ $[M+H]^+$ 488.0676 found 488.0678; HPLC purity grade. 254 nm: 92%; 300 nm: 99%

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-(4-chlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol 9c. mp 118–119 °C; δ_H(300 MHz, DMSO) 9.24 (s, 1H), 8.98 (s, 1H), 8.91 (s, 1H), 7.53–7.39 (m, 4H), 6.08 (d, $J = 5.5$ Hz, 1H), 5.80 (s, 2H), 5.57 (br s, 1H), 5.25 (br s, 1H), 5.15–5.11 (br t, $J = 5.5$ Hz, 1H), 4.68–4.63 (m, 1H), 4.24–4.19 (m, 1H), 4.02–3.98 (q, $J = 3.8$ Hz, 1H), 3.77–3.68 (m, 1H), 3.63–3.56 (m, 1H); δ_C(75 MHz, DMSO) 152.2 (CH), 151.6 (C), 146.7 (C), 145.1 (CH), 142.4 (C), 134.9 (C), 133.0 (C), 130.1 (2 × CH), 129.7 (C), 128.9 (2 × CH), 128.4 (CH), 87.8 (CH), 85.7 (CH), 73.7 (CH), 70.2 (CH), 61.2 (CH_2), 52.2 (CH_2); HRMS (ESI) calcd for $C_{19}H_{19}N_7O_4Cl$ $[M+H]^+$ 444.1182 found 444.1181; HPLC purity grade. 254 nm: 96%; 300 nm: 99%

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-(4-fluorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol 9d. mp 97–98 °C; δ_H(300 MHz, $CDCl_3$–MeOD = 1/1) 8.88 (s, 1H), 8.77 (s, 1H), 8.50 (s, 1H), 7.40–7.35 (m, 2H), 7.09–7.03 (m, 2H), 6.04 (d, $J = 6.2$ Hz, 1H), 5.65 (s, 2H), 4.80 (br dd, $J = 5.9$ and 5.5 Hz, 1H), 4.38 (dd, $J = 5.1$ and 2.7 Hz, 1H), 4.24–4.22 (m, 1H), 3.93 (dd, $J = 12.7$ and 2.3 Hz, 1H), 3.76 (dd, $J = 12.7$ and 2.3 Hz); δ_C(75 MHz, $CDCl_3$–MeOD = 1/1) 163.4 (d, $J = 248.2$ Hz, C), 152.3 (CH), 151.8 (C), 145.9 (CH), 143.7 (C), 130.8 (d, $J = 3.3$ Hz, C), 130.6 (d, $J = 8.3$ Hz, 2 × CH), 127.6 (CH) 116.5 (d, $J = 22.1$ Hz, 2 × CH), 90.8 (CH), 87.3 (CH), 74.7 (CH), 71.7 (CH), 62.7 (CH_2), 54.0 (CH_2); HRMS (ESI) calcd for $C_{19}H_{19}N_7O_4F$ $[M+H]^+$ 428.1477 found 428.1481; HPLC purity grade. 254 nm : 100%; 300 nm : 100%

(2*R*,3*S*,4*R*,5*R*)-2-(hydroxymethyl)-5-(6-(1-(4-methylbenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol 9e. mp 90–91 °C; δ_H(300 MHz, DMSO) δ 9.17 (s, 1H), 8.97 (s, 1H), 8.90 (s, 1H), 7.32 (d, $J = 8.0$ Hz, 2H), 7.20 (d, $J = 8.0$ Hz, 2H), 6.07 (d, $J = 5.5$ Hz, 1H), 5.73 (s, 2H), 5.58 (br s, 1H), 5.27 (br s, 1H), 5.15–5.12 (m, 1H), 4.65 (br s, 1H), 4.21 (br s, 1H), 4.02–3.98 (m, 1H), 3.75–3.69 (m, 1H), 3.63–3.56 (m, 1H), 2.28 (s, 3H); δ_C(75 MHz, DMSO) 152.2 (CH), 151.6 (C), 146.8 (C), 145.1 (CH), 142.4 (C), 137.7 (C), 132.8 (C), 129.7 (C), 129.4 (2 × CH), 128.2 (2 × CH), 128.1 (CH), 87.8 (CH), 85.7 (CH), 73.7 (CH), 70.2 (CH), 61.2 (CH_2), 52.8 (CH_2), 20.7 (CH_3); HRMS (ESI) calcd for $C_{20}H_{22}N_7O_4$ $[M+H]^+$ 424.1728 found 424.1726; HPLC purity grade. 254 nm: 88%; 300 nm: 97%.

(2*R*,3*S*,4*R*,5*R*)-2-(hydroxymethyl)-5-(6-(1-(4-methoxybenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol 9f. mp 167–168 °C; δ_H(300 MHz, DMSO) 9.15 (s, 1H), 8.97 (s, 1H),

8.90 (s, 1H), 7.40 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 8.5 Hz, 2H), 6.07 (d, J = 5.3 Hz, 1H), 5.70 (s, 2H), 5.57 (br s, 1H), 5.26 (br s, 1H), 5.14 (br s, 1H), 4.65 (br s, 1H), 4.21 (br s, 1H), 3.99 (br s, 1H), 3.74–3.70 (m, 4H), 3.61–3.57 (m, 1H); δ_C(75 MHz, DMSO) 159.3 (C), 152.2 (CH), 151.6 (C), 146.8 (C), 145.1 (CH), 142.3 (C), 129.9 (2 × CH), 129.7 (C), 127.9 (CH), 127.7 (C), 114.2 (2 × CH), 87.8 (CH), 85.7 (CH), 73.7 (CH), 70.2 (CH), 61.2 (CH_2), 55.2 (CH_3), 52.6 (CH_2); HRMS (ESI) calcd for $C_{20}H_{22}N_7O_5$ $[M+H]^+$ 440.1677 found 440.1677; HPLC purity grade. 254 nm: 95%; 300 nm: 100%.

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-(2-bromobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol 9g. mp 80–81 °C; δ_H(300 MHz, DMSO) δ 9.17 (s, 1H), 8.99 (s, 1H), 8.90 (s, 1H), 7.72 (br d, J = 7.6 Hz, 1H), 7.47–7.42 (m, 1H), 7.37–7.29 (m, 1H), 6.07 (d, J = 5.5 Hz, 1H), 5.89 (s, 2H), 5.56 (br s, 1H), 5.25 (br s, 1H), 5.14–5.11 (m, 1H), 4.64 (br s, 1H), 4.22 (br s, 1H), 4.02–3.98 (m, 1H), 3.75–3.68 (m, 1H), 3.63–3.56 (m, 1H); δ_C(75 MHz, $CDCl_3$) 151.9 (CH), 150.8 (C), 146.7 (C), 145.5 (CH), 142.8 (C), 133.7 (C), 133.4 (CH), 130.7 (CH), 130.6 (C), 130.4 (CH), 128.4 (CH), 127.8 (CH), 91.4 (CH), 87.7 (CH), 74.0 (CH), 72.5 (CH), 63.1 (CH_2), 54.3 (CH_2); HRMS (ESI) calcd for $C_{19}H_{19}N_7O_4Br$ $[M+H]^+$ 488.0676 found 488.0676; HPLC purity grade. 254 nm: 93%; 300 nm: 96%

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-(3-chlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol 9h. mp 67–68 °C; δ_H(300 MHz, DMSO) 9.28 (s, 1H), 8.98 (s, 1H), 8.92 (s, 1H), 7.52 (s, 1H), 7.45–7.56 (m, 3H), 6.08 (br d, J = 5.3 Hz, 1H), 5.82 (br s, 2H), 5.58 (br s, 1H), 5.26 (br s, 1H), 5.16–5.13 (m, 1H), 4.68–4.63 (m, 1H), 4.22 (br s, 1H), 4.00 (br s, 1H), 3.76–3.69 (m, 1H), 3.64–3.55 (m, 1H); δ_C(75 MHz, DMSO) 152.2 (CH), 151.6 (C), 146.7 (C), 145.2 (CH), 142.4 (C), 138.2 (C), 133.3 (C), 130.8 (CH), 129.7 (C), 128.5 (CH), 128.3 (CH), 128.1 (CH), 126.9 (CH), 87.8 (CH), 85.7 (CH), 73.7 (CH), 70.2 (CH), 61.2 (CH_2), 52.2 (CH_2); HRMS (ESI) calcd for $C_{19}H_{19}N_7O_4Cl$ $[M+H]^+$ 444.1182 found 444.1183; HPLC purity grade. 254 nm: 96%; 300 nm: 99%.

(2*R*,3*R*,4*S*,5*R*)-2-(6-(1-(3,4-dichlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol 9i. mp 117–118 °C; δ_H(300 MHz, DMSO) 9.29 (s, 1H), 8.98 (s, 1H), 8.92 (s, 1H), 7.75 (d, J = 2.1 Hz, 1H), 7.68 (d, J = 8.3 Hz, 1H), 7.40 (dd, J = 8.3 and 2.1 Hz, 1H), 6.08 (d, J = 5.5 Hz, 1H), 5.81 (s, 2H), 5.57 (br s, 1H), 5.26 (br s, 1H), 5.15–5.12 (m, 1H), 4.65 (br s, 1H), 4.21 (br s, 1H), 4.02–3.98 (m, 1H), 3.76–3.69 (m, 1H), 3.63–3.56 (m, 1H); δ_C(75 MHz, DMSO) 152.2 (CH), 151.6 (C), 146.7 (C), 145.2 (CH), 142 5 (C), 136.8 (C), 131.3 (C), 131.1 (CH), 131.0$_9$ (C), 130.3 (CH), 129.7 (C), 128.6 (CH), 128.5 (CH), 87.8 (CH), 85.7 (CH), 73.7 (CH), 70.2 (CH), 61.2 (CH_2), 51.6 (CH_2); HRMS (ESI) calcd for $C_{19}H_{18}N_7O_4Cl_2$ $[M+H]^+$ 478.0792 found 478.0789; HPLC purity grade. 254 nm: 96%; 300 nm: 99%.

(2*R*,3*S*,4*R*,5*R*)-2-(hydroxymethyl)-5-(6-(1-(4-nitrobenzyl)-1*H*-1,2,3-triazol-4-yl)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol 9j. δ_H(300 MHz, DMSO) δ 9.33 (s, 1H), 8.99 (s, 1H), 8.92 (s, 1H), 8.26 (d, J = 8.8 Hz, 2H), 7.63 (d, J = 8.8 Hz, 2H), 6.08 (d, J = 5.5 Hz, 1H), 5.99 (s, 2H), 5.57 (br d, J = 5.7 Hz, 1H), 5.25 (br d, J = 4.7 Hz, 1H), 5.13 (br t, J = 5.4 Hz, 1H), 4.68–4.63 (m, 1H), 4.24–4.19 (m, 1H), 4.02–3.98 (m, 1H), 3.76–3.69 (m, 1H), 3.63–3.56 (m, 1H); δ_C(75 MHz, DMSO) δ 152.2 (CH), 151.6 (C), 147.3 (C), 146.6 (C), 145.2 (CH), 143.3 (C), 142.5 (C), 129.7 (C), 129.2 (2 × CH), 128.8 (CH), 124.0 (2 × CH), 87.8 (CH), 85.7 (CH), 73.71 (CH), 70.2 (CH), 61.2 (CH_2), 52.1 (CH_2); HRMS (ESI) calcd for $C_{19}H_{19}N_8O_6$ $[M+H]^+$ 455.1422 found 455.1424; HPLC purity grade: 254 nm: 98%; 300 nm: 100%

Drugs, reagents and cell culture medium

RPMI 1640, glucose, fetal calf serum (10%), geneticin (G418), penicillin, streptomycin (100 IU mL^{-1}), DMEM/Nut mix F12, Glutamax were purchased from Invitrogen (Cergy Pontoise, France). Isobutyl methyl xanthine (IBMX), 8 cyclopentyl-1,3-dipropylxanthine (DPCPX), and methanol were from SIGMA Aldrich (St Quentin Fallavier, France). Chem 3 growth supplement was from Millipore (Billerica MA USA). [H^3] DPCPX were from Amersham (Orsay, France).

Cell culture

Stable cell line human recombinant A_1AR, was obtained from Millipore (CHO, CHEM 3 cells; Millipore, Billerica MA, USA). Cells were cultured (5% CO_2, 37 °C) in RPMI 1640, containing 4.5 g L^{-1} of glucose, supplemented with 10% of fetal calf serum, 0.8 mg mL^{-1} of geneticin (G418), 100 IU mL^{-1} of penicillin and streptomycin and diluted (1/100) with Chem 3 growth supplement. Cells were cultured (5% CO_2, 37 °C) in DMEM/Nut mix F12 with fetal calf serum and glutamax, according to the recommendations of the manufacturer.

cAMP concentration determination

Cells were incubated (2×10^5 per well) 30 min with 1 mM of IBMX, and increasing concentration of tested drugs. Total amount of cAMP was determined by immunoassay (cAMP Biotrak Enzyme immunoasay, EIA system, Amersham Pharmacia Biotrak Orsay, France). EC 50 is the concentration of drug causing half maximal inhibition. Data obtained from the dose–response curved were analyzed by non linear regression analysis (GraphPad Prism, Software, San Diego USA).

Binding assay

Frozen CHO cells were subjected to three freeze-thaw cycles (–80 °C, +20 °C), centrifuged (4 °C, 5000 g, 20 min), suspended in 4.5 mL of binding buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4), and homogenized just prior to the binding assay. We used [^{3}H] DPCPX selective A1 adenosine receptors (A1ARs) ligand. Homogenates of CHO cell membranes (200 µl for a total volume of 250 µl) were incubated (90 min at room temperature) with [^{3}H] DPCPX at the concentration of 5nM (10X KD) and in the presence of increasing concentration of drugs tested (10^{-8} to 10^{-4} M), to obtained displacement curves. Binding experiments were performed in triplicate. Bound and free radioactivities were separated by vacuum-filtration of the sample through Whatman (Breenford, UK) GF/C glass-fiber filters. Cold binding buffer (1 mL) was added to the sample before filtering. The filter was washed three times and bound radioactivity was determined with a Beckman LS-1800 liquid scintillation spectrometer. A weighted non-linear least-square curve fitting program, Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA) was used for the computer analysis. Non-specific binding was defined as binding in

the presence of 10 μM of unlabeled ligand. EC 50 was defined as the concentration of drug that induce the half maximal effects.

Acknowledgements

The authors thank the Agence Nationale pour la Recherche (ANR-05-BLAN-0175-01), the CNRS, the Université Paul Cézanne, the Université de la Méditerranée, the Faculté de Médecine, the Assistance Publique des Hôpitaux de Marseille, the Fondation Mérieux and the Conseil Régional Provence Alpes Côte d'Azur for fundings. The Conseil Général des Bouches du Rhône is also gratefully acknowledged.

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2. Conclusion de l'article 5

Dans cette étude, nous avons testé six nouvelles molécules synthétisées par « click chemistry » et biologiquement actives sur les A_1R. Ces composés sont des dérivés C-6 1,2,3 triazole adénosine, parmi lesquels quatre sont des antagonistes et deux sont des agonistes, suivant leurs action sur la production d'AMPc par des lignées cellulaires sur-exprimant les A_1R. Ces nouveaux dérivés présentent une affinité faible dans les tests de fixation de ligands isotopiques sur les membranes, comparés à un antagoniste (DPCPX) et un agoniste (CPA) connus des A_1R. Toutefois leurs effets biologiques apparaissent à des concentrations raisonnables.

VI. Évaluation biologique d'un ligand bivalent spécifique des récepteurs aux opioïdes et des récepteurs A_1 de l'adénosine

1. Introduction

L'interaction entre les différents récepteurs transmembranaires est un domaine de recherche en expansion. En effet les « multi target drugs » possèdent un certain nombre d'avantages :

i) On peut espérer que la co-stimulation d'une cellule cible par une drogue multipolaire permette une potentialisation du signal de transduction.

ii) L'avantage d'une prise de drogue seule portant deux fonctions différentes, par rapport à la co-administration de deux drogues distinctes, réside dans l'unicité de sa pharmacocinétique.

