Design, synthesis and pharmacological evaluation of new adenosine receptor ligands

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1. Introduction

Adenosine signaling has long been a target for drug development, with adenosine itself or its derivatives being used clinically since the 1940s. In the 1970s it became clear that there are receptors for adenosine, and there was evidence that methylxanthines such as caffeine, the most commonly consumed drug in the world, produces many of its actions by acting as antagonists at adenosine receptors. Towards the end of the twentieth century it became clear that there are subtypes of adenosine receptors, now recognized as a family of four, that are present in most vertebrates. In fact, extracellular adenosine exerts its actions via specific G protein-coupled receptors (GPCRs) divided into four adenosine receptor (AR) subtypes termed A₁, A₂A, A₂B and A₃. Since the heterogeneity of ARs and their widespread expression throughout the body, the development of drugs that selectively affect specific AR subtypes and/or specific components of adenosine signaling will hopefully provide new therapeutic opportunities for a large variety of human diseases. The knowledge of the complexity of regulatory mechanism controlling the receptor biological response under physiological and pathological conditions (1) (2) represent a crucial starting point in the development of adenosine-based drugs. In fact, these receptors are widely expressed and it was demonstrated that they are implicated in several biological functions, both physiological and pathological. These include cardiac rhythm and circulation (3) (4), lipolysis, renal blood flow (5) (6), immune function (7), sleep regulation (8) (9), angiogenesis (10), inflammatory diseases (11) (12), ischemia-reperfusion (13) and neurodegenerative disorders (14). Adenosine itself is used clinically (15) for the treatment of supraventricular tachycardia (16) (15), and many clinically used drugs (including dipyridamole and methotrexate) may exert their effects by altering extracellular adenosine concentrations and signaling. In fact, several pharmacological compounds specifically targeting individual ARs have now entered the clinic or clinical studies are ongoing. However, only one AR specific agent, the A₂A AR agonist regadenoson (Lexiscan; Astellas Pharma), has so far gained approval from the US Food and Drug Administration (FDA). Many research programs focused on the adenosine signaling in order to identify bioactive compounds that could be useful for the therapy of many AR-mediated diseases (17). The complexity of adenosine signaling was primarily responsible for the failure of one of the largest clinical trials for an A₁ receptor antagonist so far (18) (19). Moreover, since its discovery in the 1983, A₂B AR lack a selective and potent full agonist that could be helpful in order to deepen the physiopathological properties of this receptor (20). In fact, until now NECA, a non-selective AR agonist, has been used as elected ligand for the activation of the A₂B AR for the understanding of the signaling pathway mediated by this receptor, but, obviously with scarce results (21).
1.1 G-Protein Coupled Receptors

The superfamily of seven transmembrane domain (7TM) G-protein coupled receptors (GPCRs) represent the largest family of membrane proteins in the human genome and nowadays the richest source of targets and then drugs for the pharmaceutical industry. There has been remarkable progress in the field of GPCR biology during the past three decades. Notable milestones include the cloning of the first GPCR genes, and the sequencing of the human genome revealing the size of the GPCR family and the number of orphan GPCRs. In fact, a recent and detailed analysis of the human genome reveals over 800 unique GPCRs, of which approximately 460 are predicted to be olfactory receptors (22). Based on sequence similarity within the 7TM segments, these receptors could be described by belonging to five families (23):

- the rhodopsin family (701 members)
- the adhesion family (24 members)
- the frizzled/taste family (24 members)
- the glutamate family (15 members)
- the secretin family (15 members).

The physiologic function of a huge part of these 800 GPCRs is nowadays unknown: these receptors are referred to as orphan GPCRs. However, deorphanization of non-olfactory GPCRs is tough process (24), as they are a promising group of targets for the pharmaceutical industry. Actually, the number of still orphan GPRS is significantly decreasing, highlighting the efforts of scientific community on this task.

1.1.1 Structure and ligands

GPCRs bear a common structural signature made of seven hydrophobic transmembrane (TM) segments, with an extracellular amino terminus and an intracellular carboxyl terminus (Figure 1). The most variable structures among the family of GPCRs are the COOH terminus, the intracellular loop between TM5 and TM6, and the NH₂ terminus, where the greatest diversity is observed. This sequence is relatively short (10–50 amino acids) for monoamine and peptide receptors, and much larger (350–600 amino acids) for glycoprotein hormone receptors, and the glutamate family receptors. The largest amino terminal domains are observed in the adhesion family receptors. Interestingly, this structural and functional similarity of GPCRs stands in contrast to the structural diversity of the natural GPCR ligands (25).
In fact, ligands can range from subatomic particles (a photon), to ions (H\(^+\) and Ca\(^{2+}\)), to small organic molecules, to peptides and proteins. While many small organic agonists bind within the TM segments, peptide hormones and proteins often bind to the amino terminus and extracellular sequences joining the TM domains. However, size of the ligand alone cannot be used to predict the location of the binding site: for instance, glycoprotein hormones, glutamate, and Ca\(^{2+}\) all activate their respective receptors by binding to relatively large amino terminal domains (25) (26). Furthermore, it has been possible to identify small allosteric modulators that bind at the TM domains for many GPCRs that bind their native agonists on the extracellular loops or the NH\(_2\) terminus (27) (28) (29). Our understanding of GPCR structure is based largely on the high-resolution structures of the inactive state of rhodopsin (Figure 2). Rhodopsin is commonly used for structural studies than most other GPCRs because it is possible to obtain large quantities of highly enriched protein from bovine retina and it is also a remarkably stable GPCR, retaining function under conditions that denature many other GPCRs.
Moreover, there are many evidences that let to affirm that GPCRs exist as dimers (or oligomers) and that these dimers may be important for G protein activation. Dimerization is an important mechanism of receptor activation for the glutamate family of GPCRs (26) (30), where ligand-induced changes in the dimer interface of the amino terminal ligand binding domain has been demonstrated by crystallography (31) (32). However, the role of dimerization in the activation of rhodopsin family members is less clear and it remains to be seen if a receptor dimer is required for G protein activation in some cases. The effect of agonist binding on the formation or disruption of dimers is not consistent among the rhodopsin family members that have been examined (33). Moreover, ligands interact with individual receptor monomers, and there is currently no evidence that ligands could stand in between receptor dimers or span the dimer. If changes in dimerization
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occur, it is likely a frequently occurring secondary consequence of ligand-induced changes in the arrangement of the TM segments. Evidence in support of this comes from biophysical studies on leukotriene B4 homodimers demonstrating that ligand binding to one protomer leads to conformational changes in its partner (34). While dimers may be important for G protein activation, it is essential to understand the agonist-induced structural changes that occur in the context of individual GPCR monomers.

1.2 Purinergic Signaling

Adenosine receptors or P1 receptors belong to the large family of purinergic receptors, described and divided in two subtypes, P1 and P2, by Geoffrey Burnstock in 1972 (35). Burnstock introduced the concept of purinergic signaling after it was shown that ATP was a transmitter in non-adrenergic, non-cholinergic inhibitory nerves in the guinea-pig taenia coli (36) and the term purinergic receptor was introduced only to describe specific membrane protein that mediate relaxation of gut smooth cells activated by adenosine triphosphate (ATP) (P2) or adenosine (P1). P2 receptors have further been divided into two families, P2Y and P2X. P2X receptors are ligand-gated ion channels, whereas the P1 or adenosine receptors (ARs) A1, A2A, A2B and A3 and P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) are GPCRs. P2X receptors are nonselective cation channels responsible for mediating excitatory postsynaptic responses, similar to nicotinic and ionotropic glutamate receptors. P2X receptors are distinct from the rest of the widely known ligand-gated ion channels, as the genetic encoding of these particular channels, indicates the presence of only two transmembrane domains within the channels. These purinergic receptors have been shown to be widely distributed throughout the CNS, being present early in evolution in neurons and glia; therefore, are arguably the most abundant receptors in living organisms (37). ATP acts as neurotransmitter while interacting with P2 receptors mainly as a fast excitatory response mediator or as a potent mediator in long-term processes like cell proliferation, growth and development, diseases and cytotoxicity. Nowadays, it is not fully clarified whether various physiological nucleotide-receptor agonists (e.g. ATP, ADP, UTP, UDP, UDP sugars and NAD⁺) are released by common mechanisms or not. The main ways through which ATP is released extracellularly at this moment known are:

- Exocytotic neuronal vesicular release, (38)
- Vesicular release of ATP from astrocytes, (39) which perhaps involves lysosomes, (40)
- ATP binding cassette transporters, (41)
- Connexin or pannexin hemichannels, (42)
- Plasmalemmal voltage-dependent anion channels, (43)
- P2X7 receptors, (44)
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- Vesicular nucleotide transporter (VNUT), secretory and synaptic vesicles in which ATP can be accumulated and released by a Cl- dependent vesicular nucleotide transporter. (45)

ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases after release. Ectonucleotidases are divided in several families and each everyone of these families are expressed in the brain:

- Ectonucleoside triphosphate diphosphohydrolases E-NTPDases, hydrolyze ATP and ADP to AMP,
- Ectonucleotide pyrophosphatase and/or phosphodiesterases E-NPPs hydrolyze ATP and ADP to AMP and Dinucleoside polyphosphates, NAD+ and UDP sugars,
- Ecto-5'-nucleotidase also called CD73 (Cluster of Differentiation 73) hydrolyze AMP to adenosine,
- Alkaline phosphatases equally hydrolyze nucleoside tri, di and monophosphates. (37)

1.2.1 Purinergic Transimission

Nucleotides and nucleosides signaling that define the purinergic transmission can be seen as a temporal sequence. (46) Nucleotides released physiologically or pathologically from intracellular sources provide either an immediate or a delayed response to a local challenge to tissues or cells. (47) The released nucleotides can act on the same or on neighboring cells, either to open fast cation channels or for signaling on a slower time scale at metabotropic GPCRs. On a more prolonged time scale, the adenine nucleotides are converted by the sequential action of ectonucleotidases to the nucleoside adenosine for activation of the ARs (Figure 3). Thus ectonucleotidases control the lifetime of nucleotide transmitters and additionally, by degrading or interconverting the originally released ligands, they also produce mediators for additional P2 receptors and nucleosides. Besides the catabolic pathways, nucleotide interconverting enzymes exert their action by nucleotide re-phosphorylation and extracellular synthesis of ATP (e.g. ectonucleoside diphosphate kinase and adenylate kinase). Furthermore, adenosine can also be sourced intracellularly, through subpopulations of neurons and/or astrocytes that release adenosine directly. (48). These transformations and sequential receptor effects are related to overall biological functions irregularly occurring, such as cytoprotection, vascular adaptations, and immune effects, which may be effects in contrast at different points in the time sequence (e.g. the rapid effects of nucleotides on P2 receptors tend to boost the immune response, while the slower AR effects generally attenuate the immune response). (46)
1.3 Adenosine Receptors

As previously said the adenosine receptors subtypes (A₁, A₂A, A₂B and A₃) are members of the superfamily of GPCRs belonging to the subfamily of rhodopsin-like receptors and thus, show the typical heptahelical structure. Within the rhodopsin-like family A GPCRs, the TM domain is more highly conserved than the extracellular loops (ELs). TM helices, consisting of 25-30 amino acid residues each, are connected by six loops, three intracellular (IL) loops and three ELs. A disulfide link is formed between two cysteine residues present in TM3 and EL2, which are conserved in most GPCRs in order to promote packing and stabilization of a restricted number of conformations of these 7 TMs. Extracellular regions contain sites exerting post-translational modifications, such as glycosilation. The A₁ and A₃ contain also sites for palmitoylation in the C-terminus domain. The A₂A AR has a long C-terminal segment of more than 120 amino acid residues, which is not required for coupling to Gs, probably useful as a binding site for “accessory” proteins. The sequence
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identity between the human (h) A₁ and A₂ ARs is 49%, and the hA₂A and hA₂B ARs are 59% identical. Some of these particular conserved residues point to specific functions. In deep, there are two peculiar His residues in TMs 6 and 7 of A₁, A₂A, and A₂B ARs: in the A₁ AR, this His residue is lacking but another His residue can be found in TM3. All this His residues have been indicated by mutagenesis studies to be important in recognition and/or activation of the receptor (1) (49). In the beginning, adenosine receptors were divided in subtypes A₁ and A₂ following their ability to increase or decrease the activity of adenylate cyclase (AC), respectively (50) (51). Then, A₂ receptors were further classified, by Daly and coworkers (52), relying on their affinity for the endogenous ligand adenosine: A₂A with high affinity (0.1-1 μM) and A₂B with low affinity (>10 μM). Moreover, A₃ AR was identified in 1991 through a polymerase chain reaction (PCR) performed on rat cDNA encoding a GPCR that showed high affinity (58%) with A₁ and A₂A AR (53). This “new” receptor revealed an unconventional low homology between its homologous in other species (e.g. 72% versus rat A₃ AR) considering the other ARs (85-95 % of homology versus rat).

1.3.1 Crystallographic structure

Despite 25 years were passed since the discover of all AR subtypes, nowadays only the crystal structure of A₂A AR has been determined. Nevertheless, ARs characterization began long before the determination of the crystal structure of A₂A AR: prior to the report of the crystallographic structure of the A₂A AR (54), the only method to represent the 3D structure of the ARs passed through molecular modeling studies. This approach relied on information from convergent sources: homology modeling of the protein and ligand docking, receptor mutagenesis (1995) (55), and both qualitative and quantitative structure activity relationship (SAR) of ligands. The structure of the A₂A AR was determined in multiple forms with different ligands. Both antagonist-bound and agonist-bound states of the receptor were solved initially by the Stevens’s group using the stabilizing fusion protein T4 lysozyme. Jaakola and coworkers (54), obtained the 2.6 Å crystal structure of A₂A AR engineered the receptor to improve on thermostability by the insertion of the very stable lysozyme from T4 bacteriophage (T4L) for most of the third intracellular loop (Leu209-Ala221), and by deletion of the larger part of the very flexible C terminus (Ala317-Ser412) (Figure 4). This construct (A₂A-T4L-C) was further stabilized during purification with high concentrations of sodium chloride and cholesterol and a receptor-saturating concentration of the antagonist theophylline, which was replaced by the high affinity agonist 4-(2-(7-amino-2-(2-furyl)-1,2,4-triazolo[2,3-α]-1,3,5-triazin-5-yl)amino)ethyl)phenol (ZM241385, Figure 14) in the final purification step. A₂A AR complexes crystallized by Heptares Therapeutics using thermostabilized receptors by systematic scanning mutagenesis (StaRs) include several other agonists and antagonists (56).
Figure 4: Crystal structure of A2A-T4L-ΔC. (A) Overall topology of A2A-T4L-ΔC. The transmembrane part of A2A-ΔC structure is colored brown (helices I to VIII), and the T4L is in cyan. The structure is viewed perpendicular to the plasma membrane. ZM241385 is colored blue, and the five lipid molecules bound to the receptor are colored red. The four disulfide bonds are yellow. The sulfate ions are omitted. The extracellular loops (ECL1 to 3) are colored green, and the intracellular loops are colored blue (54).

The differences in the particular architecture of agonist- and antagonist-bound conformations have been analyzed in detail for the A2A AR and by analogy to other ARs (57). The conformation of the A2A AR complex with UK-432097 (Figure 5) (57), although lacking a G-protein or equivalent, displays features of an active-like conformation, e.g., ionic lock spreading. TMs 5, 6, and 7 engage in most of the conformation movement that leads to receptor activation, while the only pronounced movement within TMs 1-4 is a piston-like upward movement of TM3 to accommodate an agonist.
Figure 5: Ligand UK-432097 and its binding cavity. (A) Overall view of the ligand-binding cavity. The transmembrane part of A2AAR is shown as ribbon and colored gray (helices I to VII). Ligand UK-432097 is shown as bright green stick. For comparison, ligand ZM241385 (gray stick) is also shown at the position when the two complex structures were superimposed. The binding cavity is represented by orange. (B) Schematic representation of the hydrogen-bonding interactions (green dashed lines) between A2AAR and UK-432097. (C) Molecular interactions within the ligand-binding cavity for UK-432097 around the adenine moiety. Interacting residues are shown as sticks with side chains colored orange. Hydrogen bonds between A2AAR and ligand are shown as black dotted lines. (D) Molecular interactions around the ribose moiety. (E) Molecular interactions around the ligand substitution sites. A water molecule is shown as purple sphere. In (B)-(E), residue mutations reported to disrupt agonist and/or antagonist binding are indicated with orange squares (57).
The agonist-bound structure of A$_{2A}$AR features a contracted ribose binding region in which hydrophilic residues (His) of the pocket are drawn toward H bonding groups of the ribose (Figure 6).  

![Figure 6: Structures of the hA$_{2A}$R in cartoon representation are shown bound to the following ligands: (a) ZM241385; (b) NECA; and (c) adenosine.](image)

### 1.3.2 Homomers and Heteromers

Adenosine receptors, like other class A GPCRs, have been thought to exclusively occur in a monomeric state for a long time. Monomeric receptors are enough to induce signaling. At least some studies suggest signaling via dimers occurs only at higher receptor densities. More recently, however, evidence is accumulating that adenosine receptors can form dimeric or, more generally speaking, multimeric or oligomeric structures. Through self-association, homo-oligomers (“homomers”) can be formed. Hetero-oligomerization leading to “heteromers” may be the consequence of the association between adenosine receptors and preferred partners, mostly other GPCRs, including other adenosine receptor subtypes. This phenomenon has been demonstrated through a variety of experimental techniques, mostly in artificial cell lines. The use of overexpressed recombinant receptors may result in the creation of many more oligomers than naturally exist. In some cases, bioluminescence resonance energy transfer (BRET) may detect interactions between receptors that are in close proximity in a compact structure such as a coated pit, rather than forming true molecular dimers. Furthermore, GPCRs contain hydrophobic regions that can oligomerize, even after solubilization in sodium dodecyl sulfate (SDS). Hence, receptor dimerization or oligomerization may occur after solubilization in detergent and not be representative of receptor structure and organization in the membrane (1).
In theory, four homomeric pairs can be predicted for adenosine receptors, but only reports with experimental evidence for the occurrence of A1-A1 and A2A-A2A homomers have been published.

A1-A1. Both Ciruela (58) and Yoshioka (59) used antibodies against the wild-type adenosine A1 receptor to show how immunoprecipitation experiments analyzed with Western blotting revealed higher order bands in some instances. In another recent study by Briddon (60), A1-A1 homomers, predominantly located at the cell surface, were identified with Bimolecular Fluorescence Complementation (BiFC) techniques in CHO cells expressing yellow fluorescent protein (YFP) tagged receptors.

A2A-A2A. The first evidence for A2A receptor homodimerization was provided by Canals et al. (2004)60. The authors used both FRET and BRET techniques as well as immunoblotting to show that in transfected HEK293 cells, overexpressed recombinant A2A AR exist as both homodimers and monomers. They demonstrated, by means of cell surface biotinylation experiments, that after detergent solubilization, approximately 90% of the cell surface recombinant A2A AR species exists as a homodimer. The same pattern of dimer formation was observed for an engineered A2A AR lacking the C terminus, whereas this receptor mutant was no longer able to dimerize with the dopamine D2 receptor. A2A AR homodimerization was also demonstrated with BiFC techniques by Vidi (61), who also used a combination of fluorescence resonance energy transfer (FRET) and BiFC techniques to demonstrate that recombinant A2A AR exist as higher order oligomers, consisting of at least three monomers, at the plasma membrane of differentiated neuronal cells.

Available evidence points to the interaction of both A1 and A2A ARs with other GPCRs, however no direct data have been reported for adenosine A2B and A3 ARs.

A1-A2A. The two receptors are co-localized in striatal glutamatergic nerve terminals, both pre- and post-synaptically. This was demonstrated in immunogold blotting and, after detergent solubilization, co-immunoprecipitation experiments (62). In HEK293 cells transfected with tagged A1 as well as A2A ARs, evidence in BRET and FRET experiments was found for a direct interaction between the two recombinant receptors. Radioligand binding studies in membranes of these Human Embryonic Kidney 293 cells (HEK293) demonstrated that agonist binding to the A2A AR influenced the affinity of (R)-N6-phenylisopropyladenosine (R-PIA, Figure 11) for the A1 AR, but not vice versa. These findings constitute a mechanism for fine-tuning the release of glutamate by adenosine.

Nevertheless, heteromerizations such as A1-P2Y1 (59), A1-P2Y2, A1-D2, A2A-CB1 (61) and A2A-D2-CB1, A2A-D3, A1-Thromboxane A2 (1) have been demonstrated in many studies in literature.

A2A-D2 The heteromeric pair of A2A AR and D2 receptors is probably the most studied combination. In fact, double immunofluorescence experiments with confocal laser microscopy showing substantial co-localization of recombinant A2A AR and dopamine D2 receptors in cell membranes of SH-SYSY human neuroblastoma cells stably transfected with human D2 receptor, as well as in cultured striatal cells have been performed (63) (64)
There are evidences that heteromerization may also occur between adenosine receptors and proteins belonging to other superfamilies, in particular adenosine deaminase and calmodulin, like A₁-D₁ with adenosine deaminase and A₂A-D₂ with calmodulin. There is thus very good evidence that recombinant adenosine receptors can form homo- and heteromultimers. It is less clear, however, whether this has important pharmacological consequences in physiological tissues. In a dimeric complex, both receptors may not be simultaneously active and signaling. This implies that the signaling pathway and consequentially the derived response can be very complex. It is unclear which rules determine how partnerships are formed. Thus, the fact that adenosine receptors can form multimeric complexes with different members will necessitate much more work on signaling in intact cells in vivo (65).

1.3.3 Desensitization

Continued or repeated exposures to an agonist usually result in a flat receptor-mediated response followed by decreasing of this response despite the continual presence of agonist. Kinetically, this process is called desensitization (66). GPCRs undergo desensitization through a complex phenomenon involving multiple and temporally distinct components. The mechanisms underlying a rapid desensitization frequently involve receptor phosphorylation by a family of G protein-coupled receptor kinases (GRKs) resulting in their preferential binding to arrestins, that promote desensitization by uncoupling the receptor from its G-protein, leading to impaired receptor function (67). Following desensitization, many GPCRs internalize by an arrestin-dependent process via clathrin-coated pits which leads to the subsequent intracellular dephosphorylation of the receptor, and its re-insertion into the cell membrane leading to a resensitized state (68). More prolonged agonist activation generally leads to a subsequent down-regulation by lysosomes (69). These processes that form the physiological life of a receptor are deeply involved in the receptor-related response. It has been already shown that activation of all four AR subtypes eventually leads to their desensitization by different mechanisms. Actually, most of these observations are made following administration of non-hydrolysable agonists to cell cultures and animals and it is yet unclear how significant a role receptor desensitization plays in regulation of ARs by adenosine in vivo under physiological conditions. Studies have shown that upon addition of agonists, the A₁ AR is phosphorylated and internalized slowly, with a half-life of several hours. In contrast, A₂A and A₂B ARs demonstrate a more rapid rate of down-regulation, usually lasting about an hour. Finally, the down-regulation and desensitization of the A₃ AR occurs within minutes (70). Thus, it is important to understand their regulation in order to design drugs that can exploit or avoid the receptor-mediated signaling to treat diseases.
**A<sub>1</sub> AR:** Several studies have been carried out to understand the molecular mechanisms underlying A<sub>1</sub> AR desensitization. In an early study, Parson and Stiles (71) showed that the A<sub>1</sub> AR in rat adipocytes desensitized upon chronic administration of A<sub>1</sub> AR agonist R-PIA (Figure 11), over a period of six days by Alzet minipumps (Alza Corporation, Vacaville, CA, USA). A decrease in A<sub>1</sub> AR levels and reduced inhibition of isoproterenol-stimulated adenylyl cyclase activity was detected following R-PIA treatment. These changes were associated with decreased levels of pertussis-sensitive G proteins, but an increase in Gs proteins in the plasma membranes. Furthermore, adipocyte membranes obtained from R-PIA-treated rats showed enhance isoproterenol and forskolin-stimulated adenylyl cyclase. These studies suggest that adipocytes increase the stimulatory response (i.e., increase in adenylyl cyclase activity) because of a chronic activation of the A<sub>1</sub> AR. A subsequent study performed in cultured adipocytes confirmed the in vivo findings and further demonstrated that desensitization of the A<sub>1</sub> AR was associated with desensitization of insulin-dependent glucose transport (72). This suggests a common link between desensitization of the A<sub>1</sub> AR and insulin receptor. A later study by Longabaugh and coworkers (73) confirmed the previous findings that desensitization of the A<sub>1</sub> AR to R-PIA was linked to reductions in A<sub>1</sub> AR and Gi proteins and an increase in Gs<sub>a</sub> proteins, but showed that the changes in Gi<sub>a</sub> proteins were not dependent by alternations in their mRNA levels. Jajoo et al. (74) deepened the role of β-arrestin 1/extracellular signal-regulated kinase (ERK1/2) MAPK pathway in the regulation of A<sub>1</sub> AR desensitization and recovery in ductus deferens smooth muscle (DDT1-MF2) cells. They reported that applying the A<sub>1</sub> AR agonist R-PIA for 24 h resulted in a decrease of A<sub>1</sub> AR on the membrane which was associated with an unexpected 11-fold increase in A<sub>1</sub> AR mRNA. This effect was dependent on β-arrestin 1, knocked-down by siRNA blocked R-PIA-mediated down-regulation of the A<sub>1</sub> AR. In addition, β-arrestin 1 knockedown by siRNA suppressed R-PIA-dependent ERK1/2 and activator protein-1 (AP-1) activities and reduced the induction of A<sub>1</sub> AR mRNA. Interestingly, withdrawal of the agonist after a 24 h exposure resulted in rapid recovery of plasma membrane A<sub>1</sub> AR, which was dependent on the de novo protein synthesis and on the activity of ERK1/2 but independent of β-arrestin1 and NF-κB. These findings suggest that the β-arrestin 1/ERK1/2 pathway, which contributes to the desensitization and down-regulation of A<sub>1</sub> AR, is also able to prime the transcriptional machinery for rapid synthesis of the A<sub>1</sub> AR after withdrawal of the agonist. Chick embryo retina cultures of eight days treated for 48 h with CGS 21680 and N6-(2(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl)-adenosine (DPMA), selective A<sub>2a</sub> AR agonists, showed an increased expression of A<sub>1</sub>AR. This effect was blocked by the selective A<sub>2a</sub> AR antagonist, ZM 241385 (Figure 14), and PKA inhibitor N-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H89) (75). These in vitro findings were confirmed in an in vivo study where chick embryo retinas were treated for 48 h with A<sub>1</sub> AR agonist CGS 21680 (Figure 13). In this study, CGS 21680 was able to increase the expression of A<sub>1</sub> AR which was blocked by selective A<sub>2a</sub>AR antagonists, SCH 58261 and ZM 241385. Interestingly, under normal conditions, endogenous adenosine produced and released into the environment of the retina appears to control A<sub>2a</sub> AR-mediated
expression of A₁ AR, since the treatment with ZM 241385 and SCH 58261 aa alone was capable of reducing the expression of A₁ AR (76). These findings indicate that up-regulation of A₁ AR by long-term activation of A₂A AR depends on classical AR signaling pathway.

**A₂A AR:** Short-term agonist exposure induced a rapid desensitization of A₂A AR-stimulated AC activity which was associated with a diminished receptor-Gs coupling, and agonist-stimulated phosphorylation of the A₂A AR. Longer agonist treatment, on the other side, resulted in down-regulation in total receptor number and up-regulation of α-subunits of Gᵢ (77). The structural requirements necessary for the agonist-induced desensitization of A₂A AR resides in its COOH-terminus. Palmer and Stiles (78) introduced various mutations in the 95 amino acid sequence of the COOH-terminus of A₂A AR which contains ten different phosphorylation sites and identified Thr298 to be essential in agonist-induced receptor phosphorylation and short-term desensitization, being, at the same time, not involved in long-term desensitization of A₂A AR function. These findings confirm that short-term and long-term desensitization of A₂A AR could be mediated by structurally distinct regions of the receptor protein and may involve different mechanisms.

**A₂B AR:** A serine residue (Ser329), close to the COOH-terminus, was revealed as critical for the rapid agonist-induced desensitization and internalization of the receptor (79) through point mutation or deletion studies of rat A₂B AR stably expressed in CHO cells. Due to a GRK-2 and arrestin-dependent mechanism, A₂B AR undergoes rapid agonist-induced desensitization and internalization. Expression of a dominant negative mutant of GRK-2 in NG108-15 cells (80) or antisense-induced inhibition of non-visual arrestins (arrestin-2 and -3) in human embryonic kidney (HEK-293) cells (81) efficiently reduced the rate of agonist-induced desensitization and internalization of endogenous A₂B AR. In another study, A₂B AR was shown to internalize in an arrestin- and dynamin-sensitive manner (82). On the other hand, second messenger-dependent kinases, such as PKA and PKC, did not seem to be involved in agonist-mediated phosphorylation and subsequent desensitization of A₂B AR (83). Human astrocytoma cells which endogenously expressed A₂B AR showed that tumor necrosis factor α (TNFα), a pro-inflammatory cytokine, markedly reduced agonist-dependent receptor phosphorylation on threonine residues and attenuated agonist-mediated A₂B AR desensitization (84). TNFα-induced inhibition of A₂B AR desensitization could result in prolonged A₂B AR responsiveness and may contribute unregulated activation of astrocytes that eventually occurs in neurodegenerative diseases. In a recent study, it was proposed that A₂A AR is involved in the expression of A₂B AR in human embryonic kidney cell line, AD-293, transfected simultaneously with mouse A₂A AR and A₂B AR. It was found that newly synthesized A₂B AR is retained in the endoplasmic reticulum (ER) and is eventually targeted for degradation by the proteasomes that, when inhibited, was not sufficient to enhance maturation and surface expression of the receptor. These findings support the notion that A₂A AR and A₂B AR form heterodimer complexes for trafficking and function (85).
**A3 AR:** A3 AR undergoes rapid desensitization of receptor function after prolonged application of an agonist, as a result of phosphorylation of the receptor protein by members of the family of GRKs (86) (87). It was demonstrated (88) that a triple mutant, Thr307/Ala307, Thr318/Ala318 and Thr319/Ala319 within the COOH-terminus, showed marked reduction in agonist-stimulated phosphorylation and desensitization of rat A3 AR. Individual mutations of each residue showed that Thr318 and Thr319 are the two major sites for phosphorylation where Thr318 appeared to be necessary to observe phosphorylation at Thr319, but not vice versa. Moreover, mutation of two palmitoylation sites of rat A3 AR within the COOH-terminus, Cys302 and Cys305, which controls the GRK phosphorylation sites, revealed basal phosphorylation even in the absence of an agonist. This suggests an important regulatory role of these palmitoylation sites in receptor desensitization. Receptor kinetics of A3 AR was studied (89) in human astrocytoma cells. Short-term exposure to the agonist 2-chloro-N6-(3-iodobenzyl)-N-methyl-5'-carbamoyl-adenosine (Cl-IBMECA, Figure 17) caused rapid receptor desensitization followed by internalization within 15 and 30 min, respectively. Agonist removal resulted in recycling of the A3 ARs to the cell surface within 120 min. Long-term exposure (1–24 h) resulted in a higher receptor down-regulation and the restoration of receptor levels was also slow (24 h). MAPK was shown to regulate agonist-induced phosphorylation of A3 AR where its stimulation mediated activation of ERK1/2 within 5 min of agonist exposure in CHO cells stably expressing A3 AR. Treatment with PD98059, a well-characterized MAPK kinase inhibitor, showed impaired receptor phosphorylation, desensitization and internalization by inhibiting GRK-2 translocation from cytosol to the plasma membrane. These data suggest that A3 AR activation is regulated by ERK1/2 in a feedback mechanism which controls GRK-2 activity and prevents receptor phosphorylation (90). These findings could explain the dual and opposite role of A3 AR in neuroprotection and neurodegenerative diseases.

### 1.3.4 Physiopathological role of ARs

<table>
<thead>
<tr>
<th>Receptor name</th>
<th>Human gene</th>
<th>G proteins</th>
<th>Localization</th>
<th>Potency of adenosine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 AR</td>
<td>ADORA1</td>
<td>G&lt;sub&gt;1I&lt;/sub&gt;</td>
<td>Broad distribution: high in nerves, heart, kidney and adipose tissue</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt; to 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>A2A AR</td>
<td>ADORA2A</td>
<td>G&lt;sub&gt;1I/0&lt;/sub&gt;</td>
<td>Broad distribution: very high in basal ganglia; high in nerves, blood vessels and immune cells</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt; to 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>A2B AR</td>
<td>ADORA2B</td>
<td>G&lt;sub&gt;4i&lt;/sub&gt; (G&lt;sub&gt;q11&lt;/sub&gt;; G&lt;sub&gt;12/13&lt;/sub&gt;)</td>
<td>Broad distribution, but generally low abundance</td>
<td>3 × 10&lt;sup&gt;-7&lt;/sup&gt; to 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>A3 AR</td>
<td>ADORA3</td>
<td>G&lt;sub&gt;1I&lt;/sub&gt;</td>
<td>Restricted distribution, varying in different species: high in mast cells</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt; to 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 1: Adenosine receptors distribution and signaling. *The potency (in mol/l) is determined by the effect of the agonist in cells expressing approximately 2 × 10<sup>5</sup> receptors per cell.
Adenosine is fully considered a homeostatic regulator due to the wide range of effects mediated by the binding at adenosine receptors. ARs play an important role in several key physiological processes, ranging from neuromodulation to immune regulation, and from vascular function to metabolic control. One approach that has proved to be particularly effective in uncovering the normal physiological roles of adenosine receptors is genetic knockout mouse models (91) for all four adenosine receptors (Adora1, Adora2a, Adora2b and Adora3 are the genes encoding A1, A2a, A2b and A3 ARs, respectively, see Table 1 above) generated by the targeted deletion of either of the two critical exons of the adenosine receptors (92). Although detection of the pathophysiological roles of adenosine signaling requires models of disease in the genetically modified organism, the available genetic knockout mouse models have actually provided important evidences. Two lines of Adora1-knockout mice have been generated (93) and Adora1-knockout mice exhibit decreased fertility, a significant decrease in lifespan and an increased risk of seizures (6). In the kidney, studies conducted on knockout mice have confirmed that the stimulation at A1 AR on the glomerular afferent arteriole reduces renal blood flow and the glomerular filtration rate, and stimulation of the A1 receptor on the proximal tubules increases sodium and water reabsorption. These studies led to the rational approach for developing A1 receptor antagonists to control renal dysfunction in patients with acute heart failure. A2a AR are expressed at high levels in the dorsal striatum, a critical basal ganglia structure involved in motor control, where they are co-localized with dopamine D2 receptors: they inhibit D2 receptor binding in the striatum and immediate-early gene expression. The behavioral effect given by the application of A2a AR antagonists partly overlap with those of D2 receptor agonists, useful for the treatment of Parkinson’s disease (PD), in which D2 receptors are fully involved, and also influencing working memory (94), reversal learning (95) and goal-oriented behavior (96), although leaving spatial reference memory, motor function and anxiety-like behaviors intact. Regarding A2a AR, three strains of Adora2a-knockout mice have been generated exhibiting reduced exploratory behavior and an higher score in anxiety tests, with male mice being much more aggressive towards intruders (97). Due to the reduced activity they gain weight, especially as fat. Their response to acute pain stimuli is slower and their blood pressure and heart rate are elevated (98). Genetic Adora2a-knockout models have uncovered complex roles of the A2a AR in tissue protection: in fact, A2a AR activation confers tissue protection in peripheral organs (99), while its inactivation confers neuroprotection against brain injury (100). Genetic knockout models have shown that both A1 and A2a AR are involved in mediating the sleep-promoting properties of adenosine in the brain (101) and the arousal effects of caffeine seen in wild-type animals are dampened in Adora2a-knockout mice (9) lead to consider that AR ligands could be used as normal cognitive enhancers or sleep promoters. Moving on A2b AR, several strains of Adora2b-knockout mice have also been generated (102) (103). Assumed A2b AR as a low-affinity receptor that is mostly expressed at low levels, it could be quiet unexpected that Adora2b-knockout mice have very strong phenotypes, especially in relation to the vasculature (103). Adora2b-knockout mice show reduced inflammation of the vascular tissue at the baseline and suffer from increased vascular leakiness in several
Introduction

Adenosine signaling is not largely noticeable in physiological condition, but aberrant adenosine signaling is implicated as a common disease mechanism underlying inflammatory and ischemic tissue damage (117). This includes excessive inflammatory tissue damage such as in acute liver injury, ischemic kidney injury, acute lung injury, traumatic brain injury, ischemic brain injury, epilepsy and some neurodegenerative disorders such as Parkinson’s disease and Huntington’s disease (91) (118) (100). As such, enhanced adenosine signaling is crucial in these pathological conditions associated with tissue inflammation and remodeling.

Together with dramatic increases in extracellular adenosine levels, conditions of tissue hypoxia are associated with enhanced expression of adenosine receptors, particularly the A<sub>2A</sub> AR (119) and A<sub>2B</sub> AR, and a marked induction of ENTPD1 (120) (121) and NTSE, as these pathways are tightly controlled on a
transcriptional level. During ischemia, activation of A\textsubscript{2B} AR has been shown to promote ischemia tolerance, improve oxygen-efficient metabolism and protect against ischemia-reperfusion injury of the heart, kidneys or intestine (122). Triggering A\textsubscript{2B} AR can reduce the activation and transmigration of inflammatory cells into post-ischemic tissues and protect against sepsis-induced mortality by dampening an excessive inflammation response to the damage (123). Moreover, activation of A\textsubscript{2A} receptors on inflammatory cells invading the tissue can be an efficient treatment for reperfusion injury (124) (125): for example, activation of A\textsubscript{2A} AR expressed on T cells or dendritic cells (126) has been associated with the attenuation of organ injury during hepatic or renal ischemia, respectively. Moreover, A\textsubscript{1} AR are downregulated by hypoxia in C6 glioma cells (127).

Adenosine receptor expression may be different as physiological levels in cancer cells, but the significance of this is yet unclear. Hypoxia in solid tumors is associated with increased levels of ENTPD1-and NT5E-dependent adenosine, causing A\textsubscript{2A} AR-mediated attenuation of the immune response against cancer. On the other hand, A\textsubscript{2A} AR inactivation in the brain has been consistently associated with protection against brain damage after ischemia, excitotoxicity, traumatic brain injury (128) (129) and neurodegeneration in Parkinson’s disease (130) and Alzheimer’s disease (131). In some cases, both sides of the A\textsubscript{2A} AR modulation have a protective effect, including in animal models of Huntington’s disease (132) (133) and spinal cord injury.

Several transcriptional mechanisms contribute to the induction of A\textsubscript{2A} AR, A\textsubscript{2B} AR and ecto-nucleotidase expression in response to stress, hypoxia and inflammation and other pathological insults as well as a local increase in adenosine levels. Hypoxia is associated with a transcriptional program that results in the induction of ENTPD1 and NT5E thereby elevating the production of extracellular adenosine production. Moreover, ENTPD1 and NT5E have been implicated in the conversion of ATP and ADP to adenosine on regulatory T cells, thereby providing an autocrine feedback loop to enhance the anti-inflammatory functions of this subset of T cells (134).

1.3.5 Adenosine receptors as therapeutic targets

In the last decades many efforts have been made in order to fully understand AR signaling pathways and many AR ligands have been developed as potential therapeutics useful for a wide range of diseases. Many of the synthetized compounds nowadays are very potent (in the low nanomolar range or even subnanomolar) and selective (100 to 300 times more potent versus the others AR subtypes). However, the biggest issue regards druggability, in particular what may concern tissue distribution and bioselectivity. In fact, ARs have a broad distribution in the human body and some tissues have a larger “receptor reserve” than other tissues (e.g. adipocytes). Decades of expertise and clinical studies gave the chance to better understand how ARs
Introdu
cation

ligands could be useful in therapy and give a clearer view of a very complex scenario, concerning the openly wide physiopathological range of effects in which ARs are involved in. In theory, ARs ligands application in therapy are partially summarized in Figure 7 and in Table 2 but few of these disease or therapeutic indications are targeted by clinical studies and even less are AR ligands in therapy.

Figure 7: disease targets for selective adenosine receptor ligands (16).

Summarizing, ARs are potential therapeutic targets for Parkinson’s disease, chronic heart failure, Inflammatory diseases, autoimmune disorders, chronic neuropathological pain, sickle cell disease and cancer.
### Effects

<table>
<thead>
<tr>
<th>Adenosine A_1 receptors</th>
<th>Physiology and pathophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased renal blood flow, tubuloglomerular feedback and inhibition of renin release</td>
<td>Physiology</td>
</tr>
<tr>
<td>Inhibition of lipolysis</td>
<td>Physiology</td>
</tr>
<tr>
<td>Vasoconstriction</td>
<td>Pathophysiology?</td>
</tr>
<tr>
<td>Bronchoconstriction</td>
<td>Pathophysiology?</td>
</tr>
<tr>
<td>Inhibition of neurotransmitter release</td>
<td>Extreme physiology*</td>
</tr>
<tr>
<td>Inhibition of insulin and glucagon release</td>
<td>Physiology</td>
</tr>
<tr>
<td>Reduced heart rate</td>
<td>Physiology</td>
</tr>
<tr>
<td>Osteoclast activation and bone resorption</td>
<td>Physiology?</td>
</tr>
<tr>
<td>Reduced respiration</td>
<td>Extreme physiology*</td>
</tr>
<tr>
<td>Sleep</td>
<td>Physiology</td>
</tr>
<tr>
<td>Analgesia</td>
<td>Extreme physiology*</td>
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<tr>
<td>Cardiac preconditioning</td>
<td>Pathophysiology</td>
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</table>

### Adenosine A_2A receptors

<table>
<thead>
<tr>
<th>Effects</th>
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<tbody>
<tr>
<td>Wakefulness and locomotion</td>
<td>Physiology</td>
</tr>
<tr>
<td>Neurodegeneration (including Parkinson’s disease, stroke, traumatic brain injury and Alzheimer’s disease)</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>Extreme physiology* or pathophysiology</td>
</tr>
<tr>
<td>Vasodilation and hypotension</td>
<td>Physiology or extreme physiology*</td>
</tr>
<tr>
<td>Blood–brain barrier integrity</td>
<td>Pharmacology</td>
</tr>
<tr>
<td>Coronary vasodilation</td>
<td>Physiology or extreme physiology*</td>
</tr>
<tr>
<td>Inhibition of platelet aggregation</td>
<td>Extreme physiology*</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Extreme physiology*</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>Pathophysiology</td>
</tr>
</tbody>
</table>

### Adenosine A_2B receptors

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<th>Effects</th>
<th>Physiology or extreme physiology*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular integrity</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Cardiac preconditioning</td>
<td>Extreme physiology*</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Pro-inflammation (acute injury) and antiinflammation (some chronic disease states)</td>
<td>Physiology and pathophysiology</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>Pathophysiology</td>
</tr>
</tbody>
</table>

### Adenosine A_3 receptors

<table>
<thead>
<tr>
<th>Effects</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Increased mast cell activation</td>
<td>Extreme physiology* or pathophysiology</td>
</tr>
<tr>
<td>Airway contraction</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Inflammatory pain</td>
<td>Extreme physiology* or pathophysiology</td>
</tr>
<tr>
<td>White cell chemotaxis</td>
<td>Extreme physiology* or pathophysiology</td>
</tr>
<tr>
<td>Chronic neuropathic pain relief</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>Anticancer (melanoma)</td>
<td>Pathophysiology</td>
</tr>
</tbody>
</table>

Table 2: Physiological and pathological effects of adenosine receptors (135). *Refers to such conditions such as heavy exercise, being at high altitude and unusually high activity in the pathways of the nervous system.
A<sub>1</sub> AR:

- Chronic heart failure

In patients with acute heart failure is definitely common an impaired renal function directly contributing to deterioration of the heart and associated with an adverse outcome, including increased mortality. Measurement of local adenosine production showed that is often increased in the kidneys of patients with heart failure as a result of hypoxia caused by reduced renal perfusion and by stimulation with diuretics (136).

Based on the demonstrated control of renal function and the implication of A<sub>1</sub> AR in the mechanisms associated with renal dysfunction, A<sub>1</sub> AR antagonists were developed. These antagonists reduced the risk of persistent worsening renal failure by more than 50% in a Phase IIb study involving 301 patients with acute heart failure, and improved renal plasma flow in 63 ambulatory patients with chronic heart failure (137).