Notre choix, pour ce projet, s'est porté sur la synthèse et l'évaluation de molécules bipolaires ayant à la fois une activité sur les récepteurs mu aux opiacés (MOR) et sur les A_1R. Ce choix a été dicté par plusieurs arguments :

i) Dans le système nerveux périphérique, les A_1R et les MOR sont co-localisés au niveau des afférences nociceptives (Silinsky, 2004).

ii) Il est bien établi que l'adénosine et les morphiniques ont une action synergique au niveau du contrôle de la douleur. Ainsi l'action des morphiniques passe, en partie, par la libération locale d'adénosine (Halimi et al., 2000).

Ainsi, moduler les deux types de récepteurs simultanément pourrait avoir des applications thérapeutiques intéressantes pour certaines pathologies dans lesquelles l'implication de ces deux récepteurs est particulièrement avérée : outre la douleur chronique citons également le choc septique, le choc hypovolémique et le syndrome de sevrage aux opiacés. Partant de ces concepts, un ligand hétéro-bivalent A_1R/MOR (figure 11), qui est un composé unique à double mode d'action sur ces deux récepteurs, a été synthétisé par nos

collaborateurs chimistes. Ces derniers ont lié de façon covalente par l'intermédiaire d'un « linker » l'adénosine et le fentanyl [*N*-phenyl-*N*-(1-phenethyl-4-piperidinyl)propanamide], un des plus puissants analgésiques opioïdes.

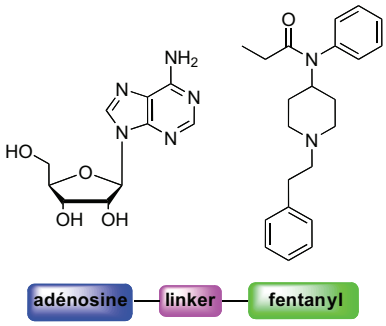

Figure 11 : Concept d'un ligand hétéro-bivalent A_1R et MOR

Après sa synthèse, nous avons évalué l'activité de ce composé hétéro-bivalent pour, d'une part, ses propriétés sur la production d'AMPc de lignées cellulaires sur-exprimant les MOR ou les A_1R et, d'autre part, ses propriétés anti-nociceptives chez la souris, évaluées par les tests « Hot-plate » et « Tail-flick » décrits précédemment.

Ce travail est détaillé dans l'article « **Design, synthesis and biological evaluation of a bivalent μ opiate and adenosine A_1 receptor antagonist** » ci-dessous.

Publication 6

"Design, synthesis and biological evaluation of a bivalent μ opiate and adenosine A_1 receptor antagonist"

Smitha C. Mathew, Nandita Ghosh, **Youlet By**, Aurélie Berthault, Marie-Alice Virolleaud, Louis Carrega, Gaëlle Chouraqui, Laurent Commeiras, Jocelyne Condo, Mireille Attolini, Anouk Gaudel-Siri, Jean Ruf, Jean-Luc Parrain, Jean Rodriguez and Régis Guieu

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Design, synthesis and biological evaluation of a bivalent μ opiate and adenosine A1 receptor antagonist

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ABSTRACT

The cross talk between different membrane receptors is the source of increasing research. We designed and synthesized a new hetero-bivalent ligand that has antagonist properties on both A_1 adenosine and μ opiate receptors with a K_i of 0.8 ± 0.05 and 0.7 ± 0.03 μM, respectively. This hybrid molecule increases cAMP production in cells that over express the μ receptor as well as those over expressing the A_1 adenosine receptor and reverses the antalgic effects of μ and A_1 adenosine receptor agonists in animals.

The cross talk between different G protein-coupled receptors (GPCRs) is the source of increasing research in the area of simultaneous targeting of more than one GPCR.[1] Most of the cross talk between different GPCRs concerns cAMP production via the modulation of adenylyl-cyclase activity. There is a great number of GPCRs that either stimulate or inhibit adenylyl-cyclase activity, depending on the nature of the G protein (G_s, G_i or other). Among GPCRs, there is evidence that A1 adenosine receptors (A_1ARs) and μ opioid receptors (MORs) are implicated in such cross talk and their activation leads to a decrease in cAMP levels in the target cells.

In peripheral nervous system, there is a cross-tolerance and cross withdrawal between A_1ARs and MORs, indicating that these receptors are localised on the same primary afferent nociceptors and that A_1ARs and MORs cooperate as a multiple receptor complex.[2] Our group has also demonstrated that the activation of MORs increases adenosine concentration in the extra cellular spaces of the central nervous system, suggesting that most effects of opioids are due to adenosine release.[3]

Based on the A_1AR/MOR cross talk, the aim of this study was to synthesize a potential hetero-bivalent A_1AR/MOR ligand and to evaluate its biological effects. A hetero-bivalent ligand is a single chemical entity that is composed of two covalently linked pharmacophores with a dual mode of action, acting on two different receptor subtypes.[1a,f,4]

Modulating both A_1ARs and MORs could have therapeutic application in some diseases. For example, the release of adenosine aggravates the hypotension during severe sepsis or septic shock, leading to subsequent tissue hypoperfusion and ischaemia. Most of these effects are secondary to the activation of A_1 adenosine receptors, explaining the absence of response to pressor amines in these patients.[5] Furthermore, naloxone, a μ receptor antagonist has been successfully used against the drop of blood pressure during septic shock[6] or hypovolemic shock,[7] suggesting that the release of endogenous opioids participates into the severity of these syndromes. Thus, blocking both A_1ARs and MORs may be of a great interest in these pathologies. Modulating both these receptors may also be of interest in the area of drug-withdrawal therapy.[8]

Fentanyl [*N*-phenyl-*N*-(1-phenethyl-4-piperidinyl)propanamide] is one of the most powerful opioid analgesics, with a potency approximately eighty times that of morphine and forty times that of oxycodone.[9] As such, we planned to associate fentanyl with adenosine to generate an appropriate hetero-bivalent ligand (Fig. 1). Herein, we report the synthesis of such a compound **10**, its MOR and A1AR binding affinities and biological properties.

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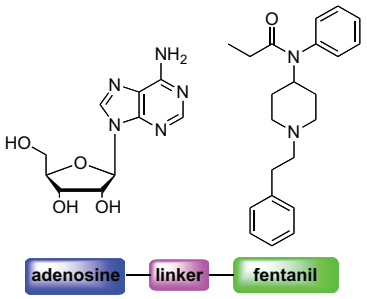


Figure 1. Concept of hetero-bivalent A_1AR/MOR ligand.

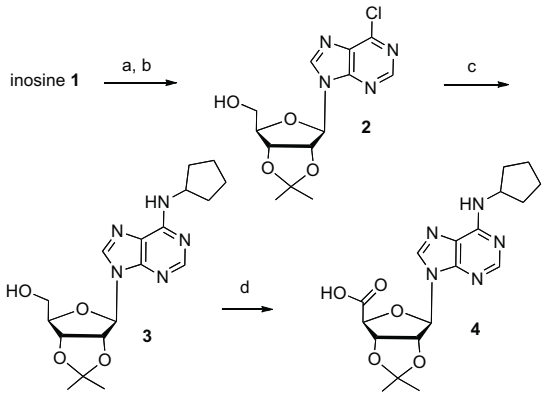
Considering that the main approach for discovering A_1AR agonists has been the modification of adenosine itself and that the N_6-cyclopentyl adenosine (CPA) derivative displays high A_1AR selectivity,[10] we first turned our attention to the synthesis of compound **10** containing an adenosine N_6-cyclopentyl group and an amide bond linker at the 5′-position.

Our approach for this potential hetero-bivalent ligand would involve amide bond formation between the adenosine and the fentanyl derivatives **4** and **8** respectively.

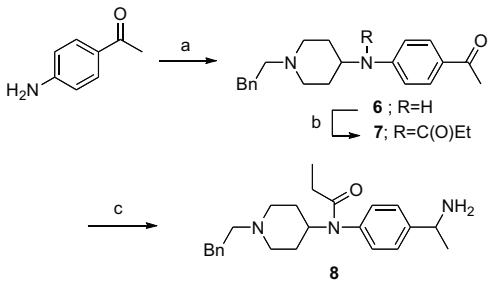
The synthesis of acid **4** (Scheme 1) commenced with the conversion of inosine (**1**) into the corresponding chloro-acetal derivative **2**. Acetylation of inosine with acetic anhydride in the presence of triethylamine and DMAP afforded the corresponding triacetylinosine in quantitative yield.

Conversion to the chloride ($SOCl_2$ in DMF) was directly followed by deacetylation using a solution of ammonia in methanol to give the expected chloroinosine (90% yield over three steps). Selective protection of the two secondary alcohols as the corresponding acetonide was achieved using dimethoxypropane in presence of *p*-TSA. Treatment of **2** with cyclopentylamine in the presence of Hunigs base provided the corresponding secondary amine **3** in 98% yield. Finally the primary alcohol was oxidised to the corresponding acid **4** under TEMPO-iodobenzene diacetate conditions[11] in 65% yield. Attention was next turned to the fentanyl subunit, as part of target molecule **8** (Scheme 2). Reductive amination of 4-aminoacetophenone with commercially available 1-phenethylpiperidine-4-one (**5**) afforded amine **6**.[12]

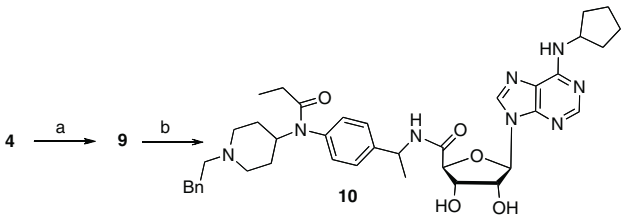
This secondary amine was next subjected to propionylation to provide **7** in 95% yield. Further reductive amination of **7** with ammonium acetate and sodium cyanoborohydride then furnished subunit **8** in 55% yield.[11]



Scheme 1. Synthesis of acid **4**. Reagents and conditions: (a) (1) Ac_2O, DMAP, Et_3N, ACN, rt, quant.; (2) $SOCl_2$, DMF, DCM, 0 °C then reflux, 90%; (3) NH_3/MeOH, 0 °C then −20 °C, quant.; (b) dimethoxypropane, *p*-TSA, acetone, rt, 70%; (c) cyclopentylamine, DIEA, EtOH, 80 °C, 98%; (d) BAIB, TEMPO, ACN/H_2O, rt, 65%.



Scheme 2. Synthesis of aminofentanyl **8**. Reagents and conditions: (a) 1-phenethylpiperidine-4-one (**5**), $NaBH(OAc)_3$, AcOH, DCE, rt, 77%; (b) propionyl chloride, Et_3N, DCM, reflux, 95%; (c) NH_4OAc, $NaBH_3CN$, MeOH, 50 °C, 55%.



Scheme 3. Synthesis of adenosyl fentanyl **10**. Reagents and conditions: (a) **7**, BOP, Et_3N, THF, rt, 77%; (b) 80% aq TFA, rt, 32%.

Completion of the targeted ligand **10** (Scheme 3) was achieved in two steps by using a BOP-assisted coupling between acid **4** and amine **8**[13] to give **9** then followed by acetal deprotection using aqueous trifluoroacetic acid. Using this approach, the potential hetero-bivalent ligand **10** was efficiently synthesized on 30 milligram scale (93% HPLC purity grade) in eight steps.[14]

This new hetero-bivalent ligand **10** was then submitted to biological evaluation. Adenosyl fentanyl **10** was first tested using nociceptive tests in mice ($n = 12$ per group, using intra cerebro ventricular route (icv)). Ligand **10** alone had no effect on latencies. However it is worthy of note that DAMGO, a well known synthetic opioid peptide with μ agonist properties, co-injected with **10** showed shorter latencies than DAMGO alone both in hot plate and tail flick tests.

Adenosyl fentanyl **10** also significantly reversed the increase in latency induced by CPA, an A1 AR agonist (Fig. 2A). Ligand **10** also reversed the increase in latencies induced by injection of both CPA and DAMGO (Fig. 2B). Comparable results were obtained using intraperitoneal (ip) administration (data not shown).

We also evaluated the acute toxicity of **10** in animals: LD 50 value was >50 μg/mouse (when icv administered) and >500 mg/kg (when ip administered).

We tested the properties of **10** to modulate cAMP production in cell culture. Compound **10** activated cAMP production in a dose dependent manner both in CHO K1 cells that over expressed MORs and in CHO Chem 3 cells that over expressed A1ARs, with a maximal stimulation of 29 ± 7% and 17 ± 3%, respectively. Comparatively, naloxone, a MOR antagonist, increased cAMP production with a maximal stimulation of 50 ± 16% while 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX), a A_1AR antagonist, increased cAMP production with a maximal stimulation of 45 ± 8% (Fig. 3). We also evaluated the affinities (K_i) of the drugs tested, using binding assay on cell membranes (Table 1). Compound **10** had substantially lower affinities for the A_1ARs as compared with A_1AR antagonist DPCPX and/or lower affinities for the MORs as compared with the MOR antagonist naloxone or with the MOR agonist DAMGO. However, its affinity was sufficient to produce biological effects at reasonable concentrations.

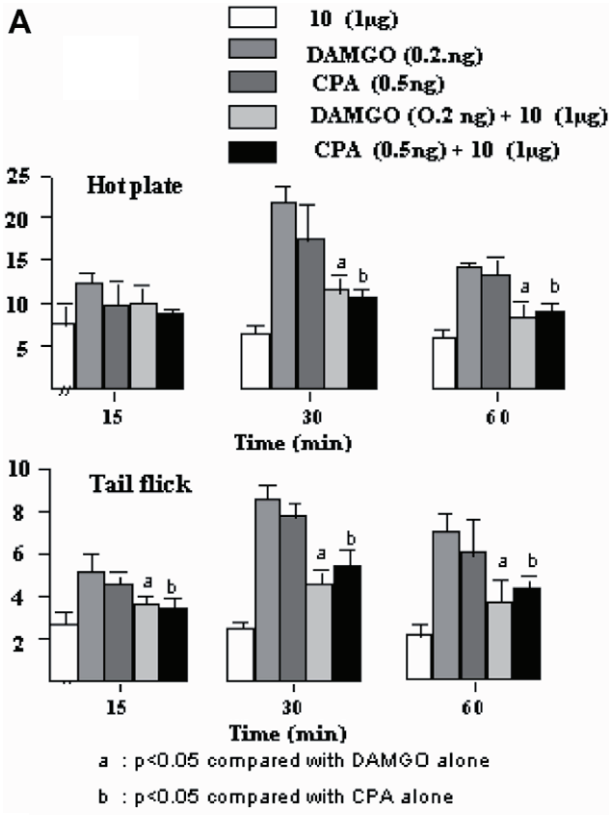


Figure 2. Results of nociceptive tests in animals.

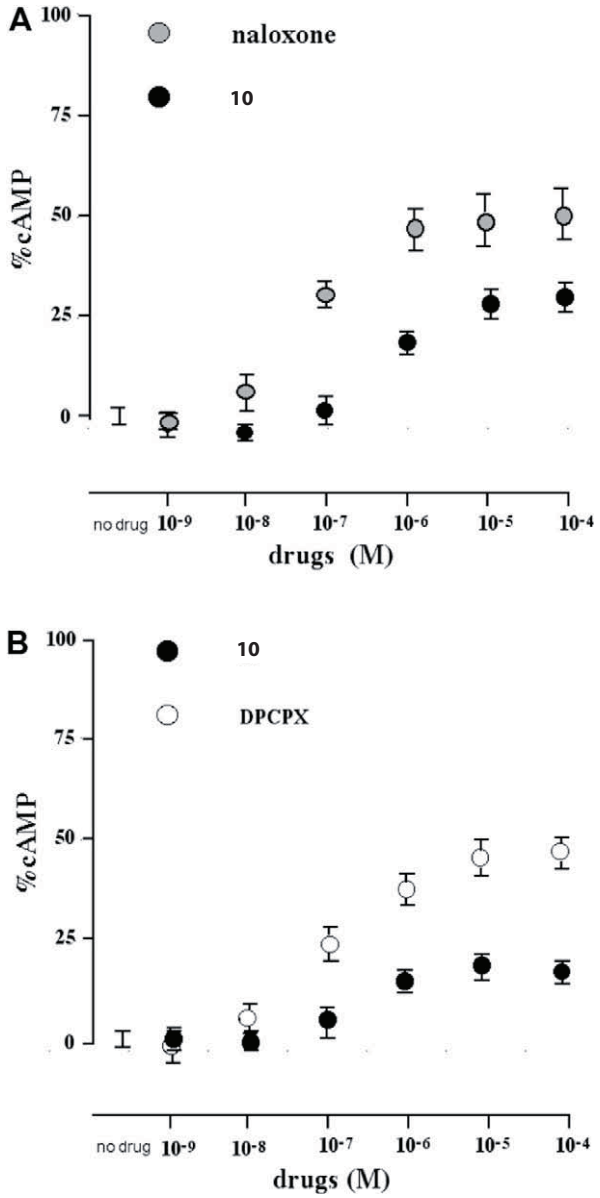


Figure 3. Comparative effects of **10**, naloxone and DPCPX on cAMP production.