Thus, a placebo-controlled, randomized Phase III trial involving 2,033 patients with acute heart failure (the PROTECT study) was carried out with the A<sub>1</sub> AR antagonist rolofylline (Figure 129; this was the largest study to date involving the use of A<sub>1</sub> AR antagonists targeting renal function. Unfortunately, the results were disappointing and rolofylline did not prevent persistent worsening renal function (18) (138). The reason for this absence of the kidney protective effects is possibly to be due to an enhanced diuretic effect (as evident by more pronounced weight loss), which may have counterweighted the positive effects of rolofylline on the preservation of renal function (19). Moreover, pharmacological and genetic studies have extensively demonstrated that A<sub>1</sub> AR exerts protective effects against ischemic kidney injury and brain injury, which is consistent with the increased frequency of stroke and seizure activity in clinical trials of A<sub>1</sub> AR antagonists (19), thus leading to assert that A<sub>1</sub> AR antagonist develop as chronic heart failure therapeutics should proceed with caution. It is under this framework where a partial A<sub>1</sub> AR agonist could be useful for a potentially hemodynamically neutral therapy that could simultaneously improve cardiomyocyte energetics, cardiac structure and function, and prevent further tissue injury while being well tolerated when added to background medical therapy. Although drug development of a partial A<sub>1</sub> AR agonist remains at an early stage, compelling biologic rationale and encouraging preclinical evidence support continued attention be given to ongoing and future trials with these agents in heart failure (139).

- Cardiovascular system

A<sub>1</sub> AR activation has many effects in the cardiovascular system, including a reduction in heart rate and atrial contractility, and the attenuation of the stimulatory actions of catecholamines on the heart (140) (141). Activation of the A<sub>1</sub> AR by intravenous infusion of adenosine is used to restore normal heart rhythm in patients with paroxysmal supraventricular tachycardia (PSVT). However, for more long-term indications, selective A<sub>1</sub> AR modulators are needed to avoid side effects related to other AR subtypes such as hypotension.
Pain

Adenosine exerts multiple influences on pain transmission at peripheral and spinal sites. At peripheral nerve terminals in rodents, A1 AR activation produces antinociception by decreasing cAMP levels in the sensory nerve terminal (144), thus A1 AR could be useful for neuropathic pain treatment. The functionally important regulation of membrane in channels seems to be the domain of A1 AR. Activation of A1 AR enhances K+ and Cl- conductance in neurons, leading to membrane hyperpolarization and postsynaptic reduction of neuronal Ca2+ influx through voltage- and N-methyl D-aspartate receptor-dependent channels. This counteracts, through synaptic and extrasynaptic receptors, nerve cell depolarization (145). A study of 2005 (146) reported that capsaicin-induced glutamate release from rat spinal synaptosomes was inhibited by adenosine and adenosine A1 AR agonists. These studies suggest that the hyperpolarizing effect of adenosine can inhibit excitation of sensory nerves and ameliorate neuropathic pain at the spinal cord level. Moreover, a 2010 study by Horiuchi et al. (147) showed how hyperalgesia induced by spinal cord injury (SCI) was significantly inhibited by the intrathecal application of 10 nmol Cl-adenosine, a nonselective adenosine receptor agonist. Cl-adenosine significantly inhibited SCI-induced hyperalgesia at concentrations between 10 and 30 nmol in a dose-dependent way. The effect of Cl-adenosine on hyperalgesia after SCI was blocked by the simultaneous application of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Figure 12), an A1 AR antagonist. This result suggests that the anti-hyperalgesic action of Cl-adenosine is mediated by the stimulation of A1 AR. Intrathecal application of R-PIA (10 nmol), an A2 AR agonist (Figure 11), inhibited SCI-induced hyperalgesia. On the other hand, intrathecal application of CGS21680, a selective adenosine A2a AR agonist, did not inhibit SCI-induced hyperalgesia. These results suggest that adenosine inhibited SCI-induced hyperalgesia through the stimulation of A1 AR. A 2015 study by Imlach et al. (148) investigated the changes in adenosine tone and A1 AR signaling, together with the actions of a novel A1 AR positive allosteric modulator (PAM), VCP171 [(2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone], on excitatory and inhibitory neurotransmission at spinal cord superficial dorsal horn synapses in a rat partial nerve-injury model of neuropathic pain. In the absence of A1 AR agonists, superfusion of the A1 AR antagonist, DPCPX, produced a significantly greater increase in electrically evoked α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor–mediated synaptic current (eEPSC) amplitude in both laminae I and II neurons from nerve-injured animals than in controls, suggesting that endogenous adenosine tone is increased in the dorsal horn.
Inhibitory GABAergic and glycineric synaptic currents were also significantly increased by DPCPX in controls but there was no difference after nerve injury. The A_1 AR agonist, N^6^-cyclopentyladenosine, produced greater inhibition of eEPSC amplitude in lamina II but not lamina I of the spinal cord dorsal horn in nerve-injured versus control animals, suggesting a functional increase in A_1 AR sensitivity in lamina II neurons after nerve injury. The A_2A AR PAM, VCP171, indeed produced a greater inhibition of eEPSC amplitude of nerve-injury versus control animals in both lamina I and lamina II neurons. Enhanced adenosine tone and A_1 AR sensitivity at excitatory synapses in the dorsal horn after nerve injury suggest that new generation PAMs of the A1R can be effective treatments for neuropathic pain.

- Parkinson’s disease

For what may concern Parkinson’s disease (PD), A_1 AR is also expressed in the striatum; based on anatomical and in vivo microdialysis studies, A_1 ARs appear to be localized presynaptically of dopamine axon terminals where they inhibit dopamine release. A_1 AR antagonists facilitate dopamine release in the striatum, and like A_2A ARs potentiate dopamine mediated responses (149). Antagonism of both the A_2A and A_1 AR would be synergistic (see Figure 8): inhibition of the A_1 receptor will facilitate dopamine release, while inhibition of the A_2A AR will enhance postsynaptic responses to dopamine. Interestingly, the A_1 receptor is also concentrated in neocortical and limbic system structures that are important for cognitive function. Pharmacological inhibition of A_1 receptors enhances neurotransmitter release in the hippocampus and enhances performance in animal models of learning and memory (150).

![Figure 8: Adenosine receptor implication in the direct and indirect pathways in the motor circuit (151).](image-url)
**A2A AR:**

- Parkinson’s disease

A2A AR antagonists have emerged as leading non-dopaminergic drugs for the treatment of Parkinson’s disease, due to many evidences accumulating in the last decades like the concentrated striatal expression of A2A AR, the antagonistic A2A-D2 receptor interaction and many preclinical studies demonstrating motor benefit in rodent and non-human primate models of Parkinson’s disease (94) (152) (153) (154). Six double-blind, placebo controlled clinical Phase IIb and Phase III trials of istradefylline (KW-6002, Figure 14), involving a total of >2,000 patients with advanced Parkinson’s disease, and one Phase IIb trial with preladenant (SCH420814, Figure 14), involving 253 patients with advanced Parkinson’s disease, have been reported (155). These clinical Phase IIb and Phase III trials have shown a little but significant reduction in the average ‘off-time’ by about 1.7 hours compared to the optimal l-DOPA (l-3,4-dihydroxyphenylalanine) dose regimen (154). However, another similar Phase III clinical trial with istradefylline in patients with Parkinson’s disease did not demonstrate a significant reduction in the ‘off-time’ compared to placebo (156). These relatively modest motor effects differed from the unambiguously motor benefit of the preclinical studies in which MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was used in order to cause permanent symptoms of Parkinson’s disease by destroying dopaminergic neurons in the substantia nigra of the primates’ brain. Clearly, further clinical investigation of A2A AR antagonism is necessary to fully understand its potential as a treatment for Parkinson’s disease, strengthened by encouraging and consistent finding in the clinical trials of A2A AR antagonists for Parkinson’s disease showing that both istradefylline and preladenant had an excellent safety profile (154) (155). In April 2007, Kyowa Hakko Kirin Pharma filed a new drug application (NDA) for istradefylline for the treatment of advanced Parkinson’s disease; however, the FDA issued a non-approval letter in 2008, citing the need for additional efficacy data. Nowadays is ongoing a Phase III long term clinical study in subjects with moderate to severe Parkinson’s disease. Currently, several Phase IIb and Phase III trials for A2A AR antagonists (other than istradefylline) are still underway and these agents remain one of the leading non-dopaminergic treatment candidates for Parkinson’s disease (157).

- Fibrosis

Both A2A and A2B AR signal for regulation of matrix production via classic GS-mediated activation of cAMP and its downstream signaling molecules. The regulatory actions of the receptors likely involve multiple interconnected pathways (Figure 9), and one of the more interesting aspects of this regulation is the opposing effect of the different levels of cAMP generated (158). Whether this relates to incorporation of alternative signaling pathways at higher levels of cAMP or the activation of discrete signaling pathways at different sites in the cell remains a subject for further investigation. Additionally, adenosine signaling also contributes to fibrosis in organ-specific ways and may have opposite effects in different organs. The deposition of collagen
by fibroblasts is an essential part of wound healing but also contributes to pathologic remodeling of organs, which can lead to substantial morbidity and mortality. Many small molecules contribute to the pathogenesis of fibrosis including the purine adenosine which signals primarily through A<sub>2A</sub> and A<sub>2B</sub> AR. These receptors are helpful in order to reduce inflammation and promote wound healing. On the other side, excessive activation leads to architectural disruption and loss of organ integrity in a variety of fibrosing disorders. The development of drugs that selectively target these receptors will disrupt the pathogenesis of fibrosis and slow or arrest the progression of these important diseases.

![Diagram of adenosine receptor signaling pathways](image)

**Figure 9:** Cross-talk of adenosine receptor signaling pathways leading to collagen deposition and fibrosis. Collagen III is separately regulated by p38, inhibition of ERK via PKA, and Akt pathways. Collagen I is shown being positively regulated by Epac2. Additionally, A<sub>2A</sub>R inhibits Fli1, which promotes CTGF transcription and fibrosis (158).

- **Sickle cell disease**

Treatment with an A<sub>2A</sub> AR agonist has been indicated to attenuate sterile inflammation and T cell activation in this disorder (159). A clinical trial in patients with sickle cell disease is currently being conducted using the
FDA-approved A$_{2A}$ AR agonist, 1-[6-amino-9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]purin-2-yl]-N-methylpyrazole-4-carboxamide (Regadenoson, CVT-3146).

- Pain

Assumed that A$_{2A}$ AR is known to exert an hyperalgesic modulation in spinal cord (see A$_1$ AR above), another study of 2007 by Hussey et al. (160) deepen the role of A$_{2A}$ AR concerning peripheral nociception, monitoring the response of NMDA and NK1 receptor in adora2 knockout mice. The results showed that there was a significant reduction in nociceptive behaviour as measured by both bites/licks and flinches in response to formalin injection. This pattern was similar when using the selective A$_{2A}$ AR antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH58261). Although the response to SCH58261 observed in this study was not dose-dependent, 3 mg/kg and 10 mg/kg were chosen on the basis of their selectivity for the A$_{2A}$ AR. In our study this is also suggested by the lack of significant difference between results obtained using the A$_{2A}$ AR knockout mice and SCH58261. There was a significant reduction in time spent biting/licking the formalin-injected paw during the first phase only, with a reduced number of flinches observed during both the first and second phases of the test. Although the mechanisms underlying the formalin test are complex it was chosen as it consists of two distinct phases, with the first phase being generally accepted to be mediated primarily by direct stimulation of peripheral sensory nerves whilst the second phase is due to an inflammatory component and involves more complex pain circuitry. The reduced response observed during the first phase in A$_{2A}$ AR knockout mice further suggests that peripheral adenosine A$_{2A}$ AR are involved in stimulation of nociceptive afferent fibres projecting to the spinal cord. This contrasts with Borghi et al. (161) who showed that administration of the selective A$_{2A}$ AR agonist CGS21680 (Figure 13) caused a reduction in nociceptive behavior during the first phase which might suggest that the receptor has an antinociceptive role. The reduced response as measured by flinches observed during the second phase of the formalin test in both A$_{2A}$ AR knockout mice and mice treated with SCH58261 further implicates the A$_{2A}$ AR in nociceptive circuits and could be linked to altered NMDA glutamate receptor signalling. The NMDA glutamate receptor is a key proponent of central sensitization which is a feature of the formalin test and occurs as a result of repetitive stimulation of afferent fibres projecting to the spinal cord. This repetitive stimulus induces activation of the NMDA glutamate receptor and leads to calcium entry through the receptor and the consequent changes in the post-synaptic membrane. Thus, the loss or blockade of A$_{2A}$ AR causing a reduction in nociceptive behaviour in the first phase will be associated with reduced direct activation of primary afferent fibres and therefore decreased activation of NMDA glutamate receptors located centrally and this is likely to result in a reduction of the central sensitization which characterizes the second phase. In conclusion, the decrease in NMDA glutamate receptor binding in A$_{2A}$ AR knockout mice and the reduced response to the formalin test in A$_{2A}$ AR knockout mice and mice treated with the selective A$_{2A}$ AR antagonist SCH58261 were most probably caused by changes occurring in peripheral nociceptive transmission as a
consequence of the genetic knockout or blockade of A2a AR at sensory terminals. These results support a key role for the A2a AR in peripheral nociceptive pathways.

**A2b AR:**

- **Inflammation**

Much research has focused on the regulation of the inflammatory response by the A2b AR. In a study of 2006, was generated an A2b AR knockout (KO) mouse model (103) showing that the A2b AR is highly expressed on macrophages and that A2b AR KO mice have a mild increase in plasma levels of the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), and decreased plasma levels of the anti-inflammatory cytokine, IL-10, following lipopolysaccharide (LPS) stimulation as compared to wild type (WT) mice. These results suggest that the A2b AR dampens the inflammatory response. Further studies have shown an anti-inflammatory role for the A2b AR. For example, activation of the A2b AR with BAY60-6583, a specific A2b AR agonist, inhibits superoxide generation by neutrophils (162). Importantly, in vivo studies have elucidated mechanisms in addition to alteration of cytokine levels that explain how the A2b AR affects the pathogenesis of inflammation. A2b AR KO mice have increased expression of adhesion molecules intracellular adhesion molecule (ICAM-1) and E-selectin, which increases leukocyte rolling and adhesion (103). Furthermore, A2b AR KO mice exhibit enhanced neutrophil infiltration into tissues under hypoxic conditions (104). These studies suggest that the A2b AR prevents inflammatory cell transmigration into the tissue of interest, one of the first steps in the inflammatory response. In addition, the A2b AR affects alternative macrophage activation. Adenosine and NECA enhance the expression of markers of alternative macrophages (arginase 1, tissue inhibitor of matrix metalloproteinase-1, and macrophage galactose-type C-type lectin-1). Pharmacologic inhibition of the A2b AR or genetic lack of the A2b AR prevents NECA-induced expression of these alternative macrophage markers (163), suggesting that A2b AR promotes alternative macrophage development. Furthermore, Ryzhov et al. generated an A2b AR KO mouse using a different targeting construct and determined the ability of the A2b AR to mediate two actions of NECA: (1) stimulation of IL-6 release and (2) inhibition of LPS-induced TNF-a release (164). In this study, NECA increases plasma IL-6 levels in WT but not A2b AR KO mice, suggesting that the A2b AR promotes inflammation by increasing IL-6 levels. They also found that NECA decreases LPS-induced TNF-a levels in both WT and A2b AR KO mice, suggesting that the A2b AR does not dampen inflammation in response to LPS. Despite these results that suggest that A2b AR activation by NECA promotes inflammation, this study also found that macrophages derived from A2bAR KO mice secrete more TNF-a than WT mice. These studies underlie important considerations in studying the A2b AR as well as other receptors. First, genetic lack of a receptor does not always predict the results of pharmaceutical modulation of the receptor. Knockout mouse models are helpful in determining the role of
a receptor in pathologic processes; however, developmental effects of genetic alteration can impact the interpretation of the results. Lack of the A$_{2b}$ AR throughout development may upregulate other proteins to compensate for lack of the A$_{2b}$ AR. Pharmacologic modulation of a receptor, unlike knockout mouse models, determines how alteration of cellular signaling affects a particular point in time, but may not mimic lifelong lack of a receptor. Second, use of pharmacologic modulators requires thorough understanding of the action of the agents on potentially other targets. The presence and activity of other receptors may influence the effect of A$_{2b}$ AR on particular process. In fact, NECA is a non-specific adenosine receptor agonist that can activate all four of the adenosine receptors, which makes it more difficult to conclude which receptor is responsible for certain actions of the compound. Different cell types, each expressing the A$_{2b}$ AR may have opposing downstream outcomes in response to modulation of the receptor. The initial in vivo studies that supported an anti-inflammatory role for the A$_{2b}$ AR showed that TNF-α levels following LPS injection are greater in A$_{2b}$ AR KO mice, than control counterparts (103). In a different disease model, that of cecal ligation and puncture-induced sepsis, A$_{2b}$ AR signaling on non-hematopoietic cells was the main contributor to reducing cytokine and chemokine production (123). Furthermore, the A$_{2b}$ AR is protective in acute inflammation as evidenced by the findings that A2bAR KO mice have increased pulmonary inflammation following acute injury from hypoxia (104) or ventilator-induced lung injury (107). Overall, when interpreting studies on the role of the A$_{2b}$ AR in inflammation-induced processes, it is important to consider timing of A$_{2b}$ AR activation or inhibition (pharmacologic or genetic), specificity of pharmacologic agents, model systems, and cell types as well as acute versus chronic responses studied. In addition, when studying inflammation in vivo, the influence of housing facilities with potentially somewhat different pathogen exposure, may impact experimental outcomes.

- Cancer

Growing evidence indicates that A$_{2b}$ AR potentially plays a pathophysiological role in human cancer (Figure 10) and might serve as a target for novel therapies or co-therapies for cancer. First, A$_{2b}$ AR is highly expressed in various types of tumor cell sort issues and promotes tumor-cell proliferation. For instance, A$_{2b}$ AR was found to be overexpressed in colorectal carcinoma cells and tissues, and inhibition of A$_{2b}$ AR blocked the proliferation of colon cancer cells (165). In prostate cancer, A$_{2b}$ AR increased cancer-cell proliferation in both ligand- dependent, and ligand-independent manners (166) (167). In human oral cancer, A$_{2b}$ AR was shown to be upregulated in oral squamous carcinoma cells, and A$_{2b}$ AR knockdown reduced the proliferation of oral cancer cells through HIF-1α activation (168). Moreover, A$_{2b}$ AR was reported to foster bladder and breast tumor growth in syngeneic mice (110). Second, A$_{2b}$ AR modulates tumor-cell metastasis. A$_{2b}$ AR was implicated in promoting breast cancer cell migration in vitro and lung metastasis in vivo (169) (170), although the underlying molecular mechanism was not fully elucidated. However, the results of a subsequent study suggested a possible explanation: A$_{2b}$ AR activation suppressed the prenylation of the small GTPase Rap1B.
and diminished Rap1B-mediated cell adhesion, which promoted cell migration (171). Third, A2b AR might regulate the tumor microenvironment, including the surrounding blood vessels, immune cells, fibroblasts, and the extracellular matrix. Ryzhov and colleagues provided the first genetic evidence indicating that A2b AR regulates vascular endothelial growth factor (VEGF) production from tumor-infiltrating host immune cells and thereby promotes tumor growth (172). Concomitantly, other groups suggested that A2b AR alters angiogenesis by regulating the production of a wide array of pro- or anti-angiogenic factors such as basic fibroblast growth factor (bFGF), angiopoietin 2, and a subset of cytokines (173) (174) (175). In addition to affecting angiogenesis, A2b AR regulates dendritic-cell differentiation and function (176) (177) and alternative macrophage activation (163) and thus contributes to cancer progression. Thus, A2b AR exerts various effects on tumor progression and metastasis. Notably, most of the aforementioned evidence was collected using in vitro systems, and it is critical to further confirm the role of A2b AR in cancer by using in vivo models before A2b AR is used as a potential cancer therapeutic target.

- **Cardiovascular system**

Cardiovascular disease (CVD) comprehends coronary artery disease (myocardial infarction, angina pectoris, heart failure), cerebrovascular disease (stroke, transient ischemic attack), peripheral artery disease, and aortic atherosclerosis and aneurysm. CVD is the leading cause of death in the world (178). Atherosclerosis is an underlying etiologic factor in many types of cardiovascular disease. The pathogenesis of atherosclerosis involves endothelial dysfunction, subendothelial accumulation of low density lipoprotein (LDL) and subsequent oxidation (179). Oxidized LDL promotes endothelial cell expression of adhesion molecules (like ICAM-1 and E-selectin) and secretion of chemokines (like TNF-a), leading to the intimal accumulation of lipid-laden macrophages, called foam cells. Further accumulation of cholesterol, inflammatory cells, and apoptotic cells forms the necrotic core of the atheroma, which is encapsulated by collagen and proliferating smooth muscle cells (179). Intervention at one or several of these steps in the development of atherosclerosis may prevent subsequent thrombotic events. The function of the A2b AR in the pathogenesis of atherosclerosis has been studied. In one approach it was investigated the effect of adenosine on foam cell formation (180) in hypoxic conditions (1% O2) using an in vitro model of foam cell formation, which involved differentiating the human myelomonocytic cell line U937 or human peripheral blood mononuclear cells into macrophages and then incubating the cells with oxidized LDL to form foam cells. The authors found that hypoxia selectively induces A2b AR expression in U937 cells, differentiated macrophages, and foam cells. Moreover, adenosine promotes hypoxia-inducible factor-1a (HIF-1a) protein and HIF-1a induces expression of vascular endothelial growth factor (VEGF) and promotes foam cell formation via the A2b AR and A3 AR specifically (180). These results suggest that antagonists to the A2b AR and A3 AR may be beneficial in preventing atherosclerotic plaque formation. In contrast, a later study using an in vivo mouse model of atherosclerosis found that the A2b AR is protective against plaque formation (109). To promote atherosclerosis development, A2bAR KO
mice were bred onto an ApoE background (A2B AR, ApoE double KO) and the mice were fed Western diet. A2B AR, ApoE double KO mice have increased plasma and liver cholesterol and triglycerides and larger atherosclerotic plaque lesions as compared to ApoE mice. Importantly, injection of the A2B AR specific agonist, BAY60-6583 (Figure 15), into ApoE mice reduces atherosclerotic plaque formation and plasma lipid levels. As a mechanism to explain the effect of the A2B AR on lipid metabolism, hepatocytes isolated from A2B AR, ApoE double KO mice express higher levels of sterol regulatory element binding protein-1 (SREBP-1), a key transcription factor in lipogenesis and cholesterol synthesis, while BAY60-6583, reduces hepatocyte expression of SREBP-1. As such, this study identified the A2B AR as atheroprotective secondary to modulation of lipid metabolism and plaque formation.