Table 1
K_i values for drugs at the A_1 AR, A_{2A} ARs and the MORs DPCPX: 8-cyclopentyl-1,3-dipropylxanthine (A1 AR antagonist)

	K_i A_1ARs (nM)	K_i A_{2A}ARs (nM)	K_i MORs (nM)
DPCPX	12.9 ± 3		>100,000
Naloxone	>50,000		1.5 ± 0.2
DAMGO	>100,000		3.8 ± 0.7
10	800 ± 57	>40,000	728 ± 37

Naloxone: MOR antagonist; DAMGO: ([D-Ala2, N-Me Phe4, Gly-ol]enkephalin)(MOR agonist).

In summary, we have synthesized a hetero-bivalent ligand **10** that has antagonist properties on both A_1ARs and MORs. Its affinity remained low for the two receptors but sufficient to have biological effects at reasonable concentration. Compound **10** reversed significantly the antalgic effects of DAMGO or CPA and activated cAMP production in both cell lines tested. To the best of our knowledge, compound **10** constitutes the first drug that blocks both MORs and A_1ARs. Multivalent-ligands are also called hybrid molecules, and are defined as chemical entities having different biological effects.[4c] There is evidence that adenosine via A_1ARs, is implicated in the modulation of opiate action in the central nervous system.[15] Morphine[16] and adenosine[17] inhibit Ca^{2+} dependent neurotransmitter release; this inhibition is blocked by theophiline an adenosine receptors antagonist.[18] A_1ARs and MORs are both implicated in septic or hypovolemic shock,[5–7] withdrawal,[8,19] mood regulation[20] and pain[21,22] Thus drugs which modulate both A_1ARs and MORs could prove highly interesting in these therapeutic areas.

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2. Conclusion de l'article 6

Dans cette étude, nous avons testé un ligand hétéro-bivalent qui s'est révélé avoir des propriétés de type antagoniste sur les A_1R et sur les MOR. Le test sur les lignées cellulaires sur-exprimant les MOR ou le A_1R montre que ce composé augmente la production d'AMPc et présente une affinité faible pour ses deux récepteurs avec un K_i de l'ordre du micromolaire, affinité toutefois suffisante pour induire des effets biologiques à des concentrations raisonnables. Les tests Hot-plate et Tail-flick chez la souris montrent que ce ligand inhibe significativement les effets antalgiques de DAMGO et CPA, agonistes spécifiques respectivement des MOR et des A_1R. À notre connaissance, cette molécule hybride, provenant d'une liaison entre le fentanyl et l'adénosine, est la première molécule qui bloque en même temps l'action des MOR et des A_1R. Ainsi, elle pourrait représenter une nouvelle drogue dans les pathologies où la modulation de ces deux récepteurs est impliquée.

Chapitre troisième

DISCUSSION et PERSPECTIVES
CONCLUSION

DISCUSSION et PERSPECTIVES

Les travaux réalisés au cours de cette thèse corroborent l'implication des A_1R et $A_{2A}R$ dans les pathologies humaines telles que les syncopes, l'inflammation et la douleur. Ces récepteurs de l'adénosine représentent donc des cibles thérapeutiques pour des drogues qui les modulent directement ou indirectement. En effet, le développement d'agonistes et d'antagonistes synthétiques, spécifiques des récepteurs de l'adénosine, reste un sujet prometteur dans le domaine de la recherche médicale. À côté des ligands synthétiques dérivés de la chimie organique, les anticorps monoclonaux (mAb), dont la spécificité pour leurs cibles est particulièrement grande grâce à leur reconnaissance épitopique bien définie, représentent une alternative protéique intéressante. En effet, ces molécules ne sont pas susceptibles de dégradation par l'ADA et elles peuvent être « humanisées » pour satisfaire aux exigences d'une molécule médicament.

1. Adonis comme outil de diagnostic

La production de mAb anti-$A_{2A}R$ paraît indispensable si nous voulons utiliser les connaissances apportées par l'étude de ces récepteurs en vue d'une application clinique. Avant d'établir mon projet de thèse, un seul mAb anti-$A_{2A}R$ était disponible sur le marché. Ce mAb reconnait un épitope situé dans la troisième boucle intra-cellulaire ce qui ne permet pas de cibler les $A_{2A}R$ à la surface des cellules intactes. Nous avons produit Adonis, un nouveau mAb anti-$A_{2A}R$ humain de type IgM, κ qui reconnait un épitope linéaire de sept acides aminés (LFEDVVP) à la position 167-173, situé dans la partie C-terminale de la seconde boucle extra-cellulaire des $A_{2A}R$. Dans cette courte séquence d'acides aminés, les résidus F-168 et E-169 ont été récemment identifiés comme ayant un rôle central dans la fixation des ligands à leurs récepteurs (Jaakola et al., 2010). Adonis présente une bonne affinité sur des cellules de mammifère transfectées avec les $A_{2A}R$ humains (A_{2A}-Chem-3) et des PBMC humaines. La spécificité d'Adonis a été confirmée, par Western blotting effectué sur des lysats de PBMC humains, par la révélation d'une bande de 45 kDa dont l'intensité était modulée par l'effet de la caféine, antagoniste des $A_{2A}R$. Ceci suggère qu'Adonis pourrait être un outil de diagnostic utile dans la mesure de l'expression des $A_{2A}R$ sur les PBMC de

patients atteints de pathologies impliquant ces récepteurs (Carrega et al., 2007a; Carrega et al., 2007b; Giaime et al., 2006; Kerbaul et al., 2008; Varani et al., 2003).

Notre deuxième étude, a permis d'établir une relation entre l'expression des $A_{2A}R$, l'APL et les tests HUT et ATP chez des patients atteints de syncopes neurocardiogéniques. Les résultats de cette étude ont montré que l'expression des $A_{2A}R$, mesurée par Western blotting sur les PBMC de patients, est plus faible chez les patients ayant des tests ATP(+) et HUT(-) par rapport aux autres groupes de patients mais n'est pas différente de celle des volontaires sains. Les patients ayant un test HUT(+) présentent une surexpression des $A_{2A}R$ par comparaison aux autres groupes de patients et aux volontaires sains. Cela confirme que l'expression des $A_{2A}R$ est modifiée dans les syncopes inexpliquées (Carrega et al., 2007b). En ce qui concerne les APL, nous avons trouvé une association entre un APL élevé et la probabilité d'avoir un test HUT(+) d'une part, et entre un APL faible et la probabilité d'obtenir un test ATP(+) d'autre part. D'une manière encore plus intéressante, les patients ayant les deux tests positifs ou les deux tests négatifs présentent un APL intermédiaire. Nos résultats suggèrent l'existence, parmi les patients souffrant de syncopes neurocardiogéniques, de sous-groupes de patients dont le profil est différent. D'un côté, les patients ayant un test ATP(+) sont assez souvent des femmes âgées ayant un APL faible et un faible niveau d'expression des $A_{2A}R$; d'un autre côté les patients ayant un test HUT(+) sont plus jeunes et ils ont des APL élevés ainsi qu'un fort niveau d'expression des $A_{2A}R$. Les différences en APL entre patients ayant des tests HUT(+) et ATP(+) nous laissent penser que les récepteurs dont l'activation conduit aux manifestations cliniques pourraient être différents : les $A_{2A}R$ de faible affinité seraient impliqués dans les tests HUT(+), les A_1R de forte affinité dans les tests ATP(+). Cette hypothèse est en cours de confirmation.

À côté de cette aide au diagnostic, Adonis possède une activité fonctionnelle qui agit sur les $A_{2A}R$ à la surface cellulaire. D'après sa caractérisation, Adonis se comporte comme un agoniste qui stimule les $A_{2A}R$ et augmente la concentration d'AMPc dans les cellules A_{2A}-Chem-3.

2. Activités fonctionnelles d'Adonis

Le fait qu'Adonis puisse se fixer aux $A_{2A}R$ à la surface cellulaire, grâce à son épitope situé dans la seconde boucle extra-cellulaire du récepteur, ouvre la possibilité d'étudier les activités fonctionnelles d'Adonis. Notre étude sur la désensibilisation des récepteurs CXCR4 et CCR5 des cellules T $CD4^+$ vient y apporter un éclairage nouveau (By et al., 2010b). Il a été rapporté que la stimulation des $A_{2A}R$ par leurs agonistes induisait une désensibilisation hétérologue des CCR5 et diminuait l'infectiosité du virus HIV-1 sur les monocytes humains (Zhang et al., 2006). Le virus HIV-1 infecte les cellules du système immunitaire par fixation sur ses récepteurs principaux, CD4, puis couplage avec un des deux autres co-récepteurs CXCR4 ou CCR5 (Dimitrov, 1997). Ainsi, dans le deuxième article, nous avons proposé d'étudier la possibilité de moduler CXCR4 et CCR5 via les $A_{2A}R$, ces trois récepteurs étant des récepteurs transmembranaires de la même famille, tous couplés aux protéines G. Nous avons montré dans cette étude qu'Adonis, grâce à ses propriétés d'agoniste, stimulait les $A_{2A}R$ d'une lignée de cellules T $CD4^+$ qui expriment de façon endogène les CXCR4 et CCR5, avec comme conséquence l'augmentation de la concentration en AMPc entraînant une inhibition de la prolifération cellulaire. Ceci laisse envisager l'utilisation possible d'Adonis comme agent anti-inflammatoire ou pour lutter contre certaines pathologies d'origine auto-immune (Palmer and Trevethick, 2008). Nous avons montré par ailleurs que cette stimulation des $A_{2A}R$ induisait une « down-régulation » de l'expression des CXCR4 et des CCR5 avec une « up-régulation » des $A_{2A}R$, le taux des CD4 restant inchangé. Il est vraisemblable que les $A_{2A}R$ s'expriment en homo-dimère à la surface cellulaire (Canals et al., 2004). Puisque Adonis est une IgM pentamérique, il pourrait stimuler les $A_{2A}R$ en stabilisant leurs homo-dimères actifs à la surface cellulaire, ce qui a été déjà rapporté dans le cas d'un mAb anti-récepteur β_2 adrénergique (Mijares et al., 2000), et donc empêcher leurs internalisation. Par contre, la diminution de l'expression des CXCR4 et CCR5 en terme de protéines totales en Western blotting, suggère un processus d'internalisation puis de dégradation des récepteurs (Marchese et al., 2008). Adonis semble être le premier anticorps monoclonal qui inhibe de façon indirecte, via la stimulation des $A_{2A}R$, l'expression à la surface des deux co-récepteurs utilisés par les virus HIV-1 pour pénétrer dans les cellules. Il serait intéressant, comme perspective de ce travail, de tester Adonis, tout d'abord *in vitro*,

sur des cellules infectées par des virus HIV-1, avant de passer, peut être, à l'expérimentation animale puis aux essais cliniques chez les malades atteints de SIDA.

Au niveau du contrôle de la nociception, nous avons vu que les $A_{2A}R$ sont impliqués non seulement dans l'inflammation mais aussi dans la douleur. L'adénosine est reconnue comme un modulateur des voies de la nociception aussi bien au niveau spinal qu'au niveau supra-spinal via les A_1R et $A_{2A}R$. Bien qu'il soit établi que l'activation des A_1R puisse avoir un effet anti-nociceptif, le rôle des $A_{2A}R$ dans ce domaine reste encore très controversé. Notre étude sur l'effet anti-nociceptif d'Adonis vient apporter un éclairage nouveau sur ce sujet pas encore bien élucidé (By et al., 2010a). Bien qu'Adonis soit un mAb anti-$A_{2A}R$ humain (By et al., 2009), il reconnaît un épitope linéaire également présent sur les $A_{2A}R$ des souris, ce qui permet d'utiliser ces dernières pour faire de l'expérimentation animale. Par contre, Adonis ne montre pas de réactivité croisée avec les A_1R puisqu'il ne stimule pas les A_1R exprimés par les cellules A_1-Chem-3. Dans les tests d'exploration de la nociception, nous avons montré que l'injection intra-cérébro-ventriculaire d'Adonis chez les souris conduisait à une augmentation significative et dose-dépendante des temps de latences des tests Hot-plate et Tail-flick. Cet effet anti-nociceptif d'Adonis pouvait être renversé par le pré-traitement des souris par la caféine ou ZM241385, deux antagonistes des $A_{2A}R$. Ces effets sur les latences étaient également partiellement renversés lors de l'administration de naloxone, un antagoniste des récepteurs aux opioïdes. Ces résultats suggèrent que la stimulation des $A_{2A}R$ a un effet anti-nociceptif et que cet effet est accompagné d'une libération d'opioïdes endogènes. L'activation des $A_{2A}R$ conduit également à une diminution de l'état d'éveil et de vigilance et inhibe la locomotion via les voies dopaminergiques des noyaux gris de la base du crâne (El et al., 2000a; El et al., 2000b). Ainsi l'action d'Adonis pourrait procéder non pas d'effets anti-nociceptifs, mais plutôt d'effets sur la motricité et la vigilance. Nous avons exclu cette hypothèse pour au moins deux raisons :

- D'une part, aux doses utilisées (pM), nous n'avons observé chez l'animal aucune réaction allant dans le sens d'un état de somnolence ;

- D'autre part, le fait que les augmentations des latences soient renversées par l'administration de naloxone, démontre que les effets d'Adonis, au moins en partie, n'ont pas de rapport avec les effets moteurs.

La signalisation des $A_{2A}R$ passe majoritairement par la voie de l'AMPc, mais d'autres voies pourraient également intervenir (Fredholm et al., 2007). Au niveau supra-spinal, la substance grise périaqueducale (PAG) joue un rôle important dans la modulation de la nociception via les récepteurs de la dopamine (Meyer et al., 2009) et ceux des opioïdes (Williams et al., 1995). Dans notre étude, puisque nous avons utilisé la voie d'administration *i.c.v*, nous ne pouvons pas exclure qu'Adonis, via les $A_{2A}R$, active la PAG, en induisant la libération d'opioïdes endogènes. Toutefois, la PAG n'est pas la seule région du cerveau où les opioïdes endogènes pourraient être libérés et des investigations ultérieures sont nécessaires pour déterminer les effets d'Adonis sur le système opioïde. En plus de ce mécanisme, les $A_{2A}R$ peuvent aussi agir sur des canaux ioniques membranaires, via différentes sous-unités de protéines G, indépendamment de la voie de l'AMPc. Par exemple, il a été montré précédemment que les canaux potassium activés par le calcium (SKCa) sont impliqués dans les effets anti-nociceptifs du CGS21680, un agoniste des $A_{2A}R$ (Regaya et al., 2004). Un autre mécanisme de signalisation possible passerait par l'hétéromérisation des $A_{2A}R$ avec les A_1R ou les récepteurs D_2 dopaminergiques (Cunha, 2005; Fredholm et al., 2005).