In addition, adenosine signaling has been implicated in mediating cardioprotective cellular responses to myocardial ischemia (181). Ischemic preconditioning, which involves repeated short episodes of ischemia and reperfusion prior to myocardial infarction (MI), has been shown to reduce infarct size after MI (182). Interestingly, A2B AR gene expression is elevated in the hearts of individuals with ischemic heart disease as compared to healthy controls (102) and A2B AR expression increases after ischemic preconditioning (183). Using a model of murine in situ preconditioning, Eckle et al. (184) showed that A2B AR signaling is necessary for the cardioprotective effect of ischemic preconditioning. Ischemic preconditioning reduces infarct size in WT, A1 AR KO, A2A AR KO, and A3 AR KO mice, but not in A2B AR KO mice after 2 hours of reperfusion. Furthermore, activation of the A2B AR with BAY60-6583 reduces infarct size after ischemia. Further studies by the same group showed that A2B AR signaling stabilizes the circadian rhythm protein, period 2 (Per2) within 2 hours to promote a protective metabolic switch in carbohydrate utilization (102). The group also showed that A2bAR signaling in bone marrow-derived cells is primarily responsible for the cardioprotective effect in ischemia-reperfusion injury, suggesting an additional role for A2B AR regulation of inflammation in mediating cardioprotective effect (185). In contrast, amore recent study did not find a role for the A2B AR in acute ischemic preconditioning (186). The authors report no difference in infarct size following ischemic preconditioning in A2B AR KO mice as compared to WT mice. However, consistent with the original report, they find that treatment with BAY60-6583 reduces infarct size and conclude that the A2B AR may play a role in a later phase of ischemic preconditioning.

Moreover, the current management of symptomatic coronary artery disease includes coronary reperfusion with percutaneous coronary intervention (PCI), which involves balloon angioplasty and stent placement at the site of the previous arterial blockage. The pathogenesis of restenosis is believed to involve arterial injury and neointimal proliferation with macrophage accumulation (187). Research into medical therapy that can reduce the rate of restenosis after PCI has gathered interest. One such agent that is being investigated is cilostazol, a phosphodiesterase inhibitor that results in an increase in intracellular cyclic AMP (cAMP). The A2B AR, which when activated also increases cAMP levels, has been studied as a therapeutic target in
restenosis. The A2B AR has been shown to be protective against restenosis following vascular injury. In a 2008 study, a guidewire-induced femoral artery injury model in mice was used to mimic angioplasty (188) A2B AR KO mice have increased neointimal formation following femoral artery injury as compared to WT mice. In addition, the chemokine receptor CXCR4, which promotes cell migration and neointimal hyperplasia, is upregulated in platelets, macrophages, and leukocytes derived from A2B AR KO mice. Furthermore, the A2B AR plays a role in vascular smooth muscle cell (VSMC) proliferation, one of the pathologic steps of restenosis, as VSMCs from A2B AR KO mice proliferate faster than those derived from WT mice. These studies suggest that A2B AR activation may be beneficial in preventing restenosis following PCI, a conclusion that was recently tested. In accordance, a study of 2012 by Bot et al. (189) showed that activation of the A2B AR with BAY60-6583, the A2B AR specific agonist, for 18 days following wire-induced vascular injury of the left common carotid artery in Apolipoprotein E (ApoE) deficient mice reduces lumen stenosis and media size. As a mechanism to explain this result, the authors noted that in vivo and in vitro treatment with BAY60-6583 decreases VSMC proliferation. Moreover, the authors also demonstrated that BAY60-6583 increases collagen content, which suggests that A2B AR activation may also promote plaque stability. Overall, these studies show promising effects of A2B AR agonism on reducing restenosis.

Vascular smooth muscle cells (VSMCs) are important in the regulation of vascular tension and have high expression of the A2B AR (103). VSMCs contract or dilate in response to changes in calcium (Ca\(^{2+}\)) levels that result from ligand stimulation of surface receptors (190). It is known that activation of the A2B AR by coupling to Gs and Gq results in downstream increases in cAMP and IP3, respectively. IP3 results in elevated cytosolic Ca\(^{2+}\) and contraction of VSMCs whereas increases in cAMP results in decreased Ca\(^{2+}\) and relaxation of VSMCs. These changes in Ca\(^{2+}\) levels cause perturbation of the cytoskeleton leading to VSMC contraction or relaxation. Ca\(^{2+}\) bound to calmodulin in VSMCs activates myosin light chain kinase (MLCK), which phosphorylates the regulatory light chains of myosin by adding a phosphate group to Ser-19. The phosphorylation of myosin light chains causes cycling of myosin heads on actin, which culminates in VSMC contraction. Thus, the specific nature of downstream signaling of the A2B AR (via IP3 or cAMP) in VSMCs alters the final impact of A2B AR stimulation on modulation of VSMC contractility and vascular tension. Several in vitro studies have examined the role of the A2B AR in relaxation of VSMCs, using A2-type AR agonist-treated, precontracted isolated aortas using a force transducer to measure tension. One study showed that NECA, the nonspecific adenosine receptor agonist as well as CGS21680, the A2A AR specific agonist, elicit vasodilation in Prostaglandin F2\(\alpha\) (PGF2\(\alpha\))-precontracted porcine arterial rings (191). When potassium chloride (KCl) is used to precontract the arterial rings, neither NECA nor CGS21680 (Figure 13) treatment causes vasodilation. This study suggests that the A2A AR (and perhaps the A2B AR) are important for vasodilation of porcine arterial rings. In vivo studies have also been undertaken to interrogate the role of the A2B AR in controlling vascular tension and blood pressure. In one study, blood pressure was measured by tail cuff and by arterial catheterization in young (8–12 weeks old) WT and A2B AR KO mice at baseline as well as
following adenosine infusion and showed no difference between genotypes, suggesting that the A$_{2B}$ AR plays no role in blood pressure regulation in young mice (103). In a mouse model of bleomycin-induced pulmonary hypertension secondary to pulmonary fibrosis, A$_{2B}$ AR signaling was found to play a role in vascular remodeling and proliferation. Another study found A$_{2B}$ AR KO mice showed decreased systolic and mean arterial blood pressure with angiotensin II infusion as compared to WT mice (192). Furthermore, angiotensin II-induced ET-1 production in the kidneys of WT mice but not in A$_{2B}$ AR KO mice or mice treated with an A$_{2B}$ AR antagonist. Furthermore, the authors found increased levels of A$_{2B}$ AR mRNA in kidney biopsies of hypertensive chronic kidney disease patients. Together, these in vivo models show that the A$_{2B}$ AR may promote vascular remodeling via factors such as ET-1. As noted, there are seemingly conflicting reports on the role of the A$_{2B}$ AR in regulating vascular dilation and resistance, possibly due to differences in downstream signaling (Gs and/or Gq), cell type, and model system tested. In addition, results are likely influenced by the examined species, as well as the methods employed to measure vascular tension and resistance.

- Renal diseases

Several studies have indicated a critical role of A$_{2B}$ AR in mediating the progression of diabetic nephropathy. Patel et al. and Valladares et al. observed that inhibition of A$_{2B}$ AR activation suppressed VEGF production in glomeruli and further attenuated renal dysfunction in diabetic nephropathy; these data suggested a protective role of A$_{2B}$ AR antagonists in VEGF-induced diabetic nephropathy (193) (194). However, this view was challenged by Tak et al., who reported elevated VEGF levels in diabetic A$_{2B}$ AR-knockout mice (195); concordantly, diabetic nephropathy was highly severe in mice with global or vascular endothelial tissue-specific A$_{2B}$ AR deletion, but not in mice with tubular-epithelial A$_{2B}$ AR deletion. Therefore, Tak et al. suggested that vascular A$_{2B}$ AR signaling is the key mediator of kidney protection during diabetic nephropathy (195). The methods used and the specific tissues studied by the aforementioned groups were distinct, which might explain their conflicting observations on the role of A$_{2B}$ AR during diabetic nephropathy. Moreover, the different time window in which A$_{2B}$ AR inhibition was induced pharmacologically and genetically might also contribute to the discrepancy in the results (196). In addition to playing a role in diabetic nephropathy, A$_{2B}$ AR has been suggested, based on studies on several mouse models, to protect against renal fibrosis. In ADA-deficient mice, a high level of adenosine in kidney tissues resulted in proteinuria and renal fibrosis, and treatment with A$_{2B}$ AR antagonists attenuated renal dysfunction and fibrosis (197). Moreover, genetic deletion of A$_{2B}$ AR protected against renal fibrosis in both mice infused with angiotensin II and mice subjected to unilateral ureteral obstruction (197). Furthermore, renal biopsy samples from patients with chronic kidney disease (CKD) showed higher levels of A$_{2B}$ AR expression than did samples from patients without CKD (192).

All of these data suggest that A$_{2B}$ AR could serve as a potential therapeutic target in the treatment of CKD (Figure 10). Acute kidney injury, a devastating kidney disease, is often caused by renal ischemia. Rigorous studies from different laboratories have suggested a pivotal role of A$_{2B}$ AR in acute kidney injury. For example,
Grenz et al. used genetic and pharmacological approaches to reveal a role of A2B AR in protecting against renal injury resulting from ischemia, although the underlying molecular mechanism was not fully clarified (198). Subsequently, the same group proposed two possible explanations for how A2B AR might provide renal protection: one, A2B AR reduces neutrophil-dependent TNF-α production and suppresses inflammation (199); and two, A2B AR promotes optimal post ischemic blood flow within the kidney and thereby ensures the maximal return of blood flow, tissue oxygenation, and removal of waste products from the ischemic kidney through the A2B AR-ENT1 (equilibrative nucleoside transporter) pathway (200).

- Diabetes

Recently, A2B AR in particular has been suggested to function as a critical regulator in diabetes mellitus (DM) (201) (202) (203) (196) (204) (205). In a type I DM model, the non-selective receptor agonist NECA blocked diabetes development, and this appeared to be mediated by A2B AR-dependent suppression of proinflammatory cytokine production (206). These data suggest that A2B AR represents a potential target for the treatment of type I diabetes (Figure 10). Conversely, some of the evidence obtained using a type II DM model indicated that A2B AR plays a pro-diabetic role. Figler et al. suggested that A2B AR activation increases insulin resistance by elevating the production of proinflammatory mediators such as IL-6 and C-reactive protein (207). Deletion of the A2BAR gene and selective blockade of A2B AR in mice reduced hepatic glucose production and enhanced glucose disposal into skeletal muscle and brown adipose tissue (207). By contrast, other studies suggested an anti-diabetic role of A2B AR. Johnston-Cox and colleagues showed that A2B AR plays an essential role in high fat diet (HFD)-induced insulin resistance in mice, and mice lacking A2B AR displayed diminished glucose clearance and elevated insulin resistance and inflammatory cytokine production (202). The underlying cellular mechanism here is mediated by A2B AR expressed in macrophages: reinstatement of macrophage A2B AR expression in A2B AR-null mice restored HFD-induced insulin tolerance and tissue insulin signaling to the level in control mice. The molecular mechanism involves A2B AR altering cAMP signaling and the levels of macrophage cytokine expression and secretion, and this regulates the levels of insulin receptor-2 and downstream insulin signaling (203). Similar results were obtained by Csoka et al., who suggested that A2B AR plays a crucial role in sustaining glucose homeostasis and preventing insulin resistance under normal dietary conditions by regulating alternative macrophage activation. Insulin- and glucose-induced glucose clearance was impaired in A2B AR-knockout mice that were fed chow diet, and these knockout mice also exhibited a low level of physical activity, which might contribute to decreased insulin sensitivity in skeletal muscles. Csoka et al. also highlighted the complex role of A2B AR in regulating liver metabolism (208).
In a 2016 study, Hu et al. (209) report that sustained elevated circulating adenosine signaling via A2B AR is required for chronic pain behavior and neuronal hypersensitivity in three independent animal models, including adenosine deaminase (ADA) -/-, sickle cell disease (SCD), and complete Freund’s adjuvant-injected (CFA) mice. Surprisingly, adenosine-dependent pain behavior did not involve direct and acute actions of A2B AR on primary nociceptors. Instead, elevated adenosine required A2B AR signaling in myeloid cells to stimulate sensory hyperexcitability and hypersensitivity through IL-6 and sIL-6R, and consequent trans-activation of gp130, which stimulates a STAT3-dependent enhancement of TRPV1 gene expression in DRG neurons. These findings reveal an unexpectedly important role for prolonged adenosine-A2B AR signaling in several models of chronic pain and show that these adenosine effects depend upon IL-6 signaling from myeloid cells to transactivate sensory neurons. A particularly important finding was that pain-related behavior induced by prolonged elevation of adenosine levels was not exerted by direct action of adenosine on sensory neurons. Instead, adenosine-induced pain behavior was produced by activating A2B AR in myeloid cells, possibly including microglial cells. Although microglia are well recognized to promote local inflammation and chronic pain (210) (211), emerging evidence indicates that increased peripheral inflammatory responses contribute
to chronic pain (212) (211). Several studies show the specific infiltration of monocyte-derived macrophages under pathological conditions (213), including multiple sclerosis, experimental autoimmune encephalomyelitis, or irradiation prior to BMT. It is possible that A$_{2B}$ AR positive SCD mouse bone marrow derived cells of the myeloid lineage may infiltrate the CNS after irradiation, differentiate into A$_{2B}$ AR positive glia, and contribute to the pain behavior observed in the A$_{2B}$ AR -/- recipient mice. Moreover, Hu et al. (209) have provided considerable genetic and pharmacological evidence that elevated circulating adenosine signaling via myeloid cell A$_{2B}$ AR (including microglial cells) contributes to chronic inflammatory pain and their study lead to a working model for the induction of persistent pain in which activation of A$_{2B}$ AR on myeloid cells by prolonged exposure to adenosine in the blood causes the release of IL-6 from these cells, transactivation of gp130 on nociceptive sensory neurons by a sIL-6R/IL-6 complex, and a consequent pSTAT3-mediated increase in the nociceptive function of sensory neurons that involves enhanced TRPV1 gene expression. These mechanistic findings suggest therapeutic approaches for treating chronic pain including polyethylene glycol-ADA (an FDA approved drug) and application of A$_{2B}$ AR specific antagonists.

**A$_3$ AR:**

- Pulmonary system diseases

Adenosine regulates respiratory system through well-known processes. Elevated levels of the nucleoside have been found in bronco-alveolar lavage, blood, and exhaled breath condensate of patients with asthma and chronic obstructive pulmonary disease (COPD). A$_3$ ARs it is known to be involved in both pro or anti-inflammatory responses depending on the cell type involved (214). In particular, the functional role of A$_3$ AR in mast cell activation is highlighted by genetic A$_3$ AR and mast cell KO mice where degranulation appears dependent on A$_3$ AR activation, thus suggesting that adenosine exposure can result in A$_3$ AR-dependent airway inflammation (215). Likewise, adenosine fails to induce histamine release from lung mast cells obtained from A$_3$ AR-KO mice and mediated airway hyper responsiveness by both A$_3$ AR-dependent and -independent mechanisms in rodents (216). In A$_3$ AR-KO mice the reconstitution of wild-type mast cells restores the airway hyper responsiveness (217). In contrast, in a bleomycin model of pulmonary inflammation and fibrosis, A$_3$ ARs have provided anti-inflammatory functions, regulating production of the mediators involved in fibrosis (218) (219). Although A$_3$ AR protein has not been found in human lung mast cells, but it is highly present in human eosinophils with anti-inflammatory functions (220) (11). As A$_3$ ARs inhibit degranulation of eosinophils, it has been speculated that specific A$_3$ AR agonists might be useful in eosinophil-dependent allergic disorders, such as asthma and rhinitis (221). However, adenosine deaminase KO mice treated with selective A$_3$ AR antagonists have shown a marked attenuation of pulmonary inflammation, decreased eosinophil infiltration, and reduced airway mucus production (222). Interestingly,
COPD patients have shown reduced A_{2B} AR density and an increase in A_{2A} and A_{3} ARs in peripheral lung compared with normal lung function in smokers (223). An increase in A_{3} ARs found in bronco-alveolar lavage macrophages from COPD patients appears to be closely associated with the presence of high levels of proinflammatory cytokines. In contrast, A_{3} AR agonists have been suggested as a potential effective therapy for ischemia-reperfusion-induced lung injury (224). Indeed, inosine has been seen to have anti-inflammatory effects in allergic lung inflammation by recruiting A_{3} ARs (225). Accordingly, A_{3} AR activation was recently found to attenuate lung ischemia-reperfusion injury through a neutrophil-dependent mechanism, suggesting the use of A_{3} AR agonists as a novel therapeutic strategy to prevent lung ischemia-reperfusion injury and primary graft dysfunction after transplantation (226) (227). The role of A_{3} AR in the human lung has still to be clarified, although data in recent literature would appear to lean toward a protective effect.

Rheumatoid arthritis and osteoarthritis

Knowledge of the effects of adenosine has revealed that ARs could be a useful target for rheumatoid arthritis (RA) therapy. Adenosine production has emerged as an important cell mechanism to regulate inflammation due to an increase in receptor density and/or functionality. A_{3} AR are upregulated in RA, psoriasis, and Crohn’s disease (228). Interestingly they are also overexpressed in untreated RA patients and in methotrexate treated RA patients, and administration of anti–TNF-α drugs normalizes their number and functionality (229). Furthermore, A_{3} AR density inversely correlates with Disease Activity Score in 28 or 44 joints, suggesting a direct role of the endogenous activation of these receptors in the control of RA inflammation (230). The overexpression of A_{3} AR in RA has been directly linked to the increase in NF-kB, a key player in the pathogenesis of arthritic diseases (231) (228). In RA patients, adenosine suppresses the elevated levels of proinflammatory cytokines such as TNF-α and IL-1β, and clinical evidence has shown that A_{3} AR agonists modulate an improvement in signs and symptoms of the disease (232) (233). Furthermore, A_{3} AR agonists prevent cartilage damage, osteoclast/osteophyte formation, bone destruction, and markedly reduce pannus and lymphocyte formation in rat models (234) (235). The anti-inflammatory effect of A_{3} ARs has also been observed in fibroblast like synoviocytes derived from the synovial fluid of RA patients and is closely associated with a decrease in NF-kB and TNF-α release (236). Oral treatment with CF101 (IB-MECA, Figure 17) has led to amelioration and a marked decrease in clinical manifestations of the disease. In a phase I study in healthy subjects, CF101 proved safe and well tolerated, offering linear pharmacokinetic activity (237). This was confirmed in a now successfully concluded phase II study in RA patients in which CF101 mediated improvement in signs and symptoms, thus suggesting an opportunity for its development as an antirheumatic agent (233). The most common form of arthritis is osteoarthritis (OA), which is the most important cause of disability in older adults. The current recommended treatment of OA involves weight loss, physical therapy, and the use of pain relievers. However, these drugs do not reverse the degenerative process in OA and show some adverse effects on cartilage metabolism (238). It is well known that p38 MAPKs are involved in
controlling such cellular responses as adenosine-mediated proinflammatory cytokine release (239). Accordingly, a pathway involving the reduction in p38 MAPKs, NF-kB, TNF-a, and IL-8 by A3 AR activation was observed in human synoviocytes (240). It was also reported that the NF-kB signaling pathway is deregulated by the presence of IB-MECA and is involved in OA pathogenesis. In addition, CF101 induces inflammatory cell apoptosis and acts as a cartilage protective agent, suggesting that it may be a suitable candidate for the treatment of OA (235). The safety and efficacy of CF101 has also been evaluated in a phase II clinical study with patients suffering from OA of the knee (241). Interestingly, a link has been found between ARs and their modulation by such physical agents as pulsed electromagnetic fields (PEMFs). In vitro studies on joint cells have suggested that exposure to PEMFs mediates a significant protection against the catabolic effect of proinflammatory cytokines and an anabolic action increasing matrix synthesis and cell proliferation (242).

Eye diseases

A3 ARs have been widely implicated in many ocular diseases including dry eyes, glaucoma, and uveitis. In the past, A3 AR KO mice showed lower intracellular pressure, thus suggesting that A3 AR antagonists could play a role in the treatment of glaucoma (243). In addition, the use of A3 AR antagonists appears to be a specific, alternate approach for treating ocular hypertension in patients affected by the pseudoexfoliation syndrome in open angle glaucoma, which is typically associated with anterior chamber hypoxia and elevated intraocular pressure (IOP) (243). Accordingly, a series of nucleoside-derived antagonists have been found to lower IOP across species (244). Recently, it was reported that adenosine may trigger oligodendrocyte death via activation of A3 AR, suggesting that this mechanism contributes to optic nerve and white matter ischemic damage (245). CF101, a prototypical A3 AR agonists, entered a clinical phase of drug development. Studies from a phase II clinical trial revealed that it was well tolerated and induced a statistically significant improvement in patients with moderate to severe dry eye syndrome. Interestingly, in the same clinical trial, CF101 decreased IOP, thus demonstrating its efficacy as an IOP-lowering agent. These data, and the anti-inflammatory characteristics of CF101, support pursuing study of this drug as a potential treatment of the signs and symptoms of dry eye syndrome and glaucoma (246) (247). Nowadays, CF101 undergoes a phase II, randomized, double-masked, placebo-controlled, parallel group study of the safety and efficacy of daily IB-MECA administered orally in subjects with elevated intraocular pressure. CF101 has also been effective in an experimental model of autoimmune uveitis, supporting further exploration of this molecule for the treatment of uveitis (248). Oral treatment with CF101, initiated upon disease onset, improves clinical uveitis fundoscopy score and ameliorates the pathologic manifestations of the disease. In fact, in parallel with IOP phase II, CF101 undergoes also a phase II, randomized, double-masked, placebo-controlled study of the safety and efficacy of daily CF101 administered orally in subjects with active, sight-threatening, noninfectious intermediate or posterior uveitis.
Pain

The contribution of $A_3$ AR in pain states has been evaluated in different studies. A nociceptive and proinflammatory response resulting in edema formation was first demonstrated after activation of $A_3$ AR; this effect was mediated by both serotonin and histamine release, most likely from mast cells and subsequent actions on the sensory nerve terminal (249). However, recently, the treatment of chronic neuropathic pain was proposed as a novel potential application of $A_3$ AR agonists (250). $A_3$ AR agonists blocked the development of mechanically and chemotherapy-induced neuropathic pain in a dose-dependent fashion and significantly augmented the analgesic effects of currently used analgesics (251). Accordingly, in an animal model of surgery-induced metastasis CI-IB-MECA significantly reduced bone cancer pain, providing the pharmacologic rationale for using selective $A_3$ AR agonists as a new approach in chronic neuropathic pain (252). Interestingly, very recently, the first mechanistic insight into beneficial effects of $A_3$ AR agonists against neuropathic pain has been explored (253) (254). In particular, it has been found that these effects were due to the inhibition of an astrocyte-associated neuroinflammatory.