Les résultats de cette étude renforcent le rôle des $A_{2A}R$ dans le contrôle de la douleur et ouvre une opportunité intéressante pour de nouvelles approches thérapeutiques. Comme perspectives, nous proposons, en collaboration avec une équipe américaine, de tester Adonis pour son action au niveau spinal sur un modèle animal de douleur neuropathique (Loram et al., 2009).

Ultérieurement, Adonis, un mAb de type IgM pentamérique, pourrait être fragmenté par digestion enzymatique puis réduction/alkylation en vue d'obtenir sa forme divalente $(Fab)_2$ puis monovalente (Fab) pour étudier la variation de ses caractéristiques en fonction de sa valence, c'est-à-dire son nombre de sites de liaison au récepteur. Ainsi une forme multivalente d'Adonis ayant une activité stimulante (agoniste) pourrait devenir antagoniste sous une forme monovalente (Mijares et al., 2000). Le fait de posséder le clone producteur d'Adonis, donne accès, par RT-PCR, au clonage de son ADN. Adonis pourrait ainsi être « humanisé » par des techniques de biologie moléculaire visant à greffer les régions liantes de l'anticorps murin sur des vecteurs d'anticorps humains comme le « CDR grafting », technique consistant à remplacer les régions hypervariables (Complementary Determining

Region, CDR) d'un anticorps humain par des régions CDR d'origine murine (Hou et al., 2008) ou le « chain shuffling », technique consistant à remplacer successivement la chaîne légère puis la chaîne lourde de l'anticorps murin par des chaînes d'anticorps humains en criblant les associations donnant la meilleure affinité (Christensen et al., 2009) pour, *in fine*, être mieux accepté par l'organisme et trouver sa place comme médicament potentiel.

3. Ligands synthétiques des A_1R

La modulation des A_1R reste une voie thérapeutique d'avenir pour une grande diversité de pathologies qui nécessite sans cesse la recherche de nouveaux ligands. D'un côté, l'objectif est l'obtention de nouveaux ligands des A_1R qui soient suffisamment affins et suffisamment spécifiques, mais sans trop d'effets secondaires, pour pouvoir être utilisés chez l'homme. D'un autre côté, les chimistes cherchent aussi à affiner de nouvelles approches de synthèses rapides, comme la « click chemistry », qui puissent également éviter les inconvénients des méthodes classiques comme, par exemple, la susceptibilité aux dégradations enzymatiques.

Notre étude sur les nouveaux ligands des A_1R dérivés de C-6 1,2,3-triazole adénosine, en collaboration avec des chimistes, ont produit six molécules intéressantes (Mathew et al., 2010). Parmi ces molécules, quatre se comportent comme des antagonistes et deux autres comme des agonistes des A_1R, suivant leurs activités sur la production d'AMPc des cellules A_1-Chem-3. Nous avons montré que les IC_{50} étaient comprises entre 0.29 et 1.5 µM pour les antagonistes et entre 11 et 42 µM pour les agonistes dans les tests effectués à partir de membranes cellulaires. Bien que ces nouvelles molécules présentent une affinité plus faible comparées aux ligands classiques comme DPCPX ou CPA (respectivement antagoniste et agoniste des A_1R avec une IC_{50} de l'ordre du nanomolaire), leurs effets biologiques se produisent à des concentrations raisonnables, c'est à dire très en dessous des LD_{50} évaluées chez l'animal, et compatibles avec une posologie utilisable chez l'homme.

Notre perspective dans ce domaine est de tester, en concertation avec les chimistes, des molécules, dérivées des premières publiées, mais modifiées pour améliorer leur biodisponibilité et leur affinité.

En ce qui concerne notre dernière étude sur une molécule de synthèse bivalente, spécifique des A_1R et des récepteurs μ des opioïdes (MOR) (Mathew et al., 2009), nous avons montré que cette molécule hybride se comportait comme un antagoniste vis-à-vis de ces deux types de récepteurs en augmentant la production d'AMPc aussi bien sur les cellules CHO K1, surexprimant les MOR, que sur les cellules A_1-Chem-3, surexprimant les A_1R. Cette molécule présente des affinités de l'ordre du micromolaire pour chacun des récepteurs. Ces affinités sont, certes, plus faibles que celles décrites pour les ligands classiques mais suffisantes pour produire des effets biologiques à des concentrations raisonnables.

Par ailleurs, cet antagoniste bivalent renverse les effets anti-nociceptifs induits par DAMGO (un agoniste des MOR) et CPA (un agoniste des A_1R) dans les tests *in vivo* chez la souris. Le fait de pouvoir bloquer simultanément les MOR et les A_1R par une même molécule pourrait trouver des applications thérapeutiques dans des pathologies impliquant une interaction entre ces deux récepteurs telles que les chocs septiques ou hypovolémiques (Faden and Holaday, 1979; Martin et al., 2000; Peters et al., 1981), les syndromes d'abstinence (Hack and Christie, 2003; Khorasani et al., 2006), les dérégulations de l'humeur (Kaster et al., 2007). Par contre, le développement d'agonistes bivalents pourrait être utile dans le traitement des douleurs neuropathiques (Guieu et al., 1996; Pham et al., 2003).

CONCLUSION GÉNÉRALE

Le but de cette thèse était d'améliorer nos connaissances sur la physiopathologie humaine des récepteurs de l'adénosine et d'apporter, d'une part, de nouveaux outils pour le diagnostic, et d'autre part, de nouvelles drogues à visée thérapeutique. Ainsi nos travaux ont produit un anticorps monoclonal anti-$A_{2A}R$ humain de l'adénosine, ayant une bonne affinité et une bonne spécificité pour mesurer l'expression des $A_{2A}R$; cet anticorps monoclonal ayant, par ailleurs, une activité de type agoniste, pourrait être utilisable, après humanisation, dans le traitement de pathologies touchant au domaine de l'inflammation, de l'immunité ou de la douleur. Notre travail confirme l'implication des $A_{2A}R$ dans la régulation des processus inflammatoires et dans le contrôle de la nociception. Outre nos études sur la régulation adénosinergique, une thématique de recherche en pleine expansion, les travaux décrits dans cette thèse auront également contribué au développement de nouveaux ligands synthétiques spécifiques pouvant agir au niveau des A_1R. Les A_1R et $A_{2A}R$ représentent des cibles thérapeutiques d'excellence pouvant agir au carrefour des voies de signalisation cellulaires et donc impacter diverses pathologies humaines.

ANNEXES

Annexe 1

"Adenosine A_{2A} Receptor Hyperexpression in Patients With Severe SIRS After Cardiopulmonary Bypass"

François Kerbaul, Frédéric Bénard, Roch Giorgi, **Youlet By**, Louis Carrega, Ibrahim Zouher, Laurence Mercier, Victoria Gérolami, Vincent Bénas, Dorothée Blayac, Vlad Gariboldi, Frédéric Collart, and Régis Guieu

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ORIGINAL ARTICLE

Adenosine A_{2A} Receptor Hyperexpression in Patients With Severe SIRS After Cardiopulmonary Bypass

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ABSTRACT

Objective: Adenosine (ADO) is an endogenous nucleo-side, which has been involved in blood pressure failure during severe systemic inflammatory response syndrome (severe SIRS) after cardiac surgery with cardiopulmonary bypass (CPB). Adenosine acts via its receptor subtypes, namely A_1,A_{2A},A_{2B},orA_3.Because A_{2A} receptors are implicated in vascular tone, their expression might contribute to severe SIRS. We compared adenosine plasma levels (APLs) and A_{2A} ADO receptor expression (ie, *B*, *K*, and mRNA amount) in patients with or without postoperative SIRS.

Patients: This was a prospective comparative observational study. Forty-four patients who underwent cardiac surgery involving CPB. Ten healthy subjects served as controls.

Measurements and Results: Among the patients, 11 presented operative vasoplegia and postoperative SIRS (named complicated patients) and 33 were without vasoplegia or SIRS (named uncomplicated patients). Adenosine plasma levels, *K*, *B*, and mRNA amount (mean ± SD) were measured on peripheral blood mononuclear cells. Adenosine plasma levels, *B*,and *K* were significantly higher in complicated patients than in uncomplicated patients (APLs: 2.7 ± 1.0 vs 1.0 ± 0.5 μmol l^{-1}, $P < 0.05$; *B*:210 ± 43 vs 65 ± 26 fmol/mg, $P < 0.05$; *K*:35 ± 10 vs 2 ± 1nM, $P < 0.05$). In uncomplicated patients, APLs remain higher than in controls (1 ± 0.5 vs 0.6 ± 0.25 μmol/L; $P < 0.05$).

Mean arterial pressure was inversely correlated to APLs ($R = -0.58$; $P < 0.001$) and *B* ($R = -0.64$; $P < 0.001$) leading to an increased requirement of vasoactive drugs during the postoperative period in vasoplegic patients.

Conclusions: High expression of A_{2A} ADO receptor and high APLs may be a predictive factor of postoperative severe SIRS after CPB.

Key Words: adenosine, SIRS, A_{2A} receptors, cardiopulmonary bypass, hemodynamics

INTRODUCTION

Systemic inflammatory response syndrome (SIRS) can complicate cardiopulmonary bypass[1] after cardiac surgery. This inflammatory syndrome is often induced by a proinflammatory cytokine release and can result in organ dysfunction (severe SIRS), such as myocardial reperfusion damage, lung injury, and generalized profound vasodilation, thus increasing postoperative morbidity.[2–4]

During cardiopulmonary bypass (CPB), hemodynamic instability associated with low vascular resistance due to a systemic release of cytokines leads to a delay in extubation and a prolonged stay in the intensive care unit (ICU).[1,5] However, a lot of molecules play a role in the physiopathology of the systemic inflammatory response to cardiac surgery. Among these, adenosine (ADO) may participate in hemodynamic disturbance in critical illness, especially in severe SIRS. Adenosine is an endogenous nucleoside that is released by endothelial cells and myocytes during metabolic stress associated with ischemia or systemic inflammation.[6–8] Thus, it is not surprising that systemic ADO levels were recently been involved in blood pressure failure induced by severe SIRS after CPB[9] or septic shock.[10]

Adenosine acts on blood vessel tone and sinoatrial nodes via 4 G-protein–coupled receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) depending on their pharmacological properties.[11,12] Stimulation of cardiac A_1 and A_3 receptors is associated with cardioprotection and ischemic conditioning,[13,14] whereas activation of A_{2A} receptors results in vasodilation.[7,15] The role of A_{2A} receptors in the control of blood pressure may be crucial. Indeed,

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the lack of receptors in animals results in hypertension and tachycardia.[16] The increase in A_{2A} ADO receptor expression contributes to hypotension during hemodialysis[17] or during neurocardiogenic syncope.[18] Furthermore, A_{2A} ADO receptors are strongly expressed in brain area implicated in the control of arterial blood pressure.[19] However, the action of ADO on vascular tone depends on the number of receptors expressed at the cell surface.

Thus, the aim of this study was to evaluate adenosine plasma levels (APLs) and A_{2A} ADO receptor expression (B), receptor affinity (K), and receptor synthesis (mRNA) in patients with or without SIRS after cardiac surgery and in controls.

■ METHODS

This study protocol was approved by our institutional Ethics Committee and informed consent was obtained from every patient included in the study.

Patients

From September 2005 to October 2005, patients with no active infection, inflammatory disease, or pulmonary hypertension, undergoing valve replacement, coronary artery surgery, combined, or other (see Table 1), were prospectively and consecutively included. Among the 70 patients hospitalized during this period, 44 filled the inclusion criteria (see Inclusion criteria section). We included at the same time 10 healthy volunteers as controls, who were recruited among hospital workers with no previous cardiac surgery (6 females and 4 males, 54 ± 11 years; range, 39–68).

Patients and volunteers were instructed to avoid coffee and tea for the 72 hours preceding the study. Patients who had been treated with papaverine, dipyridamole, immunosuppressive, or antibiotic agents during the preceding 6 weeks were not included.

Inclusion Criteria

Patients were studied by Doppler echocardiography before surgery. A preoperative left ventricular ejection fraction (LVEF) was obtained from either a left ventricular angiogram or a 2-dimensional echocardiography (Teicholz method). Patients found to have a stenotic native aortic valve with a peak aortic-jet velocity of at least 4 $m \cdot s^{-1}$ and an aortic valve area less than 0.5 $cm^2 \cdot m^{-2}$ were included in the study.[20] Patients found to have a stenotic native mitral valve with a peak aortic-jet velocity more than 4 $m \cdot s^{-1}$ and a mitral valve area less than 1.4 $cm^2 \cdot m^{-2}$ were included. Symptomatic patients with one or more stenotic coronary arteries (>70%) underwent coronary revascularization. Recent myocardial infarctions were defined as an acute coronary syndrome with or without ST modification and were associated with troponin I plasma level modifications.[21] These were observed over a 6-week period after the operation. Preoperative renal function was assessed by baseline creatinine clearance, calculated according the Cockroft-Gault formula.[22]

TABLE 1. Demographic and Baseline Patient Characteristics (Means ± SD)

	Uncomplicated Course, n = 33	*Vasoplegia, n = 11*	*P**
No. patients, n (%)	33 (75)	11 (25)	NS
Age, yr	58 ± 17	57 ± 10	NS
Male, n (%)	18 (57)	6 (56)	NS
Physical findings			
Body weight, kg	73 ± 22	76 ± 19	NS
NYHA, median†	2 (2–3)	2 (2-3)	NS
Preoperative medication, n (%)			
Inhibition-converting enzyme	9 (33)	4 (36)	NS
Medical history, n (%)			
Hypertension	13 (39)	5 (45)	NS
Peripheral vascular disease	2 (6)	1 (9)	NS
Congestive heart failure	7 (22)	2 (19)	NS
Recent myocardial infarction	2 (6)	1 (9)	NS
Unstable angina	3 (9)	1 (10)	NS
COPD	2 (7)	1 (6)	NS
Diabetes mellitus	10 (31)	3 (23)	NS
Hypercholesterolemia	11 (32)	4 (32)	NS
Renal dysfunction	11 (32)	3 (29)	NS
Results of diagnostic tests, n (%)			
LVEF, %	56 ± 13	48 ± 18	NS
Mean PAP, mm Hg	22 ± 2	21 ± 4	NS
Creatinine Cl., $mL \cdot min^{-1}$	75 ± 28	72 ± 25	NS
Results of scoring tests			
EuroSCORE	5 ± 2	7 ± 2	NS
Parsonnet Index	12 ± 6	13 ± 5	NS
Type of surgery, n (%)			
Valvular surgery	20 (61)	6 (55)	NS
CABG	7 (21)	2 (18)	NS
Combined	3 (9)	1 (9)	
Other	3 (9)	2 (18)	

*$P < 0.05$ versus uncomplicated population. †Data are median values and 25-75th percentiles in parentheses. NYHA indicates New York Heart Association (classification for cardiac insufficiency); LVDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; mean PAP, mean pulmonary arterial pressure; creatinine Cl., creatinine clearance; unstable angina, acute coronary syndrome without troponin I modification; COPD, chronic obstructive pulmonary dysfunction; CABG, coronary artery bypass grafting; combined surgery, valvular surgery associated with CABG; other, Bentall Tyron-David surgery, interventricular communication closure; NS, not significant.

Exclusion Criteria

Patients with active infection, inflammatory disease, or pulmonary hypertension were excluded. Patients under immunosuppressive agents were also excluded.

Anesthesia and Surgery

Patients were premedicated with 25 mg of orally administered oxazepam 1 hour before surgery. After preoxygenation, anesthesia was induced with intravenous midazolam (Hypnovel®; Roche Laboratories, Neully sur Seine, France), etomidate (Etomidate-Lipuro®; P Braun, Boulogne, France), sufentanil (Sufenta®; Solvay Pharma, Suresnes, France), and atracurium (Tracrium®; Glaxo-Wellcome, Marly-le-Roi, France) and maintained with sufentanil, atracurium, sevoflurane (Sevorane®; Abbott, Rungis, France) (0.5–1.5 MAC), and midazolam during CPB. Lungs were mechanically ventilated via an endotracheal tube. Prophylactic antibiotics were administered intravenously, with 1.5 g of cefamandole (Céfamandole®; Panpharma, Fougères, France) given after induction and then 750 mg given ever 2 hours during surgery. Mild hypothermic (33°C) nonpulsatile CPB was performed after administration of intravenous heparin (300 $IU \cdot kg^{-1}$), always using the same model of membrane oxygenator (BARD Quantum; Bard Ltd, Crawley, UK) and a roller pump (COBE Optima Cardiovascular, Inc, Arvada, CO). A blood flow of 2.4 $L \cdot min^{-1} \cdot m^{-2}$ was maintained with the aim of keeping arterial blood pressure between 50 and 75 mm Hg during the entire CPB. Myocardial preservation was performed with intermittent infusion of colloid-crystalloid solution (Buckberg; FRESENIUS Laboratories Kabi, Sevres, France). Immediately after induction of anesthesia, patients were infused with 1,000,000 U of aprotinin. A further 1,000,000 U were added to the cardiopulmonary bypass prime solution, and patients were continuously infused with 250,000 U/hour during surgery.[23] During CPB, vasoplegia was considered to have occurred if the mean arterial pressure was less than 50 mm Hg with a duration of more than 5 minutes despite a normal blood flow rate of 2.4 $L \cdot min^{-1} \cdot m^{-2}$.[24]

All patients had pacing wires placed but none of them required permanent pacing at the postoperative period.

Hemodynamics and Blood Gas Measurements

Patients were monitored by ECG, pulse oximetry, end-tidal carbon dioxide capnography, systemic arterial line (mean blood pressure; MBP), and transesophageal echocardiography. Hemodynamics, arterial blood gases, and hemoglobin were measured during the whole study.

In patients with low preoperative LVEF (<50%), epinephrine or dobutamine was administered when postoperative mean arterial pressure was less than 50 mm Hg. In patients with normal preoperative LVEF (>50%), norepinephrine was added if postoperative MBP was less than 50 mm Hg. The dosage was adjusted to give an MBP of approximately 70 mm Hg.

Definition of SIRS and Severe SIRS

SIRS was defined, according to the classification of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference, when 2 or more of the following signs are found: body temperature abnormalities (>38 or < 36°C); persistent tachycardia (heart rate >90 beats/min); tachypnea or hyperventilation (breathing frequency >20/min or $PaCO_2$ < 32 mm Hg), and leukocytosis or leukopenia (leukocyte count >12 Gpt/L or <4 Gpt/L).[25] Tachycardia was only considered valid if it lasted for at least 2 hours with no significant modification of central venous pressure or mean systemic arterial pressure.

Severe SIRS was defined as SIRS with one or more postoperative organ dysfunction as defined in the ODIN model.[26] SIRS and severe SIRS were classified in all patients at 5:00 AM on the first postoperative day taking into account the entire postoperative condition of the patient. For patients who remained longer in the ICU, evaluation and classification were repeated daily.

Cardiac operative risk was evaluated by the same physician, blinded to the APL values, using the EuroSCORE scoring system on the day of surgery.[27]

Patients were discharged from the ICU if they met the following criteria: tracheal extubation performed since 12 hours, adequate muscle strength, hemodynamic stability without inotropic or vasopressive support, full consciousness, stable body temperature (within the range, 37–38.5°C), and adequate ventilation (breathing rate between 10 and 30/min, PaO_2/FiO_2 >60/0.21, $PaCO_2$ between 30 and 50 mm Hg). Mediastinal drainage was systematically removed before ICU exit.

Collection of Blood Samples

Timing of Sample Collection. In a previous clinical study, we performed kinetics of APLs, before surgery, during aorta cannulation, during CPB (before and after aortic cross-clamp release), and finally 30 minutes after the end of surgery. No influence of time was evidenced concerning APLs.[9] Here we have chosen collecting samples 30 minutes after surgery because it is the most easy and reproducible time for sample collection in our protocol. In volunteers, samples were collected at 8:00 AM from a peripheral vein.

Adenosine Plasma Levels

The lumen of the arterial catheter was washed out and filled with a solution of 1 mL of papaverine and 1 mL of dipyridamole, injected through the lateral entry of a 3-way stopcock just prior blood sampling.[28] Blood (3 mL each) was taken through the axial entry of the stopcock using an ice-cold syringe containing 7 mL of the cold stop solution to prevent nucleotidases action, ADO uptake by red blood cells, and deamination into inosine.[29,30]

Isolation of Peripheral Blood Mononuclear Cells and Membrane Preparation

Peripheral mononuclear cells were used to assess ADO A_{2A} receptors because it has been established that peripheral blood circulating cells express A_{2A} receptors changes that closely mirror those occurring in the heart itself.[31] Mononuclear cells were isolated from peripheral blood using Ficoll-based CPT system (Becton Dickinson, NJ). After 3 freeze-thaw cycles, the pellets were resuspended in a Tris buffer prior binding assay. Protein concentration was determined using a Beckman Synchron LX® apparatus (Beckman Coulter, Villepinte, France).

B and *K* Determinations

The methodology has been previously described.[18,31,32] We used a selective A_{2A} receptor ligand: [^{3}H]-ZM 241385.[33] Saturation binding experiments were performed in triplicate by incubating homogenates of mononuclear cell membranes (200 μL in a total volume of 250 μL; 90 minutes, 4°C) with increasing concentrations of ligand. Bound and free radiolabeled ligands were separated by vacuum filtration of the sample through Whatman GF/C glass-fiber filters. A cold binding buffer (1 mL) was added to the sample before filtering. The filter was washed 3 times, and bound radioactivity was measured with a Beckman LS-1800 liquid scintillation spectrometer. A weighted nonlinear least-square curve fitting program (Graph Pad Prism®, Graph Pad Software Inc, San Diego, CA) was used for analysis. Nonspecific binding value of [^{3}H]-ZM 241385 was defined as the binding observed in the presence of 10 μM of unlabeled ligand.

The *K* is the concentration of ligand at which binding sites are 50% occupied (equilibrium constant, a measure of affinity). *B* represents the total number of binding sites expressed as fmol/mg of protein.

Quantification of ADO A_{2A} Receptor mRNA

Total ribonucleic acids (RNA) were extracted from purified mononuclear cells using Bio-Robot, M48 (Qiagen, Courtaboeuf, France). Complementary DNA was synthesized from 250 ng of total RNA. Real-time quantitative PCR was performed with a Light-Cycler (Roche®) according to the manufacturer's recommendation. The mRNA relative amount expression was quantified using the ratio between A_{2A} mRNAs and mRNAs from housekeeping gene18S.

Statistical Analysis

Association between vasoplegia and *K* or *B* were analyzed using the *U* test of Mann-Whitney for continuous variables. χ test or Fisher exact test when appropriate and Mann-Whitney *U* test were performed to analyze association between vasoplegia and other prognostic factors. Spearman correlation coefficient was used for correlation data. For all tests, *P* values less than 0.05 (2-tailed tests) were considered as statistically significant. Statistical analysis was performed using Statistical Package for the Social Sciences for Windows (Version 13.1; SPSS, Inc, Chicago, IL).

■ RESULTS

Clinical and Biological Parameters During Surgery

Forty-four patients were prospectively included in the study and underwent cardiac surgery with CPB. We observed a preoperative vasoplegia in 11 of them. All of these 11 patients (complicated patients) developed postoperative SIRS, with 10 developing severe SIRS. Mean age, body weight, NYHA classification status, preoperative echocardiographic, and biological parameters were not significantly different in these patients compared with those without vasoplegia (Table 1). None of the patients had unstable angina.[21]

Duration of endotracheal intubation (since induction of anesthesia) was significantly longer in patients with vasoplegia who developed postoperative SIRS compared with patients without vasoplegia (224 ± 87 vs 14 ± 9 hours, respectively; $P < 0.05$). The duration of stay in the ICU was significantly longer for patients

TABLE 2. Perioperative Patient Characteristics (Means ± SD)

	Uncomplicated Course, n = 33	*Vasoplegia, n = 11*	*P*
Mean duration of CPB, min	91± 37	98 ± 38	NS
Mean duration of aorta clamping, min	64 ± 28	72 ± 37	NS
Minimal core temperature, CPB	33.3 ± 1.4	33.1 ± 1.6	NS
Postoperative inotropic support			
Dobutamine ($\mu g \cdot kg^{-1} \cdot min^{-1}$), n (%)	3.0 ± 2.1 (10)	9.0 ± 4.0 (60)	<0.05
Epinephrine ($\mu g \cdot kg^{-1} \cdot min^{-1}$), n (%)	0.04 ± 0.02 (3)	0.32 ± 0.12 (49)	<0.05
Norepinephrine ($\mu g \cdot kg^{-1} \cdot min^{-1}$), n (%)	0.0 ± 0.0 (0)	0.15 ± 0.02 (42)	<0.05
Blood transfusion >1 U, n (%)	9 (28)	3 (29)	NS
Duration of ventilation, h	14 ± 9	224 ± 87	<0.05
Duration of stay in ICU, d	3 ± 1	16 ± 13	<0.05
Death, n (%)	0 (0)	2 (18)	<0.05

CPB indicates cardiopulmonary bypass; ICU, intensive care unit; NS, not significant.

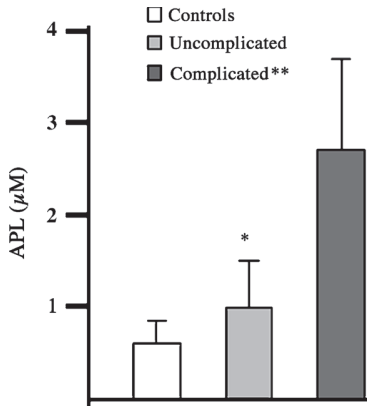

FIGURE 1. Means ± SD of adenosine plasma concentrations in complicated patients (with per operative vasoplegia or postoperative SIRS; n = 11), in patients with no complications (n = 33), and in 10 healthy subjects. *P < 0.05 versus uncomplicated group. **Preoperative vasoplegia or postoperative severe systemic inflammatory response syndrome. APL indicates adenosine plasma level.

with vasoplegia compared with patients without vasoplegia (16 ± 13 vs 3 ± 1 days; $P < 0.05$; Table 2).

The duration of CPB and aorta cross-clamping, the body temperature, and the PaO_2/FiO_2 ratio between the 2 groups were not different. Conversely, the administration of postoperative inotropic drugs was significantly higher in the patients with severe SIRS (Table 2). Dobutamine and epinephrine were simultaneously administered to 5 patients, and dobutamine and norepinephrine were simultaneously administered to 4 patients to maintain systolic arterial pressure. All these patients experienced postoperative severe SIRS.

Adenosine Plasma Levels

Adenosine plasma levels in uncomplicated patients were higher than those of healthy subjects (1 ± 0.5 vs 0.6 ± 0.25 μmol/L; $P < 0.05$). In complicated patients, APLs were significantly higher than those observed in uncomplicated patients (2.7 ± 1 vs 1 ± 0.5 μmol/L; $P < 0.05$; Fig. 1). Baseline MBPs were not different between the group of patients (80 ± 6 mm Hg in patients with vasoplegia vs 81 ± 6 mm Hg in uncomplicated). However, during surgery, MBPs strongly decreased in the vasoplegic patients (55 ± 8 mm Hg in complicated patients with vasoplegia vs 68 ± 11 mm Hg in uncomplicated patients at the end of surgery; $P < 0.002$). Among the 44 patients, MBPs were inversely correlated with arterial APL (Spearman $r = -0.58$; $P < 0.001$) and B ($r = -0.64$; $P < 0.001$).

A_{2A} Receptors Expression

Bmax and K_D were significantly higher in complicated patients compared with uncomplicated patients (Bmax: 210 ± 43 vs 65± 26 fmol/mg, $P < 0.05$; K_D: 35 ± 10 vs 2 ± 1 nM, $P < 0.05$; Fig. 2). But K_D and Bmax were not different between uncomplicated patients and healthy volunteers. Finally, mRNA amount was not different between the 2 groups of patients (mRNA A_{2A}/mRNA beta-actin; uncomplicated vs complicated patients: 19.84 ± 0.05 vs 20.05 ± 0.04; $P > 0.05$) and remains in the range of those measured in controls 19.98 ± 0.06; $P > 0.05$).

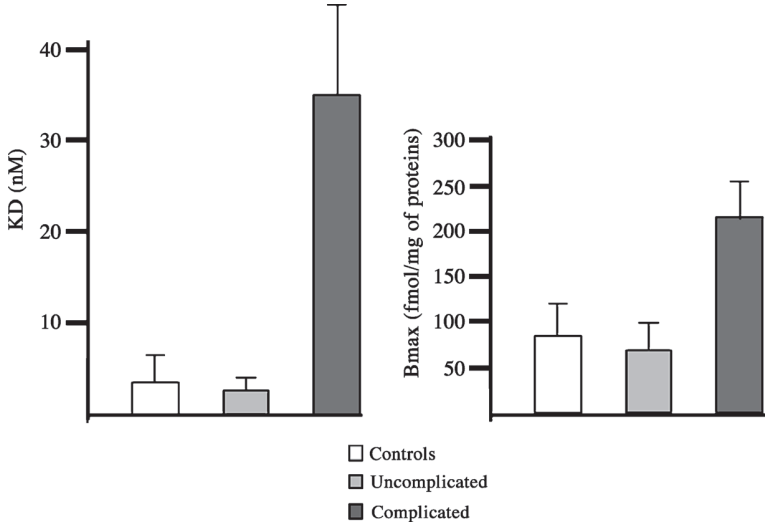

FIGURE 2. K_D and Bmax values. K_D (nM) of [^{3}H]-ZM 241385, a specific ligand or A_{2A} adenosine receptor evaluated using peripheral blood mononuclear cell membranes. Samples were collected 30 minutes after surgery. K_D was defined as the concentration of ligand at which binding sites are 50% occupied. Bmax represents the total number of binding sites expressed as femtomole per milligram of proteins. K_D and Bmax were evaluated in patients with uncomplicated operative course in patients with per operative vasoplegia or postoperative severe SIRS and in 10 healthy subjects. Data are expressed as mean and SD. *P < 0.05 versus uncomplicated group.

■ DISCUSSION

Our study showed that hyperexpression of A_{2A} receptors in a context of high APLs measured after surgery could be a predictive factor for postoperative severe SIRS. This resulted in a delayed extubation, a higher dose of catecholamines, and a prolonged stay in the ICU.

Vasoplegia during and immediately after cardiac surgery involving CPB occurs in between 5% and 20% of patients.[1,5,9] Twenty-five percent of our patients developed postoperative SIRS. However, the number of patients included was lower than in previous reports.[1,5]

The vasoplegia is one of the main factors influencing the extent of the SIRS.[1] During CPB, the exposure of blood to the extracorporeal circuit activates the complement system that initiates the proinflammatory process.[1,34] Neutrophil activation or free radical, endotoxin, or cytokine release is thought to participate in the inflammatory process of SIRS.[3,34,35] However, the role of ADO in this process has been poorly investigated. In a prospective clinical study, we have shown that high APL was found to be associated with postoperative complications after cardiac surgery using CPB.[9] But the level of A_{2A} ADO receptor expression has never been evaluated in this population.

Possible Mechanisms of High APL in Vasoplegic Patients

Adenosine is a nucleoside that is released by endothelial cells and myocytes during ischemia or oxidative stress.[6–8] Adenosine has been implicated in the drop of blood pressure that occurs during tilt-test–induced syncope[18,36] in vasoplegia of septic shock,[10] but ADO may be also an endogenous modulator that plays a role in triggering vasoplegia during heart surgery in predisposed patients. Adenosine is released by sympathetic fibers and poorly myelinated C fibers.[37,38] Moreover, ADO has been reported to modulate baroreflex activation.[39] Therefore, high APLs may lead to dysfunction of the baroreflex, as observed in some patients during surgery who were preoperatively treated with angiotensin-converting enzyme inhibitors.[9] However, the number of such patients between the vasoplegic and the nonvasoplegic groups was not different. High APLs do not seem to be related to myocardial necrosis, as we found no difference in troponin concentrations between the vasoplegic and the nonvasoplegic groups at postoperative time.