1.3.6 Adenosine receptors ligands

Medicinal chemistry approaches have been applied to all four of the adenosine receptor subtypes ($A_1$, $A_{2A}$, $A_{2B}$, and $A_3$) to create selective agonists and antagonists for each. The most recent class of selective AR ligands to be reported is the class of $A_{2B}$ AR agonists. Selective ligands have facilitated research on therapeutic applications on diseases in which ARs are involved and in some cases among the large number of AR ligands have been possible to find good clinical candidates. Prodrug approaches have been developed which improve the bioavailability of the drugs, reduce side-effects, and/or may lead to site-selective effects. The $A_{2A}$ AR agonist regadenoson (Lexiscan®), a diagnostic drug for myocardial perfusion imaging, is the first selective AR agonist to be approved. Other selective agonists and antagonists are or were undergoing clinical trials for a broad range of indications, including capadenoson and tecadenoson ($A_1$ AR agonists) for atrial fibrillation, or paroxysmal supraventricular tachycardia, respectively, BVT.115959 ($A_{2A}$ AR agonists) for pain, preladenant and istradefylline ($A_{2A}$ AR antagonist) for the treatment of Parkinson's disease, BAY 60-6583 ($A_{2B}$ AR agonist) for angina pectoris, IB-MECA (CF101) and CI-IB-MECA (CF102) ($A_3$ AR agonists) for inflammatory diseases and cancer, respectively.
\textit{A\textsubscript{1}} AR agonists

A scheme of important \textit{A\textsubscript{1}} AR agonists is shown in Figure 11. \textit{A\textsubscript{1}} AR agonists tend to be substituted at the N6 position with arylalkyl (R-PIA), cycloalkyl (CPA, CCPA, INO-8875, GR79236X and Capadenoson), bicycloalkyl (5’-Cl-5’-deoxy-(±)-ENBA), or aryl (SPA and GW493838) substituents (255). The C2 and 5’ positions also may be substituted with Cl, CCPA and 5’-Cl-5’-deoxy-(±)-ENBA, respectively, or the 5’ position with cyclic moieties (GW493838). R-PIA was one of the first \textit{A\textsubscript{1}} AR agonists to be widely used. It was previously defined as a moderately \textit{A\textsubscript{1}} AR selective agonist, but its use in that capacity is discouraged in favor of more selective nucleosides. \textit{A\textsubscript{1}} AR agonists CPA and CCPA are widely used pharmacological probes with CCPA being more selective for the \textit{A\textsubscript{1}} AR. A cautionary note is that many of the selective \textit{A\textsubscript{1}} AR agonists used routinely such as CPA and CCPA have considerable activity at the \textit{A\textsubscript{3}} AR. CPA and agents that increased endogenous adenosine mimicked the acute antinociceptive effect of acupuncture, consistent with the hypothesis that the \textit{A\textsubscript{1}} AR mediates this effect (256). The agonist INO-8875 7 (also known as Trabodenoson), which is an \textit{A\textsubscript{1}} agonist in Phase III clinical trials for glaucoma, is also intended for optic neuropathy. \textit{A\textsubscript{1}}-selective nucleoside derivative 8 failed to show efficacy in a clinical trial for dental pain and was tested in a Phase II trial for postherpetic neuralgia or peripheral nerve injury before being discontinued (257). \textit{A\textsubscript{1}}-selective non-nucleoside derivative capadenoson (BAY68-4986) is in trials for treatment of persistent atrial fibrillation and is now available as a research tool (143). Compound 10 is highly selective for the \textit{A\textsubscript{1}} AR and displayed analgesic activity in the formalin test in mice (258) (259). SPA is a peripherally-selective agonist, due to its permanently charged sulfonate group, that is moderately \textit{A\textsubscript{1}} AR selective (70-fold). GW493838 was tested in a clinical trial for peripheral neuropathic pain but discontinued (260). UNC32a displayed antinociceptive effects in mice and was suggested to cross the blood brain barrier and act directly through the \textit{A\textsubscript{1}} AR despite the 5’-phosphate group (261). Partial agonists of the \textit{A\textsubscript{1}} AR (such as the 2’-deoxy analog of 6) are effective in chronic neuropathic but not acute pain models and lack some of the cardiovascular side effects of full \textit{A\textsubscript{1}} AR agonists (262). Compound 14 is a moderately selective full \textit{A\textsubscript{1}} AR agonist that protects in some seizure models (263). It is well tolerated in rodents and does not produce the toxicity upon dose escalation that is typical of more widely used \textit{A\textsubscript{1}} AR agonists, likely from cardiovascular effects. MRS5474 was shown to induce antidepressant effects through an increase of homer1a in the brain (264).
**A₁ AR antagonists**

The widely ingested alkylxanthines theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) at physiological concentrations are weak (affinity ≈ 10 mM) nonselective antagonists at ARs. The main caffeine metabolite paraxanthine (1,7-dimethylxanthine) also acts as a central nervous stimulant and with even higher potency than caffeine at ARs and might be useful for treating hypersomnia associated with neurodegenerative diseases (265). 8-p-sulfophenyl derivatives SPT and DPSPX are moderately potent nonselective antagonists of ARs with the added benefits of water solubility and restricted entry to the brain. A₁ AR selective antagonists are derived from non-xanthines cores, e.g. adenine derivative WRC-0571 and pyrrolopyrimidine SLV320. An 8-cycloalkylxanthine derivative DPCPX is the most widely used A₁ AR selective antagonist with nM affinity, but its A₁ AR selectivity is greater in rodents than in human. Several 8-bicycloalkylxanthine derivatives KW3902 (rolofylline) and PSB36 are even more selective for the A₁ AR. As aforementioned, rolofylline was involved in the PROTECT project, a Phase III clinical study targeting renal failure in chronic heart disease (18) (138).
**A$_{2A}$ AR agonists**

A$_{2A}$ AR agonists are often substituted at the C2 position with arylalkyl (CGS21680) or ethynyl (ATL-313) substituents. CGS21680 is a widely used pharmacological probe for activation of the A$_{2A}$ AR, but its high AR subtype selectivity seen in rat and mouse is reduced at human (h) ARs, and its degree of entry into the brain is low. A single intrathecal injection of CGS21680 or ATL-313 was reported to have a long duration of protection against mechanical allodynia and thermal hyperalgesia in a model of chronic constriction injury (CCI) in mice (266). An X-ray structure of the hA$_{2A}$AR complex with agonist CGS21680 was recently reported (267). Another A$_{2A}$ AR agonist BVT.115959 (structure not disclosed) advanced to clinical trials for pain (260). UK-432097 is a selective A$_{2A}$ AR agonist that was in clinical trials for COPD, which were discontinued due to lack of efficacy. With its large molecular weight and many H-bonding groups, it is not orally bioavailable. However, these molecular features increase the stability of the A$_{2A}$AR complex, such that it was possible to obtain an X-ray crystallographic structure without extensive stabilizing point mutations (57). This was the first X-ray structure of an AR complex with an agonist to be reported. Although not detected in the X-ray structures, the A$_{2A}$ AR can form in situ various functional homo- and hetero- (e.g. with the A$_1$ AR or the D2 dopamine receptor) di- or multimers (268) (269) (270). The A$_{2A}$ AR can also heterodimerize with the P2Y$_{18}$. 

![Figure 12: Some important A1 AR antagonists in literature (20)](image)

![Figure 13: Some important A$_{2A}$ AR agonists in literature (20)](image)
**A₂A AR antagonist**

An 8-styrylxanthine derivative, Istradefylline (KW-6002, Kyowa Hakko), is approved for Parkinson’s disease treatment in Japan. The related compound CSC is selective for the A₂A AR, but also inhibits monoamine oxidase-2 (MAO-2), two activities that would be beneficial in neurodegenerative diseases. Two A₂A AR-selective antagonists, ZM-241385 and SCH-442416, are commonly used as A₂A AR-selective pharmacological probes of nM affinity. The X-ray structure of A₂A AR complex with ZM-241385 was the first antagonist-bound AR structure to be reported (54), and now a higher resolution structure of 1.8 Å resolution is available (271).

To the same family as SCH-442416 belongs Preladenant (SCH-420814, Merck), which was in clinical trials for treatment of Parkinson’s disease. A₂A AR-selective antagonists Tozadenant (SYN-115, Biotie) and Vipadenant (BII014, V2006, Biogen) were also in clinical trials for the same condition. The A₂A AR affinity of SCH-442416 was initially reported in the subnanomolar range, but the Ki value was redetermined to be 4 nM (255). The A₂A AR-selective adenine derivative ANR94, with a relatively low molecular weight, is effective in Parkinson’s disease models. A₂A AR-selective antagonists are also of interest in the context of checkpoint blockade for cancer immunotherapy, possibly by coadministration with other anticancer agents (272).

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**Figure 14:** Some important A₂A AR antagonists in literature (20)
A₂B AR agonists

Because of the widespread distribution of A₂B AR and the involvement of this receptor subtype in important (patho)-physiological processes both in peripheral tissues and in the central nervous system, many efforts have been carried out in order to identify potent and selective A₂B AR ligands yielded with noteworthy therapeutic potential. However, the lack of highly selective agents has hampered efforts to better characterize the A₂B AR subtype and consequently to fully define its therapeutic potential. 5′-(N-ethylcarboxamido)adenosine (NECA), a nonselective agonist, is currently considered to be one of the most potent agonists at the A₂B AR, with an EC₅₀ of nM (273) expressed in CHO cells and is the most frequently used ligand to activate this subtype. In order to identify selective and high affinity agonists for the A₂B AR many efforts have been devoted to modify the purine ring and ribose moiety of the adenosine. Nucleoside-based agonists are the result of modifying the endogenous ligand, adenosine, by substitution at the N6-, and C2-positions of the purine heterocycle and/or at the 5′-position of the ribose moiety. The various substitutions at these positions are designed to increase metabolic stability in biological systems, binding specificity and/or affinity at different adenosine receptor subtypes. In consequence, equally and more selective NECA derivatives, for the A₂B AR were obtained. These compounds have EC₅₀ values ranging from 82 to 450 nM for the A₂B AR and showed selectivity towards the other adenosine receptor subtypes (274). Recently, a new A₂B AR agonist 2-[6-amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-ylsulfanyl]acetamide BAY-606583, was patented by Bayer HealthCare and was used to study the cardioprotective function of A₂B receptors (275). This compound is very selective for the A₂B AR with an EC₅₀ value of 3–10 nM for the human A₂B AR and EC₅₀ values > 10 μM for the A₁, A₂A and A₃ AR subtypes, characterized by CHO cells in a gene-reporter assay expressing recombinant human receptors in high density (184). In order to identify selective and high affinity BAY-606583 has been used as a non-nucleoside agonist for selective activation of the A₂B AR, but its potency, selectivity and efficacy are less than originally reported (276) (277). Thus, it should be used cautiously and in combination with appropriate antagonists. BAY-606583 is of the same chemical series (3,5-dicyanopyridines) as A₁ AR agonist Capadenoson.

Figure 15: Some important A₂B AR agonists in literature
A2B AR antagonists

A2B AR antagonists have recently been reviewed (278). Certain modifications of the xanthine core structure at the 8-position with aryl groups have been found to result in selectivity for the A2B AR. The first antagonist to be reported was MRS1754. Later, groups at Univ. of Bonn, Germany (PSB-1115, PSB-603), University of Ferrara, Italy (MRE-2029-F20), at Gilead Sciences (CVT-6883, GS6201) and at Clinical Data Inc. (ATL802) improved on the degree of selectivity and/or the water solubility of the xanthines as A2B AR antagonists. For example, GS-6201 (CVT-6883) has a selectivity of 88-fold vs. hA1 AR, 149-fold vs. hA2A AR, and 49-fold vs. hA3 AR. PSB-603 shows a particularly high affinity and selectivity, not only in humans, but also in rodents. PSB-1115 exhibits high water-solubility and is therefore useful for in vivo studies, however its A2B AR affinity and selectivity is lower than that for other A2B AR antagonists. Besides xanthenes, 2-aminopyrimidine derivatives, such as LAS38096, 2-aminothiazolopyrimidines, benzothiazoles (279), pyridine derivatives (280), have been developed as A2B AR antagonists MRS1754 were introduced by the pharmaceutical industry for possible application to asthma, but neither remains on a clinical path.

Figure 16: A2B AR selective antagonists in literature (255)
**A<sub>3</sub> AR agonists**

A<sub>3</sub> AR agonists in Figure 17 are generally adenosine derivatives substituted at the C2, N6 and 5′ positions with various substituents. Those groups most favorable for A<sub>3</sub> AR selectivity include: N6 - benzyl or small alkyl; C2 e H, Cl, alkynyl or arylethynyl. IB-MECA (also known as Piclidenoson) and Cl-IB-MECA are widely used pharmacological probes with Cl-IB-MECA being more selective for the A<sub>3</sub> AR, and they are in clinical trials for inflammation (rheumatoid arthritis and psoriasis) and primary liver cancer, respectively (241). HEMADO has been used as a highly selective A<sub>3</sub> AR radioligand of high affinity (281). The actions of A<sub>3</sub> AR agonists IB-MECA and MRS5698 in chronic neuropathic pain and cerebroprotection have been described (282). They completely reverse or prevent alldynia and hyperalgesia in the chronic constriction injury (CCI) model but have no effect on acute pain.

![Chemical structures of A<sub>3</sub> AR agonists](image)

Figure 17: Some A<sub>3</sub> AR selective agonists in literature.
MRS5841, MRS5698 and MRS1898 contain a conformationally constrained bicyclic ring in place of ribose with a North conformation (N)-methanocarba ring system, which adds to the A<sub>3</sub> AR selectivity (283) (284) (285). MRS5698 is a peripherally-selective agonist that is highly A<sub>3</sub> AR selective (250). The pharmacokinetics of MRS5698, administered intraperitoneally in rat, indicates a t1/2 of 1.09 h (285), consistent with its duration of action in vivo in the CCI pain model. MRS5698 is also orally active in the CCI model.

**A<sub>3</sub> AR antagonists**

In an initial broad screen, several classes of non-xanthine antagonists were identified for the A<sub>3</sub> AR: 1,4-dihydropyridines, pyridines, and flavones. The pyridine derivative MRS1523 (Figure 18) has become a useful tool since it shows relatively high affinity not only for the human but also for the rat A<sub>3</sub> AR. Later it was noted that the potent non-selective AR antagonist CGS15943 could be modified to produce A<sub>3</sub> AR selectivity, and from selective modifications was obtained MRE3008-F20, one of the most potent and selective compounds at hA<sub>3</sub> AR.

![Figure 18: Some A3 AR antagonists in literature (255)](image-url)
The urea-substituted quinazoline derivative VUF5574 also possesses high $\alpha_{1B}$ AR affinity and selectivity. Some tricyclic xanthines (KF2677, PSB-10 and PSB-11) have been found to be very potent and selective antagonists at $\alpha_{1A}$ AR showing increased water-solubility due to a basic nitrogen atom in the additional imidazole ring. Recently described $\alpha_{3}$ AR-selective antagonists include pyrazolopyrimidinones. Many $\alpha_{3}$ AR antagonists are much more potent at human as compared to rat $\alpha_{3}$ AR. The principles for converting selective $\alpha_{3}$ AR agonists into selective $\alpha_{3}$ AR antagonists are based on either a conformationally constrained ribose-like ring or one that is truncated at the 4′-position (i.e., missing the ribose CH2OH group entirely). Thus, the nucleoside derivatives MRS5147 and its 3-ido analogue MRS5127 are highly selective $\alpha_{3}$ AR ligands generally across species. MRS5127 was recently reported as a radioligand selective for the $\alpha_{3}$ AR (286). The truncated 4′-thioadenosine derivative LJ-1251, which acts as a $\alpha_{3}$ AR antagonist across species, was shown to lower intraocular pressure when applied topically (287) (244).
2. Aim of the work

The work characterizing this Ph.D. thesis is part of a wider project that the research group I have joined during my Ph.D. course has undertaken in recent years and is actually developing. In fact, many compounds belonging to different heterocyclic classes were synthesized and evaluated as AR ligands (288) (289) (290) (291). In particular, the studies were focused on the development of selective antagonists for hA$_3$ and hA$_{2A}$ ARs, and also to obtain combined hA$_1$/A$_{2A}$ AR ligands with antagonistic activity (292).

As a general trend, AR ligands have been often projected through rational approaches, including molecular modeling studies based on the AR subtype structure they are targeting. In the past, the first approach that was used, usually started from the modification of the endogenous ligand, adenosine. These studies led to many different classes of ligands and several recurring structural features have been identified for defining affinity or efficacy of the compounds at the specific AR subtype (20). Antagonists are characterized by a large structural variability. They are generally nitrogen-containing heterocycles with a mono-, bi- or tricyclic core skeleton. In contrast, there is no such flexibility for the agonists. In fact, the agonist profile of AR ligands has been associated, for a long time, to an adenosine-like structure with the ribose moiety only slightly modified.

However, in the last 15 years, a major breakthrough with respect to both affinity and selectivity was achieved with the discovery of a new class of non-adenosine like compounds, the aminopyridine-3,5-dicarbonitrile series. These ligands first appeared in patent literature and the preliminary data suggested the involvement of the A$_{2B}$ AR as a target (293) (294) (295) but their intriguing characteristics emerged later. Successively, some research (296) reported that non-nucleoside AR agonists show less species differences than the adenosine-like ones and thus more versatility for pharmacological studies (255).

![Figure 19: Non-nucleoside A$_3$ and A$_{2A}$ AR agonist Capadenoson (BAY68-4986) and BAY60-6583, respectively](image-url)
Based on the aminopyridine-3,5-dicarbonitrile scaffold, Bayer Healthcare synthesized two interesting compounds belonging to this series: Capadenoson (BAY68-4986), a potent and selective A₁ AR agonist, and BAY60-6583 (Figure 19) which showed high affinity and selectivity for A₂B AR. In particular, BAY60-6583 was a real breakthrough: it was the first potent and selective AR agonist reported in literature with non-adenosine like structure. It has rapidly overcome the use of other nucleoside-like agonist in most of the pharmacological assays regarding A₂B AR. In particular it substituted the 5’-(N-ethylcarboxamido)adenosine (NECA, see Figure 15) that, since its discover in 1983, has been used as A₂B AR elected agonist, despite its scarce selectivity versus the other AR subtypes. Although BAY60-6583 has been largely studied, its real pharmacological profile is nowadays a matter of debate: in the patent describing BAY60-6583, Bayer Healthcare assessed its full agonist profile at A₂B AR. However, a 2014 study of Hinz et al. (277) reported that BAY60-6583 behaves as a partial agonist, with a maximum effect of 56% compared with the 100% of NECA as reference in cAMP accumulation assays, performed on CHO cells stably transfected with the hemagglutinin (HA)-tagged human A₂B AR (CHO-HA-hA₂B cells). In contrast, in our pharmacological assay (see Result Section below) BAY60-6583 acts as full agonist and shows to be less potent at A₂B AR than reported by Bayer Healthcare.

Contemporarily, Bayer Healthcare carried on studies on the A₁ AR agonist Capadenoson (Figure 19) which was one of the most interesting compound emerged as potentially useful for the treatment of angina being devoid of the side effect (e.g. bradycardia) proper of full agonist compounds. Since A₁ AR are the most extensively investigated among the ARs, the effects of Capadenoson emerged as so interesting that now it is in clinical trial for the treatment of persistent atrial fibrillation and also available as research tool (143). More recently, the focus of attention has moved to the antinociceptive effect exerted by A₁ AR agonist at the spinal cord level (144) (145) (146) (147) (148).

Starting from these results, in 2004 Beukers et al. (297) reported a small series of four aminopyridine-3,5-dicarbonitriles and among them LUF-5845 (Figure 20) resulted highly active at A₂B subtype though endowed with a scarce selectivity toward the other ARs.
Aim of the work

On this basis, our research group focused attention on the aminopyridine-3,5-dicarbonitrile series in order to obtain new AR ligands and to enlarge the scarcely known structure-activity relationships on this chemical class. In fact, few data are reported in the open literature, the majority of the known being included in patents (255). This kind of scaffold could allow to obtain ligands not only with a wide range of affinities but interestingly with different degrees of efficacy at the different ARs.