Effects of A_{2A} Receptor Modulation on Infectious and Noninfectious SIRS

In an animal model of infectious SIRS, deoxycoformycin, an inhibitor of ADO deaminase that increases ADO levels in the extra cellular spaces, has been shown to improve survival time and to suppress the inflammatory response indices.[40] Moreover, it was demonstrated that activation of A_{2A} receptors diminishes phagocytosis and augments secretion of anti-inflammatory cytokines in invasive bacterial infection.[41,42] Recently, Nemeth et al.[43] demonstrate that ADO A_{2A} receptor inactivation increases animal survival after polymicrobial sepsis. This protection after A_{2A} receptor blockade was paralleled by a decrease in apoptosis and proinflammatory cytokines production associated with an increase of phagocytosis. Many of the effects of ADO may also involve modulating oxyradical-mediated response. This occurs via increased oxyradical production via ADO to xanthine degradation or limiting inflammatory oxyradical generation.[44]

Adenosine has also been shown to clinically increase the systemic vasodilation that characterizes the hemodynamic profile of patients with noninfectious SIRS during CPB.[9] High APLs can result in a very low blood pressure and bradycardia, which is often resistant to vasopressive amines.[10] These are secondary effects of the activation of A_{2A} receptors, which are primarily implicated in blood pressure control.[16] A_{2A} receptor activation could therefore also result in systemic vasodilation occurring with severe SIRS.

The cardiovascular consequences of high APLs effectively seem to depend on the expression of ADO receptors. This expression may be different among patients and from 1 receptor subtype to another.[11] We observed a decrease in mean arterial pressure in vasoplegic patients with high APLs and A_{2A} receptor hyperexpression. Thus, the effects of ADO on the cardiovascular system are probably due to a balance between A_{2A} receptor hyperexpression and APL. A_{2A} receptor number was 4-fold greater in complicated than in uncomplicated patients after CPB. Furthermore, we found that in the complicated group, the ADO plasma level is increased 2.5- to 3-fold. This increase may be sufficient to activate A_2 spare receptors[45] and to precipitate vasoplegia.

Thus, the great expression of these receptors in a context of high APLs may precipitate vasoplegia in our patients.

■ CONCLUSION

These results strongly suggest that high expression of A_{2A} ADO receptor, in a context of high APLs, may be a predictive factor of postoperative severe SIRS after CPB. Further clinical studies are needed to determine if preventive blockade of A_{2A} receptors may offer a new strategy for reducing cardiovascular vasoplegia and noninfectious postoperative SIRS occurring after CPB.

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Annexe 2

"Head-up tilt induced syncope and adenosine A_{2A} receptor gene polymorphism"

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CLINICAL RESEARCH
Arrhythmia/electrophysiology

Head-up tilt induced syncope and adenosine A_{2A} receptor gene polymorphism

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Aims High adenosine plasma levels and high expression of adenosine A_{2A} receptors are observed in patients with unexplained syncope and a positive head-up tilt test (HUT). This study aimed to evaluate the single nucleotide polymorphism (SNP) (c.1364 T>C) which is the most commonly found polymorphism in the A_{2A} receptor gene, in patients with unexplained syncope undergoing HUT.

Methods and results One hundred and five patients with unexplained syncope who underwent HUT were included. Fifty-two had a positive test. Receptor genotype determinations were performed in patients and in 121 healthy subjects. Genotype (TT, CC, TC) was determined from DNA leucocytes. The distribution of the polymorphism showed significant ($P < 0.0001$) difference when the results of HUT were analysed. Fifty-two per cent of patients with a positive HUT had a CC genotype and 34.6% a TC genotype, whereas 13.2% of the patients with a negative HUT had a CC genotype and 71.7% a TC genotype. Patients with a CC genotype had a higher incidence of spontaneous syncopal episodes.

Conclusion In patients with unexplained syncope, a significant association between high incidence of syncopal episodes, positive HUT, and the presence of the CC variant in the adenosine A_{2A} receptor gene was elicited.

Keywords Adenosine • Syncope • A2A adenosine receptors • Genes

Introduction

Unexplained syncope is a common clinical problem that can alter the quality of life of affected patients. Head-up tilt testing (HUT) is an established and widely used tool for the evaluation of patients with unexplained syncope.[1] Exogenous adenosine or ATP has been used in the provocation of syncope during tilt testing in susceptible patients.[2–4] On the other hand, recent studies support the concept that endogenous adenosine may be a possible mediator in a subset of syncope patients suspected to be of neurocardiogenic origin. This is based on higher baseline plasma adenosine levels (APLs) observed during positive HUT together with a positive correlation between rising plasma adenosine concentration and the rapidity of onset of tilt-induced syncope.[5,6] It is well established that adenosine effect on blood vessel tone and on sinoatrial node occurs via activation of four subtypes of membrane receptors (A_1, A_{2A}, A_{2B}, and A_3).[7] Stimulation of A_1 and A_3 receptors is associated with cardio-protection and ischaemic preconditioning,[8,9] and activation of the adenosine A_{2A} receptors results in vasodilatation.[10,11] The role of adenosine A_{2A} receptors in the control of heart rate and blood pressure may be essential.[12,13] Moreover, these receptors are expressed in an area of the brainstem strongly implicated in the baroreflex function,[14] and the role of the baroreflex among the various mechanisms of neurocardiogenic syncope has been emphasized.[15] It has been also shown in patients with unexplained syncope that an over expression of adenosine A_{2A} receptors was observed in patients with a positive HUT.[6] The goal of this prospective study was to

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investigate the distribution frequency of the single nucleotide polymorphism (SNP) (c.1364 T>C)[16] in the adenosine A_{2A} receptor gene in patients with an unexplained syncope and a positive HUT.

Methods

Patients selection

One hundred and twenty height patients with unexplained syncope were referred to our institution for tilt testing from March 2003 to December 2007. The inclusion criteria were: (i) patients experienced two or more episodes of syncope or pre-syncope within the preceding year; (ii) The cause of syncope was unexplained despite a complete clinical work-up and was assumed to be neurocardiogenic by the referring physician; (iii) Head-up tilt was indicated for the first time and no patient had previously had an adenosine or an ATP test. Exclusion criteria were (i) the presence of orthostatic hypotension, anaemia, or endocrinological dysfunctions such as diabetes, hypoglycaemia, or thyroid dysfunction; (ii) the presence of sinus bradycardia <50 b.p.m., or bundle branch block on the electrocardiogram; (iii) the presence of abnormal electrophysiological study, implanted pacemakers, aortic stenosis, hypertrophic cardiomyopathy, pulmonary hypertension, subclavian steal syndrome, or drug induced syncope. Patients should be drug-free and were instructed to avoid coffee and tea intake 48 h prior to the study. Twenty-three of the 128 patients were not included in the study: 14 patients because they were on beta-blockers at the time of the test, 5 patients had orthostatic hypotension, 3 had a positive carotid massage subsequent to the HUT reproducing their symptoms, and one patient who developed for the first time, before tilting, a paroxysmal atrial fibrillation episode. Ultimately, 105 patients were included in the analysis. The control group consisted of healthy volunteers, members of the medical and technical staff of our institution, consecutively included during the study period. None of them had a past story of fainting or syncope. They were on no medication and were as patients, instructed to avoid coffee and tea for 48 h before blood sampling.

The investigation was conducted according to the principles outlined in the Declaration of Helsinki. All patients and control subjects gave a written informed consent to participate to the study. The study protocol was approved by the Ethical Committee for Human Research of the Centre Hospitalo-Universitaire de Marseille, Université de la Méditerranée.

Study protocol

The protocol used for performing the HUT test was consistent with the Recommendations of the European Society of Cardiology Task Force report.[17] In order to avoid false-positive diagnosis, no pharmacological provocation was allowed. Tilt testing was performed in a quiet room at 21°C equipped for cardio-pulmonary resuscitation. An intravenous catheter was inserted before the onset of the test for safety reasons and blood sampling. Patients were instructed to lie down on a tilt table for 30 min. The table was then tilted within 20 s to 60° for 45 min. The heart rate was monitored using continuous recording of six electrocardiographic leads and blood pressure was measured manually by the same operator every 2 min.

Collection of blood samples

Samples were collected and treated as previously described.[5,6] In brief, venous blood was withdrawn together with an iced cold stop solution in order to prevent adenosine degradation and uptake. Samples were collected: (i) just before tilting (baseline), (ii) immediately after tilting the table in order to have information on adenosine release during tilting (mentioned as 'Tilt' in the tables), and (iii) either during syncope, immediately after resetting the table in the horizontal position (positive test) or after 45 min of tilting (negative test). The blood samples for $ADORA_{2A}$ genotype determination were withdrawn just before tilting.

Definition

A positive test was defined as the occurrence of syncope or pre-syncope in association with bradycardia (at least 20% decrease in heart rate) and/or hypotension (systolic blood pressure <80 mm Hg).[18] Syncope was defined as a transient loss of consciousness, and pre-syncope as premonitory signs and symptoms of eminent syncope (e.g. severe light-headedness, transient hearing loss, blurring of vision, or severe weakness).

Adenosine plasma levels

Adenosine plasma measurement has been previously described.[5] After samples deproteinization, adenosine was identified and quantified using HPLC (Hewlett Packard 1100). The absence of xanthine derivatives was checked on each chromatogram. Technicians blind to the tilt-test response performed all the biological measurements.

Genotyping of the $ADORA_{2A}$ polymorphism

The T/C polymorphism located in codon 361 (rs5751876) was determined after genomic DNA extraction from blood cells and PCR amplification. The amplification was performed using forward primer F2 5′CTGAGCGGAGGCCCAATGGGTA3′ and reverse primer R2 5′CTCCCAACGTGACTGGTCAAG3′. The primer F2 was modified to introduce a *Rsa*1 restriction site when C replaced T at nucleotide 1364 in the sequence (NM_000675 GenBank) that resulted in the cleavage of the 256 bp amplicon into 22 bp and 234 bp fragments.

Statistical analysis

Statistical analyses were performed using the SPSS software for Windows 13.1. As the TT variant is not common (10–15%),[16,19,20] the recruitment of a minimum of 100 patients was necessary to include 10–15 patients with the TT genotype.

Quantitative variables are reported as mean ± standard deviation (SD) or median and interquartile range (IQR), and qualitative variables as numbers and percentages. Differences in the distribution of patient's characteristics by tilt-test results and $ADORA_{2A}$ genotypes were tested using the Mann–Whitney non-parametric test (continuous variables) or using the χ^2 or, where appropriate, the Fisher's exact test (categorical variables). Two-sided tests were performed and a *P*-value <0.05 was considered as significant. A Bonferroni correction procedure was then used to account for false positive results and to accurately determine which differences in the distribution were significant across $ADORA_{2A}$ genotypes. To evaluate the presence of a significant statistical difference, and to ensure an overall type I error rate of 5%, an adjusted $P < 0.05/3 = 0.016$ was considered significant.

To determine the independent relationship between genotype and positive HUT, a logistic regression multivariable analysis was systematically adjusted for the main predictive factors, based on epidemiological knowledge[17,18,21,22] (i.e. age, sex, number of syncopal episodes per patient, presence of vasovagal symptoms, presence of triggering factors). The number of variables was voluntary limited in order to have an appropriate ratio between the number of variables and the number of events. For each continuous variable, the linearity assumption was assessed by plotting the unadjusted relationship between variables and logit probability of HUT. The model's goodness-of-fit was

Table 1 Clinical characteristics of patients and tilt-test results

Variables	Patient population ($n = 105$)	Negative tilt test ($n = 53$)	Positive tilt test ($n = 52$)	*P*-value
Age (years)[a]	49.5 ± 18.0	52.7 ± 18.2	46.4 ± 17.4	0.05
Male (%)	60 (57)	34 (64.2)	26 (50)	0.14
Weight (kg)[a]	72.2 ± 14.1	74.6 ± 13.9	69.9 ± 169.3	0.08
Height (cm)[a]	168.7 ± 9.1	168.1 ± 8.1	169.3 ± 10.1	0.45
Heart diseases				
Moderate hypertension	18	10	8	
History of atrial fibrillation	1	1	0	
Mild mitral regurgitation	1	0	1	
History of syncopal episodes				
Time since first episode (months)[b]	6 (2–24)	4 (1–24)	9.5 (3–24)	0.10
Syncope and pre-syncope/month[b]	1.0 (0.5–2.1)	1 (0.3–2)	1.25 (0.7–2.6)	0.04
Syncopal episodes/patient[b]	2 (1–4)	1 (1–2.5)	3 (1.2–6)	0.001
Pre-syncopal episodes/patient[b]	3 (0–11)	2 (0–4.5)	6 (0–20)	0.001
Number of patient with pre-syncopal episodes (%)	70 (66.7)	32 (60.4)	38 (73.1)	0.17
Number of patients with situational symptoms (%)	34 (32.4)	15 (28.3)	19 (36.5)	0.37
Number of patients with triggering factors (%)	42 (40.0)	17 (32.1)	25 (48.1)	0.09
Number of patients with vasovagal symptoms (%)	56 (53.3)	20 (37.7)	36 (69.2)	0.001

[a]Mean ± SD.
[b]Median (IQR).

assessed by the Hosmer–Lemeshow test (larger *P*-value means better fit or reliability), and predictive accuracy was assessed by the area under the receiver-operating characteristic curve.

Linkage analyses were not performed in this study because c 1364 T>C is a silent polymorphism and no assumption concerning the mode of inheritance, dominant or recessive, is therefore necessary.

Results

Demographics and symptoms

One hundred and five patients referred for unexplained syncope and HUT were included in the study. Their mean age was 49.05 ± 1.8 years (range 13–76). The control group included 121 healthy volunteers: 71 males and 50 females with a mean age of 48.2 ± 1.23 years (range 32–69). No difference was observed between the control group and the syncope patients, neither between the patients with a positive or with a negative HUT with respect to age, sex, height, baseline heart rate, and blood pressure. The HUT was positive in 52 patients and negative in 53 patients. The clinical characteristics of the patients are summarized in *Table 1*. In the group of patients with a positive HUT test, syncope occurred in 46 and pre-syncope in 6 after a mean of 30 ± 1.8 min (range 4–45). Upon syncope, systolic blood pressure dropped by 51 ± 2% and heart rate by 33 ± 3.5% ($P < 0.0001$). Regarding the history of symptoms, the time since first episode and the frequency of spontaneous syncope or pre-syncope events per month were similar in both groups. However, the numbers of syncope and pre-syncope episodes per patient were higher in patients with a positive HUT test as shown in *Table 1*.

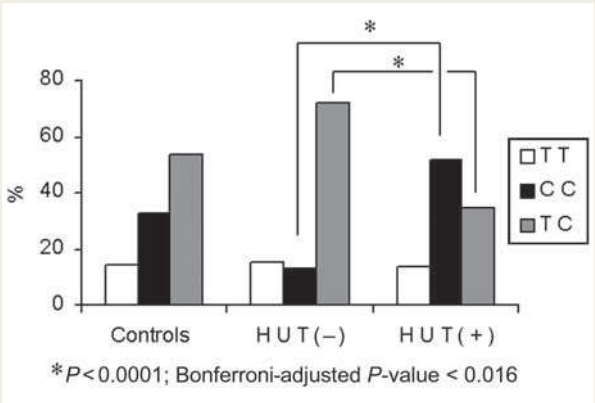

Figure 1 Distributions of genotype frequencies for adenosine A_{2A} receptors in controls and in patients with a negative or positive head-up tilt test.

Adenosine A_{2A} receptor gene polymorphism

The global distribution of genotype frequency was not significantly different ($P = 0.80$) in the 105 syncope patients and in the 121 control subjects: TT = 15 (14.3%) vs. 21 (17.3%); CC = 34 (32.4%) vs. 39 (32.2%), and TC = 56 (53.3%) vs. 61 (50.5%), respectively (*Figure 1*). As seen in *Table 2*, the patients with the CC variant were significantly younger (45.6 ± 15.6 years) than those with the TC variant (54.3 ± 18 years). The TC variant was found more commonly in men than in women. The distribution

Table 2 Clinical characteristics of the patients, adenosine levels, and tilt-test response according to the genotype of the A2A receptor

Genotype[a]	TT ($n = 15$)	CC ($n = 34$)	TC ($n = 56$)	*P*-value[b] (CC vs. TC)
Age (years)[c]	40.5 ± 20.4	45.6 ± 15.6	54.3 ± 18.0	0.01
Men (%)	5 (33.3)	16 (47.1)	39 (69.6)	0.03
History of syncopal episode				
Time since first episode (months)[d]	3 (1–24)	7 (3–24)	6 (1–24)	0.39
Syncopes and pre-syncopes per month[d]	1 (0.4–3)	1.3 (0.7–2.5)	1 (0.5–1.9)	0.07
Syncopal episodes per patient[d]	2 (1–3)	3.5 (1.7–6)	1.5 (1–3)	0.001
Pre-syncopal episodes per patient[d]	2 (0–8)	4.5 (0–18.5)	3 (0–6)	0.22
Number of patients with pre-syncopal episodes (%)	10 (66.7)	23 (67.6)	37 (66.1)	0.88
Number of patients with situational symptoms (%)	5 (33.3)	10 (29.4)	19 (33.9)	0.66
Number of patients with triggering factors (%)	5 (33.3)	17 (50.0)	20 (35.7)	0.18
Number of patients with vasovagal symptoms (%)	9 (60.0)	24 (70.6)	23 (41.1)	0.007
Adenosine plasma levels (μM)				
Baseline[c]	1.8 ± 1.8	1.9 ± 1.6	1.2 ± 1.2	0.02
Tilt[c]	2.4 ± 2.4	2.1 ± 1.7	1.3 ± 1.2	0.01
Syncopal episodes (52 positive tests)[c]	2.7 ± 1.7	2.5 ± 1.4	2.9 ± 1.4	0.20
End of test (45th min) (53 negative tests)[c]	0.3 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.53
Genotype				
Positive HUTT (%)	7 (46.7)	27 (79.4)	18 (32.1)	<0.001

[a]c.1364 T>C polymorphism, rs 5751876.
[b]Bonferroni adjusted *P*-value <0.016.
[c]Mean ± SD.
[d]Median (IQR).

of the genotype was in accordance with the Hardy–Weinberg laws of equilibrium ($P = 0.29$). Differences in the distribution of genotype frequencies were found in the patients with a negative HUT when compared with those with a positive HUT: 7 CC (13.2%) vs. 27 (52%) ($P < 0.0001$) and 38 TC (71.7%) vs. 18 (34.6%), respectively, ($P < 0.0001$). These differences were detected with a statistical power >95%. The TT variant distribution was similar in the two groups: 8 (15.1%) vs. 7 (13.4%). The number of syncopal episodes per patient was significantly higher in patients with the CC variant when compared with those with the TC variant ($P = 0.001$) (*Table 2*). On the other hand, the number of syncopes and pre-syncopes per month, the time since first episode, the number of patients with pre-syncopal episodes, with situational symptoms and triggering factors were not different. *Table 3* shows that CC genotype was associated with positive HUT, after adjustment for age, sex, the number of syncopal episodes per patient, the presence of vasovagal symptoms, and the presence of triggering factors. The model was reliable (Hosmer–Lemeshow test $P = 0.93$) and accurate (area under the curve = 0.82; 95% confidence interval: 0.74–0.90; $P \leq 0.001$).

Adenosine plasma levels

Baseline APLs were comparable in patients with a negative HUT and in those of the control group (*Table 4*). Patients with a positive test had significantly higher baseline APLs than those with a negative HUT. In patients with a negative HUT, APLs remained stable after 45 min of tilting (*Table 4*). Finally, APLs were higher in patients with CC variant compared with TC (*Table 2*).

Table 3 Independent predictors of head-up tilt-testing response (multivariate logistic regression)

Genotype	Adjusted odds ratio[a]	95% confidence intervals	*P*-value
TC	1		
TT	1.4	0.3–5.4	0.66
CC	5.1	1.7–15.4	0.004

[a]Odds ratio were systematically adjusted for age, sex, number of syncopal episodes per patient, presence of vasovagal symptoms, presence of triggering factors. Hosmer–Lemeshow test: $P = 0.93$.

Discussion

The main findings of this study were three-fold: (i) in patients with unexplained syncope, an over-representation of the genotype CC variant in the adenosine A_{2A} receptor gene was observed in the group with a positive HUT when compared with those with a negative HUT. Conversely, when the HUT was negative, the variant TC was more common; (ii) a higher number of spontaneous syncopal episodes per patient was observed in the

Table 4 Heart rate, arterial pressure, plasma adenosine levels in controls and patients

Variables	Controls ($n = 121$)	Patient population ($n = 105$)	*P*-value	Negative tilt tests ($n = 53$)	Positive tilt tests ($n = 52$)	*P*-value
Heart rate[a] (b.p.m.)						
Basal	72.8 ± 11.1	73.6 ± 11.8	0.61	73.7 ± 10.4	73.6 ± 13.1	0.83
Tilt		82.9 ± 14.8		81.4 ± 11.8	84.4 ± 17.3	0.41
End of test		66.9 ± 25.5		84.1 ± 13.7	49.4 ± 22.7	0.001
Arterial pressure[a] (mmHg)						
Basal	123 ± 17/72 ± 9	119 ± 15/72 ± 10	0.12/0.95	120 ± 13/72 ± 11	117 ± 17/71 ± 9	0.13/0.67
Tilt		121 ± 17/75 ± 12		120 ± 16/75 ± 13	121 ± 17/75 ± 11	0.88/0.84
End of test		87 ± 34.6/41 ± 36		117 ± 17/74 ± 11	57 ± 17/9 ± 19	0.001/0.001
Adenosine plasma level[a] (μM)						
Basal	0.41 ± 0.33	1.5 ± 1.5	0.0001	0.4 ± 0.2	2.5 ± 1.4	0.001
Tilt		1.7 ± 1.6		0.4 ± 0.3	2.8 ± 1.5	0.001
End of test		1.7 ± 1.5		0.4 ± 0.2	2.7 ± 1.4	0.001

[a]Mean ± SD.

group of CC genotype; (iii) the general distribution frequency of genotypes did not differ in the syncope patients from that of the control group.

The mechanisms of cardiogenic syncope have not yet been unravelled. Previous studies have suggested that endogenous adenosine may play a role in a subset of patients with unexplained syncope.[5] In brief, baseline APLs were found to be significantly higher in patients with a positive HUT than in those with a negative HUT or in those of healthy volunteers serving as a control group.[5] A positive correlation was also found in those with a positive HUT between rising plasma adenosine concentration and rapidity of symptom onset during HUT.[5,6] A_{2A} adenosine receptors are known to be implicated in the control of heart rate and blood pressure.[13,14] The expression of A_{2A} receptor at the membrane level was found to be increased in patients with positive HUT.[6]

The SNP (c.1364 T>C) is the most commonly found polymorphism in the A_{2A} receptor gene in the general population.[16,19] This study is to our knowledge the first to investigate the SNP (c. 1364 T>C) in patients with unexplained syncope. Despite the absence of a significant difference in SNP distribution frequency between the syncope patient group and the control group, the study showed differences between patients with a positive HUT when compared with those with a negative HUT, suggesting a significant association between c. T1364 T>C polymorphism and positive HUT. Consequently, the question arises whether the presence of the CC variant in the adenosine A_{2A} receptors is related to the susceptibility to develop syncope. Our results clearly identify a subset of patients with unexplained syncope, high basal APLs and with high incidence of the CC variant in the A_{2A} adenosine receptor gene. Substances, which are able to modulate the A2A receptors, deserve a trial in this subset of patients.

The group of positive HUT is also the group in which baseline APLs are high and clinically the group in which the frequency of spontaneous syncope attacks per patient is the highest. Such findings support the hypothesis that an adenosinergic mechanism may play a role in this subset of patients.

Limitations of the study

In the present report, we found a significant association between c. T1364 T>C polymorphism and positive HUT. However, because a 'silent' SNP do not produce altered coding sequence and therefore are not expected to change the function of the protein receptor, the question arises whether such a silent mutation could modify the interaction between adenosineA_{2A} receptors and adenosine in the susceptible patients. However, it was recently shown that a 'silent' polymorphism in MDR1 gene (a transmembrane protein without any relationship with adenosine receptors) changes substrate specificity by altering the folding, the insertion, and the quantity of protein expressed at the cell membrane.[23] It is possible that such a mechanism could be involved in adenosine A_{2A} receptors. The fact that the polymorphism in adenosine A_{2A} receptors gene is associated with habitual caffeine consumption[24] supports this hypothesis since caffeine is a well-known ligand for adenosine A_{2A} receptors.

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Annexe 3

“Peripheral plasma adenosine release in patients with chronic heart failure”

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Peripheral plasma adenosine release in patients with chronic heart failure

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ABSTRACT

Objective: Chronic heart failure (CHF) is accompanied by increased adenosine plasma levels (APLs). It is unknown whether adenosine release occurs at the peripheral level or whether the myocardium itself is the source of adenosine release. To answer this question, we evaluated APLs in the coronary sinus of CHF patients during a resynchronisation procedure and compared the values with those at the peripheral level. We also investigated a possible correlation between APLs and ischaemia-modified albumin (IMA) levels, a useful marker of tissue ischaemia.

Methods: 19 men and seven women were prospectively included. Blood samples for APLs were collected simultaneously from a brachial vein (peripheral) and from the coronary sinus. Blood samples for brain natriutretic peptide (BNP) and IMA were collected from a brachial vein.

Results: APLs from the brachial vein were higher than those from the coronary sinus (1.69 vs 0.75 μM $p<0.01$). IMA levels were correlated with APLs from the brachial vein ($r = 0.59$, $p<0.01$). BNP concentrations were correlated with APLs from the brachial vein ($r = 0.73$, $p<0.001$) but not with APLs from the coronary sinus ($r = 0.38$, $p>0.05$). BNP concentrations and IMA levels were correlated ($r = 0.71$, $p<0.001$).

Conclusions: In CHF patients, adenosine release occurs at a peripheral level and not at the myocardium level.

Chronic heart failure (CHF) results in the release of many neurohumoral factors, including catecholamines, renin-angiotensin and cytokines. Most of them worsen cardiac performances,[1–3] but some, like adenosine, could have primary beneficial effects on myocardial function.[4] Adenosine is a ubiquitous nucleoside that comes partly from ATP dephosphorylation and partly from cysteine metabolism.[5] Adenosine is released in the case of even minimal oxidative stress[6 7] or during ischaemia.[6 8–10]

Increased adenosine plasma levels (APLs) have been reported in CHF patients, and it has been suggested that this increase is secondary to an ischaemic process.[11 12] It is not known, however, whether the release of adenosine occurs at the peripheral level or comes from the impaired myocardium itself. To answer this question, we compared APLs measured simultaneously in the coronary sinus and at the peripheral levels of CHF patients. Furthermore, ischaemia-modified albumin (IMA) and adenosine are early and sensitive markers of ischaemia,[8 13 14] so we also looked for a relation between peripheral and coronary sinus APLs and between IMA level and APLs at the peripheral level. Indeed, establishing such a relation would support the assumption that increased APLs are related to an ischaemic process.

METHODS

Subjects and study design

Twenty-six patients with severe congestive heart failure and undergoing cardiac resynchronisation therapy (CRT) (19 men and seven women, mean age 69 (9) years; range 49–85) were included prospectively during a 6-month period and were classified according to the New York Heart Association (NYHA) functional classification. Ischaemic cardiomyopathy was defined as left ventricular systolic dysfunction associated with at least 70% narrowing of at least one of the three major coronary arteries or a documented history of myocardial infarction. Dilated cardiomyopathy was defined as left ventricular systolic dysfunction without coronary stenosis. Indications for CRT and study inclusion criteria according to the European Society of Cardiology guidelines, were (1) patients with severe heart failure (NYHA class III-IV) remaining symptomatic despite a complete pharmacological treatment, (2) low left ventricular ejection fraction $\leqslant$35% (echocardiography or ventriculography), and (3) left bundle branch block >120 ms[15]. Every patient remained in NYHA class III or IV, and no intravenous inotropic drugs were required. Heart failure was also assessed by measuring brain natriuretic peptide (BNP). Every patient had a coronary angiography in the 6 months preceding the CRT. We excluded patients in NYHA class I or II, patients with a recent acute coronary syndrome (<3 months) or a valvular stenosis, patients who had sepsis or infectious disease in the last 3 months and patients with an evolutive neoplasia. None of the patients was receiving a xanthine oxidase derivative.

Control subjects were 14 age-matched healthy staff members of the biochemistry laboratory and the cardiology department (six men and eight women, mean age 54 (6) years, range 47–65). None of the control subjects were taking medication, whether statins, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers. Patients and controls were instructed to avoid drinking coffee or tea 72 hours before the study. The investigation conformed with the principles outlined in the Declaration of Helsinki. All patients and control subjects gave written informed consent to participate in the study. The protocol was approved by the ethics committee for human research of the Centre Hospitalo-Universitaire de Marseille, Université de la Méditerranée.

Sample collection for APL measurement

Samples were collected during cardiac resynchronisation procedures. After local anaesthesia

(xylocaine 1%), a subclavian puncture was performed and an 8 F sheath (CPS Direct SL, St Jude Medical) was advanced. Then the coronary sinus was cannulated. Adenosine sample collection and treatment have been described.[9 16] For the coronary sinus samples, the lumen of the sheaths was washed out and filled with 1 ml of papaverine and 1 ml of dipyridamole, injected through the lateral entry of a three-way stopcock just before blood sampling. Blood (3 ml each) was taken through the axial entry of the stopcock using an ice-cold syringe containing 3 ml of the cold stop solution (dipyridamole 0.2 mM; Na2 EDTA 4.2 mM; (9-erythro-3 nonyl) adenine (EHNA) 5 mM; α-β-methylene adenosine 5′ diphosphates (AOPCP) 79 mM; heparin sulfate 1 UI/ml; Na Cl 0.9%) to prevent both adenosine uptake by red blood cells, deamination into inosine and ATP dephosphorylation by 5′nucleotidases.[16 17] For patients and healthy subjects, peripheral blood samples (3 ml) were collected in 3 ml of cold stop solution as previously described.[18 19] For patients, blood samples were collected simultaneously in the coronary sinus and in a brachial vein.

Sample collection for c-TnI, IMA and BNP measurement

Before sample collection for APL determination, 2 ml of whole blood was collected from a brachial vein in patients and healthy subjects.

Adenosine assay

Adenosine assay has been described.[18 20] In brief, samples were immediately centrifuged (4°C, 1500 *g*), haematocrits and proteins were evaluated, and then samples were deproteinised (perchloric acid, 70%) and lyophilised before being analysed by chromatography. A modular system with a diode array detector (Hewlett Packard, HP 1100, Palo Alto, CA, USA) was used. Lyophilised samples were dissolved in 1 ml of phosphate buffer and eluted with a methanol gradient (0–35% in 60 minutes) on a Merck LiChrospher C18 column (Nottingham, UK). Adenosine was identified by its elution time and by spectrum and quantified by comparison of peak areas with those given by known quantities of adenosine. The sensitivity threshold was 2 pMol/ml of plasma matrix. The intra-assay and inter-assay coefficients of variation ranged from 1% to 3%. Absence of xanthine derivatives was checked on each chromatogram.