In fact, in a master degree thesis developed in our research group (298) four interesting compounds belonging to this chemical class were synthesized and pharmacologically evaluated at the ARs (Figure 21):

![Chemical structures A-D](image)

\[
\text{A} \quad \begin{align*}
\text{hA}_{1} \text{AR } K_i &= 57 \pm 4 \text{ nM} \\
\text{hA}_{2A} \text{AR } K_i &= 27\% \\
\text{hA}_{2B} \text{AR EC}_{50} &= >1000 \text{ (7\%)} \\
\text{hA}_{3} \text{AR } K_i &= 29\%
\end{align*}
\]

\[
\text{B} \quad \begin{align*}
\text{hA}_{1} \text{AR } K_i &= 108 \pm 9 \text{ nM} \\
\text{hA}_{2A} \text{AR } K_i &= 17\% \\
\text{hA}_{2B} \text{AR EC}_{50} &= >1000 \text{ (4\%)} \\
\text{hA}_{3} \text{AR } K_i &= 25\%
\end{align*}
\]

\[
\text{C} \quad \begin{align*}
\text{hA}_{1} \text{AR } K_i &= 175 \pm 12 \text{ nM} \\
\text{hA}_{2A} \text{AR } K_i &= 21\% \\
\text{hA}_{2B} \text{AR EC}_{50} &= >1000 \text{ (17\%)} \\
\text{hA}_{3} \text{AR } K_i &= 177 \pm 15
\end{align*}
\]

\[
\text{D} \quad \begin{align*}
\text{hA}_{1} \text{AR } K_i &= 12 \pm 2 \text{ nM} \\
\text{hA}_{2A} \text{AR } K_i &= 26 \pm 2 \text{ nM} \\
\text{hA}_{2B} \text{AR EC}_{50} &= 1.7 \pm 0.2 \text{ nM} \\
\text{hA}_{3} \text{AR } K_i &= 65 \pm 5 \text{ nM}
\end{align*}
\]

Figure 21: Lead compounds for aminopyridine-3,5-dicarbonitrile scaffold modifications

Pharmacological evaluation of these compounds highlighted the good affinity of compounds A-D at A\textsubscript{1} AR, being A and B A\textsubscript{1} selective. In contrast, while derivative C binds also the A\textsubscript{3} subtype, D emerges as totally unselective ligand. Together with the dicyanopyridine derivatives reported in literature (Figure 19 and 20), in this Ph.D. thesis we elect compounds A-C as leads, in order to evaluate how modifications at the dicyanopyridine core could modulate the affinity and selectivity, but also the pharmacological profile of the members of this chemical class at the AR subtypes. Thus, based on preliminary molecular modelling studies (299) (300), different substituents were introduced in diverse positions on the selected scaffold (Figure 22).
Aim of the work

Modifications were performed at both R₄ and R₆ positions (see Figure 22) on the dicyanopyridine core. In particular, while various aryl and heteroaryl substituents were introduced at R₄, position R₆ generally contains groups or atoms able to engage hydrogen-bonding interactions with the receptor site, being this latter a condition that seems to be mandatory for the AR receptor interaction. Moreover, it has been investigated substitution (R₂) on the amine function with cycloalkyl, aryl and acyl moieties in order to evaluate how they could have modulated the pharmacological profile of the target compounds on the diverse AR subtypes. Further modifications regard the sulfanyl linker of the leads which has been substituted with an oxygen atom or a secondary amino group.

Two major sets of amino-3,5-dicyanopyridines were synthesized: DCP₁ series that contains compounds preferentially binding the A₁ AR (Table 3) and the DCP₂B series including compounds designed in order to target the A₂B subtype (Table 4). For DCP₁ series, SAR investigations were performed at the R₂, R₄ and R₆ positions and also at level of the side chain linker. On the basis of compounds A-C pharmacological data (Figure 21), major modifications were performed at R₆ position of the dicyanopyridine core while maintaining the 2-furanyl moiety at R₄ or inserting other heteroaryl groups.

Figure 22: Modifications performed on the aminopyridine-3,5-dicarbanitrile scaffold

CH₂OH, CN, CONH₂, CONHCH₃, CONHCH₂OH, CONHOH, COOCH₃, COPh, COOCH₂Ph, 3-(COOCH₃)Ph, 3-(COOH)Ph, 3-(CONH₂)Ph, 3-(CH₂OH)Ph, 3-(CN)Ph, 2-(5-COOCH₃)furyl, 2-imidazole, 2-imidazoline, 2-benzimidazole, 3-pyrazole, 5-(1,2,4)triazole, 3-furyl, 2-thienyl, 3-thienyl, 3-pyridyl, 4-pyridyl, benzyl, Ph, 4-(Cl)Ph, 4-(F)Ph, 4-(CH₃)Ph, 4-(SCH₃)Ph, 4-(OCH₂C₆H₅)Ph, 4-(OCH₂C₄H₉)Ph, 4-(OCH₂CH(CH₃)₂)Ph, 4-(OCH₂CH₂)Ph, 4-(OCH₃C₆H₅)Ph, 4-(OCH₃C(CH₃)=CH₂)Ph, 4-(OCH₂C(CH₃)=CH₂)Ph, 4-(OH)Ph, 4-(NHCOCH₃)Ph

X = S, NH, O

Cyclopropyl, cyclopentyl, 4-(I)Ph, benzyl, COCH₃
Aim of the work

**DCP1 series**

<table>
<thead>
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<th>n°</th>
<th>X</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>n°</th>
<th>X</th>
<th>R₂</th>
<th>R₃</th>
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<th>R₅</th>
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<td>H</td>
<td>2-furyl</td>
<td>3-(COOCH₃)C₆H₄</td>
<td>14</td>
<td>S</td>
<td>H</td>
<td>2-furyl</td>
<td>2-phenylthiazol-4-yl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>H</td>
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<td>3-(CONH₂)C₆H₄</td>
<td>15</td>
<td>S</td>
<td>H</td>
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<td>3-pyridyl</td>
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</table>

Table 3: Synthesized compounds belonging to the DCP1 series

Thus, substituents bearing H-bond acceptor and donor groups were introduced at R₆ (compounds 1-16). Firstly, aryl homologation of the R₆ of the leads A-C were performed (compounds 1-9) together with other variegated modifications (10-14). Also nitrogen-containing heterocycles were inserted at R₆ (derivatives 13-16) in order to imitate the lead Capadenoson (Figure 19). Then, the influence of the nature of the linker X (compounds 17-20) or of the presence of R₂ substituents on the amine function (21-25) was evaluated. R₂ groups were selected on the basis of the SAR of adenosine-like agonists which preferentially bind the A₁ subtype. Such modifications were performed on the lead A or on strictly correlated analogues. As for the DCP2B series is concerned, major modifications were performed at R₄ and R₅ positions in order to target the A₂B subtype.
Aim of the work

DCP2B series

Table 4: Synthesized compounds belonging to the DCP2B series
While keeping the imidazol-2yl moiety at R₆ as it in compound D (Figure 21), different substituents were introduced at R₄ position (26-37, Table 4). Firstly, various heteroaryls, poorly investigated in literature, were evaluated together with diverse para-substituted phenyl moieties and also benzyl and methyl groups.

In parallel, position R₆ was modified while maintaining the para-(cyclopropylloxymethyl)phenyl substituent at R₄ that is typical of BAY60-6583 (Figure 19). Following the imidazole moiety idea (see the lead compound D, Figure 21), preferentially five-membered nitrogen-containing heterocycles bearing H-bond donor and acceptor groups were introduced at R₆ (compounds 38-46, Table 4). Also different carboxamide bioisosters (47-50) and their aryl homologues (51-52) were evaluated by maintaining the possibility of hydrogen bonding interaction at this level. Moreover, by in progress selecting the best substituents at R₆ in term of hA₂B AR affinity and selectivity, different para-oxysubstituted phenyl groups were introduced at R₄ (compounds 53-65). The O-(cyclo)alkyl and alkenyl substituents were chosen in order to maintain similar electronic and steric hindrance of the O-cyclopropyl moiety of BAY60-6583. In addition, the para-acetamido substituent (compounds 66-67, Table 4) as containing both H-bond donor and acceptor groups was evaluated.

Moreover, the 3,6-diamino-5-cyano-4-phenyl-thieno[2,3-b]pyridine-2-carboxamide scaffold will be evaluate and obtained by an intramolecular cyclization of the 6-amino-3,5-dicyanopyridines (proved to be AR ligands) bearing a sulfanyl-acetamido substituent at position-2 of the pyridine core. Preliminary investigation was performed on BAY60-6583 (Figure 23) in order to evaluate how molecular stiffening could influence the affinity and/or the pharmacological profile of these compounds at the ARs.

![BAY60-6583](image)

Figure 23: Bicyclic compound derived from cyclization of BAY60-6583
In addition to probe a new set of compounds as AR ligands, the information obtained could be useful for SAR studies and molecular modeling rationalization.

Finally, another objective of this work was the synthesis of a small set of compounds, the 7-amino-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitriles 70-73 (Figure 24) through a procedure that can be considered brand new for the most part. These compounds can be considered as ensued from a molecular complication of the amino-3,5-dicyanopyridine scaffold by condensation with a pyrazole core. The modification was planned in order to maintain the most important hydrophobic and hydrogen-bonding interactions potentially engaged by the original pattern.

Most of the effort was directed to finalize the procedure to obtain 2-arylpyrazolo[1,5-c]pyrimidines suitably substituted at position 5 in analogy to the lead DCP1 and DCP2B amino-3,5-dicyanopyridines. However, in the preliminary exploration of different synthetic approaches yielding the target compounds, a set of 7-amino-substituted derivatives 70-73 were synthesized and evaluated for their affinity at ARs. The latter, due to their structure and to the 5-amino-functionalization can be considered as potential A2A AR antagonists if compared for example to the well known ZM-241385 (Figure 24) even if they are lacking the essential 2-furyl moiety.

Figure 24: Newly synthesized 7-Amino-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitriles and lead compounds
Aim of the work

In addition to identify a new scaffold for the development of new AR ligands, this molecular complication approach was undertaken to evaluate the effect of this modification on the pharmacological profile of the new bicyclic derivatives, in particular at A_{2B} AR subtype.

To summarize, the main aim of this Ph.D. research was the investigation of the aminopyridine-3,5-dicarbonitrile chemical class in order to obtain potent and selective AR ligands. The study was an exploration of the potentiality of this scaffold to produce AR ligands with different affinities and pharmacological profiles with an eye to the A_{2B} and A_{1} subtypes. Preliminary studies were carried out on 7-amino-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitriles (70-73) to establish whether this heterocyclic scaffold suitably decorated could lead to the discover of new AR ligands.
3. Chemistry

3.1 Aminopyridine-3,5-dicarbonitriles

In order to achieve the desired aminopyridine-3,5-dicarbonitrile derivatives a short synthetic pathway was developed, following literature and patent advices (293) (294) (295). Most of the aminopyridine-3,5-dicarbonitrile compounds were synthesized applying the same procedures with only some exceptions. In fact, some compounds were synthesized with small deviation from the usual synthetic pathway for a matter of scarce yield or reactivity.

3.1.1 Chemistry of DCP1

For what may concern the aminopyridine-3,5-dicarbonitrile derivatives targeting A1 AR, the synthesis of compounds 1-16, B starts from the one-pot cyclization of the suitable commercial aldehyde to give sulfanylphenyl compounds 74-77 (scheme 1). In order to achieve these latter, the condensation reaction was performed by using tetrabutylammonium fluoride (TBAF), producing higher yields with respect to other methods reported in literature (293) (297). Intermediates 74-77 were converted into the sulfanyl derivatives 78-81 with sodium sulfide, which were used in order to obtain compounds 1-16, A by reacting with the suitable halides in mild alkaline conditions using sodium hydrgencarbonate as base. This reaction afforded final compounds in good yields.
In order to modify the sulfanyl bridge with an amine linker, compounds 17 and 18 were synthesized starting from intermediates 74 and 76, respectively, taking advantage of the good property of sulfanylphenyl group.
as leaving group in a nucleophile substitution. The reaction was easily performed at 100°C in N,N-dimethylformamide (DMF) using 2-aminoethanol as nucleophile (scheme 2).

![Scheme 2]

Compound 19, bearing a phenyl group at position $R_4$, was obtained through the synthesis of the chlorine intermediate 82 (scheme 3), prepared in a one-pot reaction (301) involving triethylorthobenzoate, malononitrile and hydrochloric acid and then used as starting material for coupling with commercial 2-aminoethanol to afford compound 19.

![Scheme 3]

Pursuing the modifications at the sulfanyl linker, compound 20, bearing a 6-oxyethanol chain was synthesized. One-pot cyclization of commercial 2-furaldehyde and malononitrile in the presence of NaOCH$_3$, afforded in low yields intermediate 83 (scheme 4) which was converted into the corresponding hydroxy analogue 84 by treatment with concentrated hydrochloric acid and glacial acetic acid at 100°C (302). Then, 84 was used as starting material for the coupling reaction with 2-bromoethanol, performed in microwave reactor, affording final compound 20.
Replacement of the primary amine function of the lead compound B with suitable substituted amino groups (21-24) was performed starting from B which undergoes Sandmeyer reaction using isoamyl nitrite as nitrosonium-donor molecule and CuCl$_2$ (scheme 5) (303). This reaction afforded compound 85 in good yields. Thus, the 2-chlorine atom of 85 was replaced with the suitable substituted amine yielding final compounds 21-24 in high yields.

Finally, derivative 25, bearing an acetyl moiety at the amino group, was synthesized starting from the reaction of the derivative 74 with acetic anhydride in the presence of pyridine, affording the intermediate 86 (scheme 6) (304). Then, the latter was converted into the corresponding sulfanyl derivative 87 by reacting with sodium...
sulfide. Then, compound 87 was used as starting material for the coupling reaction with 2-bromoethanol affording compound 25.

\[ \text{Scheme 6} \]

3.1.2 Chemistry of DCP2B

Concerning the synthesis of the aminopyridine-3,5-dicarbonitrile derivatives targeting the A2B AR, it is pretty similar to the general procedure showed before for the preparation of compound 1-16, B (scheme 1). The synthesis of compounds 26-34 starts from the one-pot cyclization of the suitable commercial aldehyde to give sulfanylphenyl intermediates 75, 77, 88-94 (scheme 7). This reaction has been performed by using diverse cyclization alkaline adjuvant able to work in a phase-transfer system. The synthetic pathway reported in scheme 7 involves the use of sodium sulfide for the achievement of the sulfanyl intermediates (79, 81, 95-101) and the coupling reaction with the 2-bromomethylimidazole 102 (scheme 10) in the presence of sodium hydrogen carbonate for obtaining the final compounds 26-34 (scheme 7).
For what may concern the sulfanyl intermediate 104, the precursor of final compound 35, a different approach was used: acetaldehyde highlighted a scarce reactivity in the one-pot reaction showed before, so it was necessary to synthesize the intermediate 103 by reaction of trimethylorthobenzoate with malononitrile (305) (Scheme 8). This intermediate allowed an easier cyclization to the sulfanyl compound 104 by direct reaction with thiocyanacetamide. Treatment of the latter with bromomethyl-2-imidazole hydrobromide 102 (scheme 10) in the presence of sodium hydrogen carbonate afforded the final derivative 35.
Introduction of an amine function replacing the sulfanyl linker typical of most of the final compounds was performed by using the chloro-intermediate 82 obtained in scheme 3. The latter was used as starting material for coupling with commercial (1H-imidazol-2-yl)methanamine to afford compound 36 (scheme 9).

Due to the unavailability of commercial 2-bromomethyl-1H-imidazole hydrobromide 102, this product was synthesized (297) (scheme 10) starting from imidazole-2-carboxyaldehyde, which was reduced to the corresponding alcohol 105 using sodium borohydride in absolute ethanol at room temperature for 2h. The latter was transformed into the bromomethyl derivative 102 by treatment with a 33% hydrobromic acid solution in acetic acid at 100°C.
In order to achieve \( \textit{para-O-phenyl} \)-substituted aldehydes 106-111, 4-hydroxybenzaldehyde was alkylated at the \textit{para}-OH function with the suitable alkyl halide in refluxing acetone in the presence of potassium carbonate (scheme 11) (306).

![Scheme 11](image)

O-susbtituted aldehydes 106-111 following a one-pot reaction with thiophenol, malononitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in aqueous ethanol (307) afforded the sulfanylphenyl intermediates 112-117 (scheme 12). Initially, compound 112 was synthesized by using \( \text{Al}_2\text{O}_3 \) as base, which was then replaced by DBU, leading to a general procedure with an easier work-up and higher yield. Thus, intermediates 112-117 were transformed into the sulfanyl analogues 118-123 with sodium sulfide in the conditions aforementioned, and then used as nucleophile in the coupling reaction with suitable halides yielding derivatives 37-65.
Scheme 12

\[
\text{106-111} + \text{CN} \rightarrow \text{112-117}
\]

\[
\text{37-65} \rightarrow \text{118-123}
\]

\[
a = \text{Diazabicycloundec-1-ene, EtOH}_{aq}, \text{55°C, 3-18h}
\]
\[
b = \text{Al}_2\text{O}_3, \text{H}_2\text{O, reflux, 6h}
\]
\[
c = \text{Na}_3\text{S, DMF}_{dry}, \text{80°C, 3h}
\]
\[
d = \text{R}_6\text{CH}_2\text{X, NaHCO}_3, \text{DMF}_{dry}, \text{rt}
\]

R = cyclopropylmethyl (106,112,118, 37-52)
cyclobutylmethyl (107,113,119,53,54)
isobutyl (108,114,120,55,56)
ethyl (109,115,121,57-60)
allyl (110,116,122,61-63)
2-methyl-1-propene (111,117,123,64,65)

<table>
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<th>n°</th>
<th>R</th>
<th>R_6</th>
<th>R_5CH_2X</th>
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<td>38</td>
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<td>imidazol-5-yl</td>
<td>c.a.</td>
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<tr>
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<td>1,2,4-triazol-1-yl</td>
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<td>128</td>
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\[
c.a. = \text{commercially available}
\]
As previously assumed, some of the halides used to achieve final products are not commercially available, so it has been necessary to synthesize them. The 3-(chloromethyl)-1H-pyrazole 124 was synthesized starting from commercial 3-methylpyrazole, which, in the first step, was oxidized with aqueous potassium permanganate to afford the 3-carboxylic acid 125 (scheme 13). Thus, the latter was transformed into the corresponding ethyl ester 126 using thionyl chloride in absolute ethanol, in a reaction in which was involved the formation of the acyl chloride, immediately reacting with ethanol and yielding compound 126. This procedure was the preferred one since the direct reduction of the carboxylic acid 125 did not give compound 127 in good yields. In contrast, reduction of 126 with lithium aluminum hydride in anhydrous THF afforded the target 127 in good yields, which was converted into the corresponding chloromethyl derivative 124 by reacting with thionyl chloride in anhydrous dichloromethane (308).

![Scheme 13](image)

Moreover, 3-chloromethyl(1H-1,2,4-triazole) hydrochloride 128 was synthesized starting from commercial ethyl 1H-1,2,4-triazole-3-carboxylate, which was previously converted into the corresponding alcohol 129 (scheme 14) using lithium aluminum hydride in anhydrous THF. This intermediate was finally transformed into 128 through a reaction in which thionyl chloride has been used as chlorinating agent (309).

![Scheme 14](image)
Furthermore, 4-(chloromethyl)thiazol-2-amine 130 and 4-(chloromethyl)-2-phenylthiazole 131 were synthesized in a similar way, starting from thiourea and thiobenzamide, respectively, by using 1,3-dichloroacetone in dry acetone (scheme 15) (310).

The 3-(chloromethyl)benzamide (311) 132, was synthesized starting from commercial 3-(chloromethyl)benzoic acid going through 3-(chloromethyl)benzoyl chloride obtained with oxalyl chloride in DCM using DMF as catalyst (scheme 16). The acyl chloride was not isolated, but it has been immediately used for the next step by reacting with anhydrous ammonia in dioxane to afford the desired compound 132.

The synthesis of benzyl 2-chloroacetate 133 (312) starts from commercial chloroacetic acid which was reacted with benzyl alcohol in the presence of p-toluenesulfonic acid (scheme 17). Furthermore, 2-chloro-N-hydroxyacetamide 134 was synthesized (313) from ethyl chloroacetoacetate in a 50% aqueous solution of hydroxylamine.
The 2-(chloromethyl)-4,5-dihydro-1H-imidazole 135 was produced (314) by cyclization of the iminoester hydrochloride 136 which was synthesized (315) (scheme 18) from commercial chloroacetonitrile in a saturated hydrochloric acid solution in absolute ethanol and dry diethyl ether while hydrochloric acid is bubbling. After isolation, the intermediate 136 was cyclized using ethylenediamine in absolute ethanol.

In order to obtain compounds 66 and 67 which bear a 4-(para-acetamidophenyl) moiety on the aminopyridine-3,5-dicarbonitrile core, it was not possible to achieve intermediate 137 in a one-pot reaction, due to the scarce reactivity of 4-acetamidobenzaldehyde. Thus, the aldehyde was reacted with malononitrile in a straightforward Knoevenagel condensation (scheme 19) (293) in the presence of piperidine as catalyst in ethanol to give intermediate 138 which was isolated to be used in the cyclization reaction involving thiophenol and DBU to yield 137.
Then, the sulfanylphenyl derivative 137 was converted into the sulfanyl intermediate 139 using sodium sulfide as previously shown. The latter was used to synthesize the final compounds 66 and 67 by reacting with 2-bromomethyl-1H-imidazole hydrobromide 102 (66) or commercially available 2-chloroacetamide (67), NaHCO₃, DMF, rt, 18h.

In order to shorten the procedure leading to the para-oxyphenyl-substituted compounds 37-65, a further pathway had been evaluated starting from commercial 4-hydroxybenzaldehyde (scheme 20). The latter was reacted with thiophenol, malononitrile and DBU in aqueous ethanol to afford the sulfanylphenyl intermediate 140 that was converted into the corresponding sulfanyl intermediate 141 then coupled with 2-chloroacetamide to yield compound 142. Here, the O-alkylation with potassium carbonate was expected to produce compound 54, but a competitive reaction afforded a diverse compound (69). In fact, due to the long time reaction and the basicity of the medium, the nitrile group in position 3 of the aminopyridine core...
undergoes condensation with the active methylene group on the sulfanylacetamide chain affording the bicyclic compound \textbf{69}. This collateral product was characterized through $^1$H NMR, $^{13}$C NMR and IR spectroscopy confirming our hypothesis.
Consequently, cyclization of BAY60-6583 was performed forcing reaction condition \((316)\) which involved potassium hydroxide in absolute ethanol as alkaline reactant to obtain compound \(68\) (scheme 21).