Table 1 Clinical and biological characteristics of the study patients and controls

	Patients (n = 26)	Controls (n = 14)
Age (years, mean (SD))	69.0 (9.0)	54.0 (6.0)
Sex (men/women)	19/7	6/8
NYHA class (n)		
III	25	NA
IV	1	NA
Left ventricular ejection fraction (n)		
<0.20	5	0
0.20–0.24	14	0
0.25–0.30	7	0
Diabetes (n)	7	0
Chronic obstructive pulmonary disease (n)	5	0
Systemic hypertension (n)	6	0
Current smoker (n)	4	6
Atrial fibrillation (n)	4	0
Current treatment (n)		
Digitalis	2	0
Amiodarone	11	0
Aldosterone receptor antagonists	4	0
β-blockers	15	0
Diuretics	26	0
ACE inhibitors	19	0
Angiotensin II receptor blockers	6	0
Creatinine plasma levels (μM, mean (SD))	120.7 (48.0)	71.0 (12.0)
Urea plasma levels, (mg/l, mean (SD))	9.3 (4.5)	4.5 (2.7)
Coronary sinus APLs (μM, mean (SD))	0.75 (0.31)	NA
Brachial vein APLs (μM, mean (SD))	1.69 (0.68)	0.65 (0.19)
Brachial vein IMA (arbitrary units)	108.7 (15.0)	87.9 (10.6)
BNP (ng/ml, mean (SD))	506.8 (277.0)	

*p<0.05 compared with controls; **p<0.05 compared with values obtained in the coronary sinus.
ACE, angiotensin-converting enzyme; APLs, adenosine plasma levels; BNP, brain natriuretic peptide; IMA, ischaemia-modified albumin; NA, not applicable; NYHA, New York Heart Association.

Ischaemia-modified albumin measurement

IMA was evaluated as previously described.[21] In brief, the albumin cobalt binding (ACB) test is a quantitative in vitro diagnostic test that detects IMA by measuring the cobalt binding capacity of albumin in human serum (Ischaemia Technologies, Denver, CO, USA). IMA level (arbitrary units) was determined with a Synchron LX 20 analyser (Beckman Coulter, Villepinte, France). The coefficient of variation was <5%.

BNP measurement

Samples were collected on sodium EDTA. BNP assay was performed on the ADVIA Centaur analyser (Siemens Medical Solutions Diagnostics SAS, 92815, Puteaux cedex France). The ADVIA Centaur BNP assay is a fully automated two-site sandwich immunoassay using direct chemiluminescent technology, with two monoclonal antibodies. This assay measures the physiologically active BNP molecule (77–108), and antibodies are specific to the C-terminal portion. The assay range is from 2–50 ng/ml. The coefficient of variation (CV) at 2 pg/ml ranges from 10–20% according to the manufacturer's data.

Cardiac troponin I assay

Samples were collected on lithium heparin. Cardiac troponin I (c-TnI) was measured by immunoassay using the ADVIA Centaur analyser, according to the manufacturer's recommendations. This assay is three-site sandwich immunoassay using direct chemiluminometric technology. The manufacturer recommends an assay range of 0.06–50 μg/l. At 0.03 μg/l, the coefficient of variation was <10%.

Reagents

Adenosine (crystallised, 99%) and dipyridamole were supplied by Boehringer Mannheim; AOPCP, deoxycofrormycin and EDTA were supplied by Sigma Aldrich; EHNA was supplied by Burroughs Welcome; heparin sulfate was supplied by Sanofi Aventis (Paris, France); methanol and other reagents were supplied by Merck, France.

Statistical analysis

Patients' data were expressed as mean (SD). Correlations between biological parameters were quantified and tested using Spearman rank correlation coefficient. Comparisons of biological parameters between patients and controls were performed using non-parametric Mann-Whitney U test (for independent variables). the non-parametric Wilcoxon paired test was used to compare biological values obtained in the coronary sinus with those obtained at the peripheral level. All statistical tests were two-sided and p values less than 0.05 were considered statistically significant. Analysis was performed with SPSS software.

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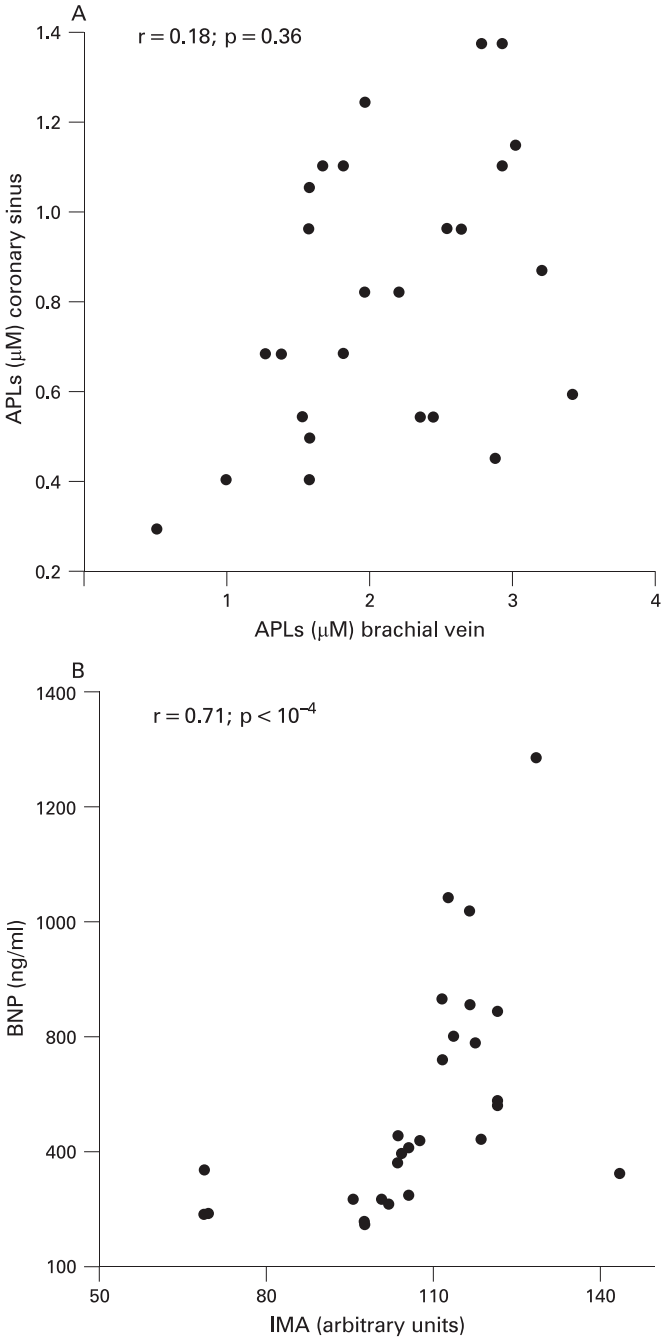

Figure 1 Correlation curves between adenosine plasma levels (APLs) (A); samples were collected simultaneously in the brachial vein and in the coronary sinus, in 26 patients with chronic heart failure. In (B), correlation curve between ischaemia-modified albumin (IMA) concentrations and brain natriuretic peptide (BNP) concentrations evaluated in samples collected from a brachial vein. r, Spearman's correlation coefficient.

RESULTS

Among patients, 25 were NYHA class III and one was NYHA class IV (see table 1). Systolic blood pressure (mean (SD)) was 131 (4) mm Hg and diastolic blood pressure was 78 (4) mm Hg. Heart rate was 72 (8) mm Hg. Ejection fraction was 25.38% (3.4%).

BNP concentrations

BNP concentration was higher than 200 ng/ml in every patient (see table 1). No significant difference in BNP concentration was found between patients with ischaemic heart disease and those with non-ischaemic heart disease (442 (228) vs 562 (311) ng/ml, p = 0.41).

c-TnI concentrations

Mean c-TnI concentrations remained low (see table 1). No significant difference was found between patients with ischaemic cardiomyopathy and those with dilated cardiomyopathy (0.09 (0.09) vs 0.08 (0.06), p = 0.32).

IMA concentrations

IMA level was significantly higher in patients than in controls (mean + 24%, p<0.01). No significant difference was found in IMA concentration between patients with ischaemic cardiomyopathy and those with dilated cardiomyopathy (100 (15) vs 116 (12.6); p = 0.14).

APLs

APLs were higher (mean 2.25-fold) in the brachial vein than in the coronary sinus (table 1). No significant difference was found in APLs between the patients with ischaemic cardiomyopathy and those with dilated cardiomyopathy (coronary sinus: 0.73 (0.35) vs 0.77 (0.30; p = 0.59; brachial vein: 1.41 (0.47) vs 1.92 (0.77); p = 0.94). On the other hand, APLs evaluated in the coronary sinus were not different from those measured in the brachial vein of healthy subjects (table 2).

Correlation data

No correlation was found between APLs in the coronary sinus and in the brachial vein (Spearman's r = 0.18, p = 0.36). In contrast, APLs in the brachial vein were correlated with both IMA and BNP concentrations (fig 1). APLs in the coronary sinus were not correlated with IMA or BNP concentrations (see fig 2). A correlation was observed between IMA and BNP concentrations, but no correlation was found between c-TnI and IMA or between c-TnI and APLs.

DISCUSSION

The present study shows that in patients with severe CHF, APLs are significantly higher at the peripheral level (brachial vein) than in the coronary sinus. This finding suggests that adenosine release in these conditions occurs mainly at the peripheral level.

Cause of high levels of adenosine and IMA

Adenosine release is likely to occur during an ischaemic process,[8 22] which can also result in an increased IMA level.[13 14] The increases observed at the peripheral level in APLs and in IMA, as well as the correlation between these two variables, support the hypothesis that the adenosine release was secondary to a peripheral ischaemic process. In a few cases, APLs were increased and IMA levels were normal. These data suggest that APLs could be a more sensitive marker of ischaemia than IMA.

At the myocardial level, most of the adenosine comes from ATP dephosphorylation and it has been established that myocardial ATP remains normal even at advanced degrees of heart failure.[23–25] Furthermore, low myocardial adenosine level was found in an animal model of cardiac failure.[26] These observations and experimental findings are consistent with our finding that the myocardium is not the source of the high APLs in CHF patients.

Compared to controls, patients with severe CHF had high IMA levels. IMA results from the modification of the N-terminus cobalt-binding sites of albumin caused by the release

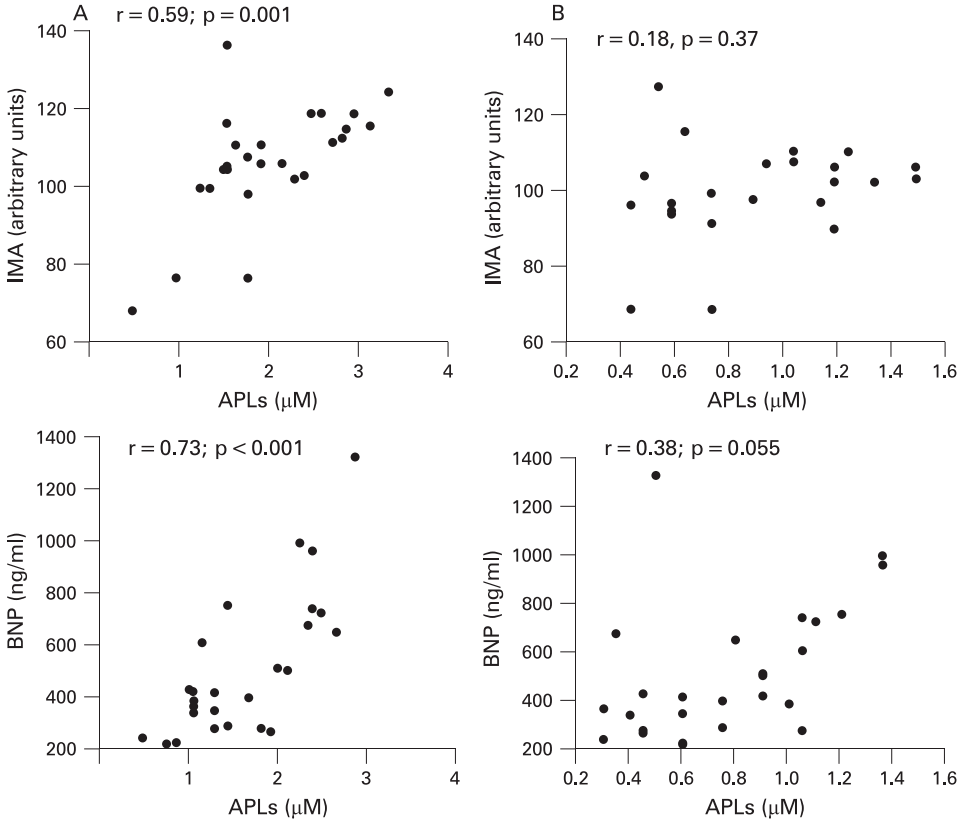

Figure 2 Correlation data between adenosine plasma levels (APLs) and ischaemia-modified albumin (IMA) or brain natriuretic peptide (BNP). Blood samples were collected from a brachial vein (A) and the coronary sinus (B). r, Spearman's correlation coefficient.

of free radicals from ischaemic tissue. The IMA concentration increases only a few minutes after the beginning of even a minimal ischaemic process, and its level is correlated with the size of the ischaemic tissue.[13] Thus, IMA can be a potential marker of transient myocardial[27 28] or non-myocardial ischaemia.[29] The present study strongly indicates that an ischaemic process occurs outside the myocardium, at a peripheral level. In CHF patients the decrease in blood flow can induce a relative ischaemic process in most tissues, which is responsible for the release of adenosine and the increase in IMA concentration. Indeed, adenosine and IMA are very sensitive markers of tissue ischaemia. The increase in BNP level is associated with the severity of CHF[30] and its concentration is inversely correlated with the left ventricular ejection fraction.[31] That IMA was correlated with BNP concentrations supports the hypothesis that a peripheral ischaemic process secondary to a decreased blood flow occurs in severe CHF.

Adenosine release can also happen at the renal level through the 5′ nucleotidase activation and dephosphorylation of AMP,[32 33] especially during an ischaemic process.[34] APLs can also be increased in patients with chronic renal failure, but this increase has been observed only in patients with end-stage renal failure under haemodialysis treatment.[35 36]

Nine patients among 26 had a weak increase in c-TnI level (between 0.1 ng/ml and 0.39 ng/ml). This could be considered in such patients without acute coronary syndrome as an independent prognostic marker of in-hospital mortality.[37] Also, CHF patients with a weak elevation in cardiac troponin T (c-TnT) level (>0.012 ng/ml) have more severe CHF and worse outcome.[38] In the present study, however, the number of patients is too small to allow any conclusion on the prognostic significance of c-TnI levels.

Possible effects of adenosine release in CHF patients

It was suggested that dipyridamole, which increases APLs,[39] improves ventricular systolic function in patients with CHF.[40 41] Extracellular adenosine has cardiac actions, mediated through the activation of adenosine receptor subtypes, called A_1, A_{2A}, A_{2B} and A_3 depending on the pharmacological properties of receptor subtypes.[42 43] It is well known that adenosine protects the heart during ischaemic reperfusion via the activation of A_1 and A_3 receptors.[44 45] However, the effects of activation of A_1 adenosine receptors on left ventricular function are debated. In patients with left ventricular dysfunction, the activation of A_1 adenosine receptors had no significant effects on haemodynamic changes.[46] A more recent experimental study indicates that activation of these receptors may attenuate cardiac hypertrophy in murine left ventricular pressure-overload model.[47] However, A_1 adenosine receptor blockade could have beneficial effects on CHF patients by improving renal function and diuresis.[48]

Adenosine increases coronary blood flow via the activation of A_{2A} and A_{2B} receptors.[49 50] Activation of A_{2A} receptors improves contractile performances of the myocardium[51–53] and inhibits cytokine production and inflammatory response.[54] Cytokine production and inflammatory response, however, are involved in cardiac depression.[55] Finally, endogenous adenosine also reduces myocardial oxygen consumption in the failing heart[48] and decreases oxygen radical release from activated neutrophils, thus preventing endothelial cell damage.[10]

Study limitations

Although the present study included enough patients to reach statistical significance, additional studies including a larger number of patients are needed. Indeed, only patients with

Heart 2009;**95**:651–655. doi:10.1136/hrt.2008.155242

severe CHF have been included. It would be of interest to include patients with less severe CHF in further studies.

CONCLUSION

High APLs in CHF patients do not result from a myocardial release but are secondary to a peripheral release. This release is probably caused by an ischaemic process, probably because of a decreased blood flow secondary to left ventricular failure.

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