![Chemistry diagram](image)

3.2 Chemistry of 7-amino-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitriles

The 7-amino-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile derivatives \(70-73\) were prepared following a new and unique synthetic pathway (scheme 22). The synthesis included a one-pot condensation of ethyl cyanoacetate with carbon disulfide in DMF dry, followed by methylation of sulfide groups with methyl iodide, to afford intermediate \(143\). This latter was cyclized \((317)\) with acetophenone in the presence of potassium hydroxide in dry dimethylsulfoxide (DMSO) yielding the oxo-pyranone \(144\) in good yields which was used as starting material for the production of the pyrazole \(145\) by reacting with hydrazine monohydrate in pyridine \((318)\). Furthermore, the pyrazole \(\text{NH}\) of compound \(145\) was acylated with a Boc protecting group in order to avoid parasite reactions on the free nitrogen atom. The reaction was performed by using tert-butyl anhydride in dry acetonitrile in the presence of triethylamine (TEA), obtaining compound \(146\). Condensation of the latter with carbon disulfide using sodium hydride in dry DMSO and following methylation with methyl iodide in a one-pot reaction allowed to obtain intermediate \(147\), which was then deprotected by using trifluoroacetic acid in DCM to yield compound \(148\). Cyclization of the latter in N-methylpyrrolidin-1-one (NMP) by reacting with a strong excess of cyanamide in the presence of 4-toluenesulphonic acid afforded compound \(149\).
The reaction was performed in microwave reactor at 160°C for 1h. Consequentially, methylsulfanyl function of compound 149 was oxidize to the methylsulfonyl group with 3-chloroperoxybenzoic acid (m-CPBA) in DCM affording compound 150. The latter was used as starting material for the reaction with the suitable primary amine in the presence of N,N-diisopropylethylamine (DIPEA) to obtain the desired final compounds 70-73 in high yields.
4. Results and discussions

All the synthesized compounds were evaluated by the group of Prof. Katia Varani at the Medical Science Department – Pharmacology section of the University of Ferrara, for their affinity at human (h) A1, hA2A and hA3 AR (expressed as Kᵢ values obtained in binding experiments) displacing the suitable radioligand for each AR subtype ([³H]DPCPX for A1 AR, [³H]ZM241385 for A2A AR and [¹²⁵I]AB-MECA for A3 AR, respectively) stably expressed on chinese hamster ovary (CHO) cells. The potency at the hA2B AR (expressed as EC₅₀ values in cAMP functional assay) was assessed in hA2B CHO cells stimulated by 200 nM NECA, at 1µM concentration of the examined compounds. Kᵢ values are means ± SEM of four separate assays each performed in duplicate while EC₅₀ values are expressed as means ± SEM of four separate cAMP experiments. Some selected derivatives (A, C, 1, 2, 4, 5, 9, 14, 15, 26, 36) were tested to assess their agonistic or antagonistic properties at hA1 and hA2A ARs. Thus their capability to modulate cAMP production was evaluated on hA1 and hA2A CHO cells.

4.1 DCP1 series

Based on the preliminary results obtained for compound A-C (Figure 21), modifications on dicyanopyridine scaffold were performed at position R₆ (Table 5). Firstly, replacement of the methyl carboxylate function of B with a nitrile group yielded compound 10 which results equipotent at the hA1 AR as the leads A-C. Aryl homologation on methyl ester function of B led to compound 1, which binds at the hA2A subtype but loses selectivity for the hA1 AR with respect to the lead B. In functional assays 1 shows an inverse agonist profile at the hA1 AR, while it acts as antagonist at the hA2A AR (Table 6). In order to enlarge information on this kind of residues at position R₆, the methyl ester of 1 was transformed into the carboxamido (2) (as aryl homologue of the lead C), carboxylic acid (3), methyl alcohol (4) and nitrile (5) analogues which maintain on the whole the same trend of AR affinity with respect to compound 1. In contrast, compound 2, 3 and 5 show some affinity for hA3 AR but in the high nanomolar range thus maintaining a good selectivity. In fact, the carboxylic acid 3 shows more than 100 fold hA1 selectivity versus hA2A and hA3 receptors, thus higher than the parents 1, 2, 4, 5. It is important to highlight that compounds 2, 4 and 5, such as 1, act as inverse agonist at hA1 AR while 2 and 4 behave as hA2A AR antagonists. An interesting result was obtained performing an isosteric replacement of the phenyl ring of compound 1 with a furane moiety obtaining the methyl 5-furan-2-carboxylate derivative 6 which is endowed with good affinity and high selectivity at the hA1 AR. Meanwhile, modifications at R₄ were performed replacing the 2-furyl moiety of 1 with a 3-furyl (7), 2-thienyl (8) and 3-pyridyl (9) substituent in order to understand if a diverse heterocycle could modulate the affinity and the pharmacological profile of the target compounds. While compound 7 bearing the 3-furyl substituent at R₄
Results and discussion

maintains the same trend of affinity of the reference 1, derivatives 8 and 9 acquire a higher selectivity versus the hA2A AR with respect to 1.

![Diagram](image)

Table 5

<table>
<thead>
<tr>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>R&lt;sub&gt;4&lt;/sub&gt;</th>
<th>R&lt;sub&gt;6&lt;/sub&gt;</th>
<th>hA&lt;sub&gt;1&lt;/sub&gt; CHO cells</th>
<th>hA&lt;sub&gt;2A&lt;/sub&gt; CHO cells</th>
<th>hA&lt;sub&gt;2B&lt;/sub&gt; cAMP assay</th>
<th>hA&lt;sub&gt;3&lt;/sub&gt; CHO cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2-furyl</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;OH</td>
<td>57 ± 4 (inverse agonist)</td>
<td>27%</td>
<td>&gt;1000 (7%)</td>
<td>29%</td>
</tr>
<tr>
<td>B</td>
<td>2-furyl</td>
<td>COOCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>108 ± 9</td>
<td>17%</td>
<td>&gt;1000 (4%)</td>
<td>25%</td>
</tr>
<tr>
<td>C</td>
<td>2-furyl</td>
<td>CONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>175 ± 12</td>
<td>21%</td>
<td>&gt;1000 (17%)</td>
<td>177 ± 15</td>
</tr>
<tr>
<td>1</td>
<td>2-furyl</td>
<td>3-(COOCH&lt;sub&gt;3&lt;/sub&gt;)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>7.22 ± 0.63 (inverse agonist)</td>
<td>68±3 (antagonist)</td>
<td>&gt;1000 (7%)</td>
<td>26%</td>
</tr>
<tr>
<td>2</td>
<td>2-furyl</td>
<td>3-(CONH&lt;sub&gt;2&lt;/sub&gt;)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1.32±0.11 (inverse agonist)</td>
<td>67±6 (antagonist)</td>
<td>&gt;1000 (6%)</td>
<td>326±31</td>
</tr>
<tr>
<td>3</td>
<td>2-furyl</td>
<td>3-(COOH)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4.12±0.38</td>
<td>581±54</td>
<td>&gt;1000 (1%)</td>
<td>311±58</td>
</tr>
<tr>
<td>4</td>
<td>2-furyl</td>
<td>3-(CH&lt;sub&gt;3&lt;/sub&gt;OH)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.89±0.09 (inverse agonist)</td>
<td>31±3 (antagonist)</td>
<td>&gt;1000 (4%)</td>
<td>25%</td>
</tr>
<tr>
<td>5</td>
<td>2-furyl</td>
<td>3-(CN)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1.02±0.11 (inverse agonist)</td>
<td>93±9</td>
<td>&gt;1000 (7%)</td>
<td>668±64</td>
</tr>
<tr>
<td>6</td>
<td>2-furyl</td>
<td>methyl 5-furan-2-carboxylate</td>
<td>82 ± 8</td>
<td>1%</td>
<td>&gt;1000 (7%)</td>
<td>676±64</td>
</tr>
<tr>
<td>7</td>
<td>3-furyl</td>
<td>3-(COOCH&lt;sub&gt;3&lt;/sub&gt;)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2.11±0.19</td>
<td>96±8</td>
<td>&gt;1000 (7%)</td>
<td>1%</td>
</tr>
<tr>
<td>8</td>
<td>2-thienyl</td>
<td>3-(COOCH&lt;sub&gt;3&lt;/sub&gt;)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2.72±0.21</td>
<td>693±68</td>
<td>98±95 (62%)</td>
<td>15%</td>
</tr>
<tr>
<td>9</td>
<td>3-pyridyl</td>
<td>3-(COOCH&lt;sub&gt;3&lt;/sub&gt;)C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1.18±0.11 (inverse agonist)</td>
<td>636±57</td>
<td>&gt;1000 (15%)</td>
<td>8%</td>
</tr>
<tr>
<td>10</td>
<td>2-furyl</td>
<td>CN</td>
<td>93 ± 8</td>
<td>887±84</td>
<td>&gt;1000 (1%)</td>
<td>582±56</td>
</tr>
<tr>
<td>11</td>
<td>2-furyl</td>
<td>COOCH&lt;sub&gt;3&lt;/sub&gt;CaH&lt;sub&gt;5&lt;/sub&gt;</td>
<td>86±7</td>
<td>8%</td>
<td>&gt;1000 (10%)</td>
<td>1%</td>
</tr>
<tr>
<td>12</td>
<td>2-furyl</td>
<td>COCaH&lt;sub&gt;5&lt;/sub&gt;</td>
<td>23 ± 2</td>
<td>224±21</td>
<td>&gt;1000 (1%)</td>
<td>1%</td>
</tr>
<tr>
<td>13</td>
<td>2-furyl</td>
<td>3-pyridyl</td>
<td>1.42±0.15</td>
<td>24±3</td>
<td>&gt;1000 (1%)</td>
<td>948±82</td>
</tr>
<tr>
<td>14</td>
<td>2-furyl</td>
<td>2-pyridyl</td>
<td>2.71±0.21 (inverse agonist)</td>
<td>108±9</td>
<td>&gt;1000 (9%)</td>
<td>134±12</td>
</tr>
<tr>
<td>15</td>
<td>2-furyl</td>
<td>2-phenylthiazol-4-yl</td>
<td>3.85±0.32 (partial agonist)</td>
<td>523±49</td>
<td>947±92 (29%)</td>
<td>438±41</td>
</tr>
<tr>
<td>16</td>
<td>2-furyl</td>
<td>2-aminothiazol-4-yl</td>
<td>1.27 ± 0.11</td>
<td>22 ± 2</td>
<td>&gt;1000 (9%)</td>
<td>193 ± 17</td>
</tr>
</tbody>
</table>

*Ki values are means ± SEM of four separate assays each performed in duplicate. Percentage of inhibition (I%) are determined at 1µM concentration of the tested compounds. EC<sub>50</sub> values are expressed as means ± SEM of four separate cAMP experiments.*
This data let us to hypothesize that the furan ring is important for the anchoring of these derivatives at the hA2A subtype. Further modifications were performed at R6 keeping the 2-furyl moiety at position R4. Thus, compound 11 and 12 were synthesized, spacing the phenyl residue at R6 from the dicyanopyridine core with respect to compounds 1-6. While compound 12 maintains the same profile of derivatives 1-5 binding at both hA1 and hA2A AR, the benzyl ester 11 showed a good affinity for hA1 AR and high selectivity suggesting the possibility to modulate A1 selectivity with respect to A2A AR by performing this modification on the sulfanylmethyl function.

Furthermore, in order to evaluate the presence of diverse heteroaryl moieties at position R6 for the binding at ARs, 3-pyridyl (13) and 2-pyridyl (14) derivatives were produced which maintain hA1 and hA2A affinity in the low and in the high nanomolar range, respectively. For compound 14 which behaves as inverse agonist (Table 5 and 6) also the binding at hA3 subtype increases. What is noteworthy is that preliminary functional data indicate that compound 13 seems to behave as a weak agonist at the hA2A AR opening the possibility to consider this compound as a lead to design new A2A agonists belonging to this series. Pursuing modifications at position R6, 2-phenylthiazol-4-yl (15) and 2-aminothiazol-4-yl (16) derivatives were synthesized. These compounds showed high affinity for hA1 AR, and compound 15, acting as a partial agonist, a good selectivity versus the other ARs. At this moment, no data are available about the functional profile of the aminothiazole compound 16, that could have been of help for understanding the potential role of a thiazolyl moiety at R6 for shifting the profile of these derivatives from inverse agonist to agonist (compare to 15).

Table 6: Functional assays at A1 and A2A ARs of selected compounds

<table>
<thead>
<tr>
<th></th>
<th>hA1 EC50 (nM)</th>
<th>hA2A CHO cells EC50 (nM)</th>
<th>hA2A CHO cells IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128 ± 11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1.43 ± 0.11</td>
<td>&lt; 20%</td>
<td>313 ± 29 (10 µM: 67%)</td>
</tr>
<tr>
<td>2</td>
<td>2.84 ± 0.27</td>
<td>&lt; 20%</td>
<td>58 ± 5 (10 µM: 49%)</td>
</tr>
<tr>
<td>4</td>
<td>0.98 ± 0.09</td>
<td>&lt; 20%</td>
<td>22 ± 2 (10 µM: 99%)</td>
</tr>
<tr>
<td>5</td>
<td>4.82 ± 0.39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>6.26 ± 0.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>34 ± 3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>4.12 ± 0.37</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Moreover, replacement of the sulfanyl linker of the lead compound A with an amino group (17) or an oxygen atom (20) results to be detrimental for affinity at hA1 AR (Table 7), thus confirming the importance of the sulfur atom as linker in this position. While keeping the aminoethanol function of 17, replacement of the 2-furyl moiety with 2-thienyl (18) or phenyl (19) substituent enhanced affinity at the A1 AR with respect to 17.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>n°</th>
<th>R4</th>
<th>X</th>
<th>[3H]DPCPX Ki (nM) or % at 1μM</th>
<th>[3H]ZM241385 Ki (nM) or % at 1μM</th>
<th>EC50 (nM) (efficacy %)</th>
<th>[125I]ABMECA Ki (nM) or % at 1μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2-furyl</td>
<td>S</td>
<td>57 ± 4</td>
<td>27%</td>
<td>&gt;1000 (7%)</td>
<td>29%</td>
</tr>
<tr>
<td>17</td>
<td>2-furyl</td>
<td>NH</td>
<td>20%</td>
<td>18%</td>
<td>&gt;1000 (1%)</td>
<td>17%</td>
</tr>
<tr>
<td>18</td>
<td>2-thienyl</td>
<td>NH</td>
<td>324 ± 28</td>
<td>33%</td>
<td>&gt;1000 (1%)</td>
<td>29%</td>
</tr>
<tr>
<td>19</td>
<td>C6H5</td>
<td>NH</td>
<td>943 ± 87</td>
<td>1%</td>
<td>&gt;1000(1%)</td>
<td>1%</td>
</tr>
<tr>
<td>20</td>
<td>2-furyl</td>
<td>O</td>
<td>632 ± 53</td>
<td>25%</td>
<td>&gt;1000 (11%)</td>
<td>3%</td>
</tr>
</tbody>
</table>

Table 7

Substitution of the primary amine function with R2 groups (Table 8) was useful to evaluate the role of this position for the binding at the others ARs. In particular, this information is important to evaluate the mode of binding of these compounds at the A1 subtype with respect to the adenosine-like ligands. Cycloalkyl groups such as cyclopropyl (21) and cyclopentyl (22) and also other moieties (compounds 23-25) have been introduced at R2 on the basis of advice coming from the SARs on the adenosine-like A1 AR agonists reported in literature. While compound 21 binds the hA1 AR as the lead A, compound 22 bearing the usual cyclopentyl substituent for selective A1 ligands was inactive at this subtype. Unexpectedly, also the aryl-substituted compound 23 showed any affinity at the ARs, although some nucleosidic A1 AR agonists bear this moiety. Nevertheless, this behavior can confirm the diverse binding mode of these non-nucleoside ligands with respect to the nucleoside ones. Compound 24 bearing a NH-benzylamino substituent is endowed with high affinity and good selectivity for the hA1 AR that are comparable to those of derivative 21. Probably, an increased length chain of this residue could be useful for the anchoring at the active site of the A1 AR. Replacement of the amine function of A with an acetamidido group (compound 25) enhances hA3 AR affinity suggesting that an acyl moiety could be useful for shifting the binding of the aminopyridine-3,5-dicarbonitrile derivatives toward the hA3 AR, as extensively demonstrated for other AR ligands in literature.
Results and discussion

4.2 DCP2B series

All the derivatives designed as A$_{2b}$ AR ligands (Table 9) behave generally as partial agonists (Table 10) with the only exception of derivatives 66, 67 which show a full agonist profile.

Previously reported compound D bearing a 2-imidazolyl moiety at position R$_6$ shows high affinity at all the AR subtypes, and in particular high activity at the hA$_{2b}$ AR behaving as a partial agonist. The trend is similar to that of the analogue LUF-5845, reported in literature (296). In order to explore the potentiality of the imidazolyl moiety at position R$_6$ for activity at A$_{2b}$ AR, the 2-furyl substituent at R$_4$ of D was replaced especially by other heteroaryl and 4-substituted-aryl residues, obtaining a set of compounds showed in Table 9. All these derivatives are endowed with a partial agonist profile at the hA$_{2b}$ AR.
Results and discussion

<table>
<thead>
<tr>
<th>n°</th>
<th>X</th>
<th>R₄</th>
<th>[³H]DPCPX Ki(nM) or I% at 1µM</th>
<th>[³H]ZM241385 Ki (nM) or I% at 1µM</th>
<th>EC₅₀ (nM) (efficacy %)</th>
<th>[¹²⁵I]ABMECA Ki (nM) or I% at 1µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>S</td>
<td>2-furyl</td>
<td>12 ± 2</td>
<td>26 ± 2 (partial agonist)</td>
<td>1.7 ± 0.2 (52%)</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>26</td>
<td>S</td>
<td>3-furyl</td>
<td>0.77±0.09</td>
<td>37±3 (antagonist)</td>
<td>2.32±0.21 (42%)</td>
<td>274±23</td>
</tr>
<tr>
<td>27</td>
<td>S</td>
<td>3-thiényl</td>
<td>1.01±0.09</td>
<td>55±6</td>
<td>3.15±0.29 (47%)</td>
<td>221±20</td>
</tr>
<tr>
<td>28</td>
<td>S</td>
<td>3-pyridyl</td>
<td>5.26±0.48</td>
<td>463±38</td>
<td>63±5 (58%)</td>
<td>19%</td>
</tr>
<tr>
<td>29</td>
<td>S</td>
<td>4-pyridyl</td>
<td>8.85±0.82</td>
<td>81±7</td>
<td>12.5±1.3 (36%)</td>
<td>26%</td>
</tr>
<tr>
<td>30</td>
<td>S</td>
<td>CH₂C₆H₅</td>
<td>31%</td>
<td>8%</td>
<td>260±24 (44%)</td>
<td>1%</td>
</tr>
<tr>
<td>31</td>
<td>S</td>
<td>4-Cl-C₆H₄</td>
<td>30±4</td>
<td>865±68</td>
<td>109±12 (32%)</td>
<td>850±62</td>
</tr>
<tr>
<td>32</td>
<td>S</td>
<td>4-F-C₆H₄</td>
<td>0.78±0.08</td>
<td>139±12</td>
<td>18±2 (76%)</td>
<td>410±38</td>
</tr>
<tr>
<td>33</td>
<td>S</td>
<td>4-CH₃-C₆H₄</td>
<td>5.32±0.42</td>
<td>47±4</td>
<td>78±6 (78%)</td>
<td>212±19</td>
</tr>
<tr>
<td>34</td>
<td>S</td>
<td>4-SCH₃-C₆H₄</td>
<td>8.32±0.78</td>
<td>227±21</td>
<td>35.7±3.4 (30%)</td>
<td>388±29</td>
</tr>
<tr>
<td>35</td>
<td>S</td>
<td>CH₃</td>
<td>74±8</td>
<td>331±32</td>
<td>1%</td>
<td>510±53</td>
</tr>
<tr>
<td>36</td>
<td>NH</td>
<td>C₆H₅</td>
<td>140 ± 12</td>
<td>115±10 (antagonist)</td>
<td>1%</td>
<td>19%</td>
</tr>
<tr>
<td>37</td>
<td>S</td>
<td>4-(OCH₂C₆H₅)-C₆H₄</td>
<td>235±24</td>
<td>764±72</td>
<td>9.52±0.91 (70%)</td>
<td>474±45</td>
</tr>
<tr>
<td>LUF-5845 (296)</td>
<td>S</td>
<td>4-(OCH₃)C₆H₄</td>
<td>7</td>
<td>214</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>BAY60-6583</td>
<td></td>
<td></td>
<td>31%</td>
<td>2%</td>
<td>3 ± 3</td>
<td>8%</td>
</tr>
</tbody>
</table>

Table 9
Substitution at R₄ with different heterocyclic residues such as 3-furyl (26), 3-thienyl (27), 3-pyridyl (28) and 4-pyridyl (29) maintains the activity at the A₂B AR, but also scarce selectivity. Various para-substituted phenyl groups were also introduced at this position: 4-Cl (31), 4-F (32), 4-CH₃ (33) and 4-SCH₃ (34) residues positively influence affinity and activity at A₁ and A₂B AR, even if in minor extent for the 4-chloro-substituent, and have different effects on selectivity versus the other AR subtypes. However, para-substitution with a cyclopropylmethyloxy, which is typical of BAY60-6583, makes compound 37 as a very potent and selective hA₂B agonist. In fact, 37 results 3-fold more active than BAY60-6583 at the hA₂B AR, and shows also very good selectivity versus the other AR subtypes (25 times vs hA₁, 80 times vs hA₂A, and 50 times vs hA₃). Furthermore, it acts as partial agonist. Indeed, substitution at R₄ of a benzyl moiety (30) produces a very good selectivity for hA₂B but also a decrease of potency is observed. Thus, it remains to be demonstrated whether a compromise between length and dimension of the appended R₄ substituent could exist, in order to target selectively the A₂B subtype. Moreover, a methyl group at position R₄ was introduced yielding compound 35 that is totally inactive at the hA₂B AR but shows nanomolar affinity for the hA₁ subtype. Replacement of the sulfanyl linker with an amine function (36) as performed on DCP1 series is extremely detrimental for hA₂B AR activity, while some affinity for hA₁ and hA₂A AR is maintained.

Thus, keeping the 4-cyclopropylmethyloxyphenyl moiety at R₄, the imidazolyl at R₆ was replaced with diverse H-bond donor/acceptor-group containing heterocycles to yield a subset of compounds showed in Table 11. All these derivatives show a partial agonist profile at hA₂B AR.

### Table 10: Functional assays on A1 and A2A ARs for selected compounds

<table>
<thead>
<tr>
<th></th>
<th>hA₁ EC₅₀ (nM)</th>
<th>hA₂B CHO cells EC₅₀ (nM)</th>
<th>hA₂B CHO cells IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>19 ± 2 (39%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Partial Agonists</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>0.77 ± 0.09</td>
<td>&lt; 20%</td>
<td>138 ± 12 (10 µM: 64%)</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
<td>&lt; 20%</td>
<td>814 ± 72 (10 µM: 93%)</td>
</tr>
</tbody>
</table>

cAMP assay
Firstly, the isomer of compound 37, which bears an imidazol-5-yl moiety (38), was produced. The latter results less active than its isomer at the hA2B AR, but but also at hA2A and hA3 ARs. Unexpectedly, replacement of the 2-imidazolyl group of 37 with an imidazoline (39) was detrimental for hA2B activity. The same is observed for the 2-benzoimidazole moiety (compound 40). In this case, the inactivity at A2B receptor could be ascribed to the excessive steric hindrance of this moiety at this level. Compound 41, bearing a 3-pyrazolyl...
group shows a good affinity and selectivity at hA$_{2B}$ AR which are comparable with those of the 1,2,4-triazol-5-yl group (43). These data, together with those of the 1,2,4-triazol-1-yl compound (42), which is totally inactive at all the ARs, suggest how important is the presence at R$_{6}$ of H-bond acceptor/donor functions. Compound 42 drastically loses activity at A$_{2B}$ AR due probably to the loss of NH function on the appended triazole moiety useful for a profitable interaction with the binding site. Tetrazolyl-substitution in compound 44 results detrimental for affinity at all ARs, probably due to the excessive acidity of the NH tetrazole moiety. Moreover, confirming data on the DCP1 series, 2-aminothiazol-4-yl (45) and 3-pyridyl (46) moieties shift the affinity toward the hA$_{1}$ AR, to the detriment of hA$_{2B}$ AR. N-methylacetamido compound 47 and its analogous N-hydroxymethylacetamido 48 result to be highly selective and active at hA$_{2B}$ AR, while hydroxamic acid 49 loses its potency, probably due to the acidity of its R$_{6}$ residue, scarcely tolerated by A$_{2B}$ AR. Hence, a methyl carboxylate function results detrimental (compound 50) for hA$_{2B}$ affinity. Also its aryl homologue 51 has low activity at hA$_{2B}$ AR confirming how both H-bond acceptor and donor functions at this position are essential for receptor-ligand interaction. In fact, 3-benzamido-substituted compound 52 is highly active at hA$_{2B}$ AR while maintaining good affinity for hA$_{1}$ subtype.

At the end, further substitutions were performed in para position of the phenyl moiety at R$_{4}$, while keeping few selected substituents (imidazol-2-yl, pyrazol-3-yl, carboxyamido and N-methylcarboxyamido) at position R$_{6}$, thus, a subset of compounds were generated whose pharmacological data are expressed in Table 12. It has to be outlined that all the aminodicyanopyridine reported until now behave as partial agonist at the hA$_{2B}$ AR with the only exception of compounds 66 and 67 that show a full agonist profile at this subtype. Preliminary data show that 4-cyclobutylmethylxylo substitution results detrimental for activity at all the ARs (compound 53), this result being quite unexpected. Furthermore, the 4-isobutyloxy derivatives 55, 56 show divergent results. In fact, compound 55, bearing a imidazol-2-yl moiety at R$_{6}$ is endowed of modest activity at the hA$_{2B}$ AR, while the carboxamido derivative 56 is completely inactive at this subtype. The 4-ethyloxy substituted compounds 57-60 are endowed from high (57,58) to modest (59,60) activity at A$_{2B}$ AR but scarce selectivity.

Balanced A$_{1}$/A$_{2B}$ AR activity is proper of the 4-allyloxy-substituted compound 61, which bears the imidazol-2-yl moiety at R$_{6}$. However, despite the same 4-allyloxy group on R$_{4}$ position, substitution with both carboxyamido (62) and pyrazol-3-yl (63) moieties resulted detrimental for hA$_{2B}$ AR activity although compound 63 binds with high activity and selectivity the hA$_{1}$ AR. Also the 4-methyallyloxy substituent (derivatives 64) shifts the affinity toward the hA$_{1}$ AR. In some case, the replacement of the para-cyclopropylmethylxylo-phenyl group at position R$_{4}$ typical of the A$_{2B}$ agonist BAY60-6583 with other para-cycloalkyl- or para-cycloalkenyl-methylxyphenyl substituents do not produce a high decrease of hA$_{2B}$ activity but preferentially an increase of affinity at the other ARs, thus reducing the selectivity. Finally, para-acetamidophenyl-substituted derivatives 66, 67, bearing respectively a imidazol-2-yl and a carboxyamido
Results and discussion

66 and 67 moieties at R₆ are endowed of high activity at hA₂B AR despite a scarce selectivity versus the others AR subtypes. However, as already outlined, compounds 66 and 67 are the only two amino-3,5-dicyanopyridines in the whole series having a full agonist profile.

<table>
<thead>
<tr>
<th>n°</th>
<th>R₄</th>
<th>R₆</th>
<th>[³H]DPCPX Ki (nM) or (% at 1µM)</th>
<th>[³H]ZM241385 Ki (nM) or (% at 1µM)</th>
<th>EC₅₀ (nM) (efficacy %)</th>
<th>[¹²⁵I]ABMECA Ki (nM) or (% at 1µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>4-(OCH₂C₃H₆)C₆H₄</td>
<td>imidazol-2-yl</td>
<td>235±24</td>
<td>764±72</td>
<td>9.52±0.91 (70%)</td>
<td>474±45</td>
</tr>
<tr>
<td>53</td>
<td>4-(OCH₂C₄H₇)C₆H₄</td>
<td>imidazol-2-yl</td>
<td>25%</td>
<td>4%</td>
<td>12%</td>
<td>574 ± 46</td>
</tr>
<tr>
<td>54</td>
<td>4-(OCH₂C₄H₇)C₆H₄</td>
<td>CONH₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>4-(OCH₂CH(CH₂)₂)C₆H₄</td>
<td>imidazol-2-yl</td>
<td>26%</td>
<td>1%</td>
<td>227±21</td>
<td>1%</td>
</tr>
<tr>
<td>56</td>
<td>4-(OCH₂CH(CH₂)₂)C₆H₄</td>
<td>CONH₂</td>
<td>603 ± 52</td>
<td>307 ± 27</td>
<td>1%</td>
<td>9%</td>
</tr>
<tr>
<td>57</td>
<td>4-(OCH₂CH₂)C₆H₄</td>
<td>imidazol-2-yl</td>
<td>8.21±0.73</td>
<td>221±19</td>
<td>11.7±1.2 (62%)</td>
<td>85±6</td>
</tr>
<tr>
<td>58</td>
<td>4-(OCH₂CH₂)C₆H₄</td>
<td>CONH₂</td>
<td>345±27</td>
<td>1%</td>
<td>38±3 (66%)</td>
<td>20%</td>
</tr>
<tr>
<td>59</td>
<td>4-(OCH₂CH₂)C₆H₄</td>
<td>CONHCH₃</td>
<td>104 ± 8</td>
<td>8%</td>
<td>211 ± 17 (48%)</td>
<td>834 ± 74</td>
</tr>
<tr>
<td>60</td>
<td>4-(OCH₂CH₂)C₆H₄</td>
<td>pyrazol-3-yl</td>
<td>21 ± 2</td>
<td>4%</td>
<td>332 ± 28 (34%)</td>
<td>18%</td>
</tr>
<tr>
<td>61</td>
<td>4-(OCH₂CH=CH₂)C₆H₄</td>
<td>imidazol-2-yl</td>
<td>16±2</td>
<td>17%</td>
<td>27±3 (56%)</td>
<td>8%</td>
</tr>
<tr>
<td>62</td>
<td>4-(OCH₂CH=CH₂)C₆H₄</td>
<td>pyrazol-3-yl</td>
<td>44 ± 4</td>
<td>8%</td>
<td>7%</td>
<td>13%</td>
</tr>
<tr>
<td>63</td>
<td>4-(OCH₂CH=CH₂)C₆H₄</td>
<td>CONH₂</td>
<td>323 ± 25</td>
<td>18%</td>
<td>11%</td>
<td>444 ± 39</td>
</tr>
<tr>
<td>64</td>
<td>4-(OCH₂C(CH₃)=CH₂)C₆H₄</td>
<td>imidazol-2-yl</td>
<td>77 ± 6</td>
<td>1%</td>
<td>14%</td>
<td>8%</td>
</tr>
<tr>
<td>65</td>
<td>4-(OCH₂C(CH₃)=CH₂)C₆H₄</td>
<td>CONH₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>NHCOCH₃</td>
<td>imidazol-2-yl</td>
<td>83 ± 7</td>
<td>38 ± 3</td>
<td>12 ± 1 (100%)</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>67</td>
<td>NHCOCH₃</td>
<td>CONH₂</td>
<td>17%</td>
<td>572±51</td>
<td>164±12 (92%)</td>
<td>742±67</td>
</tr>
</tbody>
</table>

BAY60-6583

| Table 12 |

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Results and discussion

This “result” can be considered as a real breakthrough due to the actually limited number of non-adenosine hA₂B AR full agonists reported in literature, as this information can be used to generate in the near future a new series of non-nucleosidic derivatives with this pharmacological profile. However, it has to be clarified the role of the N-acetamido function at para position of the 4-phenyl substituent of derivative 66, 67 in determininig the A₂B pharmacological profile of these compounds.

The two bicyclic compounds originated by intramolecular cyclization of both BAY60-6583 and the analogue 54, i.e. derivatives 68 and 69, respectively, bind none of the ARs (Table 13), suggesting that this kind of molecular complication, making the structure more rigid, is detrimental for activity at ARs and in particular at A₂B subtype. However, molecular docking at the ARs are on going to obtain some suggestion about the potentiality of this scaffold to be correctly modified and decorated to obtain AR ligands.

In order to evaluate how a molecular complication of aminopyridine-3,5-dicarbonitriles could modulate the affinity and the pharmacological profile of these derivatives at the ARs, a small set of 7-aminopyrazolo[1,5-c]pyrimidine-4-carbonitriles was synthesized (Table 14). The substituents firstly introduced at position-2

| Table 13 |
|---|---|---|---|
| n° | R₄ | hA₁ CHO cells | hA₂A CHO cells | hA₂B cAMP assay | hA₃ CHO cells |
|---|---|---|---|---|
| BAY60-6583 | cyclopropyl | 31% | 2% | 31 ± 3 | 8% |
| 68 | cyclopropyl | 19% | 3% | >1000 (1%) | 13% |
| 54 | cyclobutyl | 25% | 4% | >1000 (12%) | 574 ± 46 |
| 69 | cyclobutyl | 14% | 1% | >1000 (1%) | 11% |
(phenyl group) and -5 (substituted amines) of the scaffold have been selected to make easier the development of a synthetic procedure, new for the most part, that was as much as possible generalizable. Then an investigation in this regard was undertaken and it is still on-going.

The 5-amino substituents are characteristic of hA2A AR antagonists, of which ZM-241385 (see Table 14) is one of the most popular reported in literature, due to its high affinity and selectivity at this subtype. Evident is the structure similarity of the pyrazolopyrimidine scaffold of 70-73 to that of ZM-241385 and to those of many other A2A AR antagonists reported in the literature (255).

In fact, as expected, introduction of a phenyl moiety at position 2 of the bicyclic scaffold is not the best choice to obtain A2A AR antagonists. Compounds 70-73 preferentially bind the hA1 AR due to the lack of the 2-furyl moiety, generally mandatory for potent and selective hA2A AR antagonists. Interestingly, compound 71 shows a balanced A1/A3 AR affinity.

Despite the small set of synthesized compounds, preliminary information suggest that this new scaffold, suitably decorated, could be a good starting point for the design of new AR ligands.

![Chemical structures](image_url)

<table>
<thead>
<tr>
<th>n*</th>
<th>R₅</th>
<th>hA₁ CHO cells</th>
<th>hA₂A CHO cells</th>
<th>hA₂B cAMP assay</th>
<th>hA₃ CHO cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[³H]DPCPX Kᵢ (nM) or I% at 1µM</td>
<td>[³H]ZM241385 Kᵢ (nM) or I% at 1µM</td>
<td>EC₅₀ (nM) (efficacy %)</td>
<td>[¹²⁵I]ABMECA Kᵢ (nM) or I% at 1µM</td>
</tr>
<tr>
<td>70</td>
<td>C₆H₅</td>
<td>193±16</td>
<td>3%</td>
<td>&gt;1000 (1%)</td>
<td>9%</td>
</tr>
<tr>
<td>71</td>
<td>3-OCH₃C₆H₄</td>
<td>116±9</td>
<td>7%</td>
<td>&gt;1000 (1%)</td>
<td>12%</td>
</tr>
<tr>
<td>72</td>
<td>2-furyl</td>
<td>64±5</td>
<td>3%</td>
<td>&gt;1000 (2%)</td>
<td>110±11</td>
</tr>
<tr>
<td>73</td>
<td>CH₂C₆H₅</td>
<td>68±5</td>
<td>1%</td>
<td>&gt;1000 (1%)</td>
<td>1%</td>
</tr>
<tr>
<td>ZM-241385 (255)</td>
<td></td>
<td>774</td>
<td>1.6</td>
<td>75*</td>
<td>743</td>
</tr>
</tbody>
</table>

*Kᵢ from binding experiments

Table 14
5. Molecular modeling studies

In order to rationalize the results obtained from pharmacological assays, the synthesized compounds were subjected to a molecular modeling investigation. Docking simulations were performed at both hA_1 and hA_2B AR by Prof. Diego Dal Ben at the School of Pharmacy, Medicinal Chemistry Unit of the University of Camerino. Although docking studies are still ongoing some results have emerged. In particular, the newly synthesized amino-3,5-dicyanopyridine derivatives were docked into the binding site of homology models of both hA_1 and hA_2B AR built by using the crystal structure of the hA_2A receptor as a template.

Molecular modeling studies aimed at analyzing the interaction between AR binding sites and non-nucleoside agonists have been reported in literature (319) (320) (299) (300) (322) (323). While the binding mode of nucleoside agonists has been depicted from experimental results obtained through X-ray crystallography, the interaction between these receptors and agonists based on simplified scaffolds (i.e. pyridine or pyrimidine) has been only simulated by molecular docking approaches. In addition, while for the A_2A AR several crystal structures in the activated or inactivated state have been solved and reported, in the case of the A_1 and A_2B ARs subtypes the protein architecture has been obtained through homology modeling techniques and then used as target for docking studies.

![LUF-5833](image)

**Figure 25**

The binding modes obtained in this study for the AR non-nucleoside agonists may be grouped in four families, according to modeling results reported in literature. One of the first non-nucleosidic AR ligand which was evaluated in this study was **LUF-5833** (Figure 25) (323). This compound behaves as an agonist at all the AR subtypes with different degrees of efficacies and belongs to the amino-3,5-dicyanopyridine series developed in this thesis. In Figure 26 are shown the four binding modes obtained at the hA_2A receptor model for this compound. This set of conformations comprises the potential binding modes suggested not only at the A_2A receptor but also at the A_1 and A_2B ARs.

In fact, the first binding mode (Figure 26-A) presents the compounds oriented in a way similar to the one previously described for analogue agonists at the ARs (319) (321) (300) (322) (323), where the 5-cyano group
Molecular modeling studies

and the 6-amine of the pyridine derivatives giving a polar interaction with the amide function of Asn253. The phenyl group in 4-position is located in proximity of residues of TM3,6,7 and the 2-thio substituent points toward the extracellular environment getting close to residues of TM1,2,7. This binding mode was compared with the one of the AR endogenous ligand adenosine, with the nitrogen atom of the 5-cyano group and the 6-amine of pyridines being located in correspondence of the N7 atom and the N6 amine of adenosine. This binding mode is one of the most populated ones by the A1 ligands developed in this project, as well as binding mode 3.

Figure 26: Binding poses of LUF-5833 as example for non-nucleoside AR agonists at A1 AR

The binding mode 2 (Figure 26-B) is a 180° rotated version of binding mode 1 (321) (323). This arrangement makes the exocyclic amine and the proximal cyano group being located close to TM7 residues, while the 3-cyano group gets in proximity of Asn253. The 4-phenyl ring gets located at the entrance of the binding cavity, while the sulfanyl substituent is close to TM 3,5,6 residues. The binding mode 3 (Figure 26-C) makes the exocyclic amine and the proximal cyano group being located close to TM7 residues and the 3-cyano group in proximity of Asn253. The phenyl group in 4-position is located in proximity of TM 3,6,7 residues while the sulfanyl substituent points toward the extracellular environment. As such as binding mode 1, the number 3 is one of the most populated for the A1 ligands of this project. The binding mode 4 (Figure 26-D) presents
the N1 atom and the exocyclic amino group giving polar interaction with the Asn253 amide function and the 3-cyano group close to TM7 residues. The sulfanyl substituent gets in proximity of TM 3,5,6 residues. In the case of derivatives presenting small and polar groups within this substituent, polar interactions with TM3 and TM6 residues are observable. Even this binding mode was previously described in literature for non-nucleoside agonists of ARs, in particular A2B AR (320) (299) (322).

A classical strategy to improve the A1 AR affinity for nucleoside compounds has been the introduction of cycloalkyl groups at the N6 amine function of adenosine derivatives. Comparing the binding mode 1 and 3 at the A1 AR taking the A1 selective agonist 15 as template (Figure 27), it may be noticed as the introduction of alkyl or aromatic groups at the exocyclic amine function may be well admitted for binding mode 1 (Figure 27-A) as these substituents would get located at the extracellular environment. The docking of the 6-cyclopentylamino-substituted derivative 22 and of the other analogues 21,23-25 are on going. In fact, the results of binding studies show that the introduction of cycloalkyl groups or aromatic groups at this amine function leads to a decrease of A1 AR affinity, conversely to what observed for nucleoside derivatives. It may be concluded that the non-nucleoside derivatives follow a different binding strategy than the nucleoside ones. In fact, the binding mode 3 does not provide sufficient free space within the binding cavity to allow the introduction of these substituents at the 6-amine for a matter of steric hindrance, thus suggesting that binding mode 3 could present higher reliability than the binding mode 1, even if further modelling studies and a deepen knowledge in SAR at the aminopyridine-3,5-dicarbonitrile scaffold could be necessary to get a final depiction.

![Figure 27: Binding mode 1 (A) and 3 (B) for compound 15 at A1 AR](image-url)
Concerning docking at A<sub>2B</sub> AR, as preliminary step, the study starts from the rebuilding of homology models of the human A<sub>2B</sub> AR by using as templates the crystal structures of the human A<sub>2A</sub> AR that is the member of AR family presenting also the highest sequence conservation with the A<sub>2B</sub> AR. The binding mode of ligands is then simulated by molecular docking tools, followed by energy minimization and post-docking analysis. In this study, it is not possible to depict any correlation between binding scores or interaction energies and activity data, as the potencies of the compounds have been measured as EC<sub>50</sub> with functional studies and not as K<sub>i</sub> affinity with radioligand binding assays. Furthermore, the study provides an interpretation of the interaction features for both series of ligands at the A<sub>2B</sub> AR but do not consider the interaction with the other AR subtypes. To simulate the binding mode of nucleoside and non-nucleoside agonists at A<sub>2B</sub> AR and to compare the key ligand-target interaction features of the two structural families of compounds, a molecular docking analysis was performed at homology models of the human A<sub>2B</sub> AR developed by using two recently published crystal structures of the agonist-bound A<sub>2A</sub> AR as templates (pdb code: 2YDO; 3.0-Å resolution and pdb code: 3QAK; 2.7-Å resolution, in complex with Ado and UK-432097 (see Figure 5), respectively). The final A<sub>2B</sub> AR models contain a disulfide bridge given by two cysteine residues belonging to TM3 and extracellular loop (EL) 2 domains (Cys783.25 - where 3.25 indicates the residue position within helix and Cys171, respectively), in agreement with recent mutagenesis studies (324) and as observed in the case of recently reported modelling analyses on this AR subtype (320).

Figure 28: NECA docked in the crystal structure of the A<sub>2A</sub> AR
The binding sites of the two developed A2B AR models are very similar considering both receptor residues orientation and pocket volumes. Slight differences are still observable, due to diverse arrangements of some residues detectable even at the two A2A AR crystal structures templates. In order to evaluate differences regarding docking at A2B AR between adenosine-like agonist and the aminopyridine-3,5-dicarbonitriles, both NECA (Figure 15) and compound D were docked. While NECA was docked in the crystal structure of the hA2A AR (Figure 28), docking study of compound D was performed at hA2B AR homology model (Figure 29).

For summarizing, the exocyclic amino group of compound D mimics the same function in NECA, while the cyano at position-3 of the pyridine scaffold could be essential in keeping the 4-furyl substituents and the 2-sulfanylacetamide function in a non-coplanar conformation. This behavior seems to be mandatory for the agonist profile at the A2B AR of these derivatives.

In fact, considering the non-nucleoside pyridine derivatives, the lowest score and most populated family of docking conformations shows the pyridine ring located in rough correspondence to the adenine scaffold of nucleoside agonists and forming a π-stacking interaction with Phe173 in the EL2 and this conformation seems to be stabilized by the non-coplanarity of the pyridine ring and the substituent at position 2, where H-bond
acceptor and donor group help in reaching a stable conformation. In detail, the interaction of the scaffold with the $A_{2B}$ AR binding site is given as H-bonding between the N1 atom and the exocyclic amino group of pyridines and the amine and carbonyl groups of Asn2546.55 amide function, respectively. This double polar interaction is clearly present only for some derivatives, due to the nature of the 4-substituent that slightly modifies in some cases the orientation of the scaffold. Analogously to the nucleoside agonists, the exocyclic amino group of pyridines gives a H-bond interaction with Glu174 in the case of the 2YDO-based $A_{2B}$ AR model, while in the case of the 3QAK-based model the side chain of the same residue in some cases is not close enough to the ligand amino group and hence not able to provide a clear H-bonding. The sulfanylmethylimidazole group of compound 37 (Figure 30) and the sulfanylacetamide function of compound D (Figure 29) at 2-position are inserted between TM3, TM5, and TM6 residues. In the case of compound 37, the H-bond donor function of imidazole is oriented towards the oxygen atom of Thr893.36, while the acceptor feature points towards the polar hydrogen of His2516.52. The 4’-N-ethyl-carboxyamide function of the nucleoside derivative NECA (Figure 28) is in a nearly identical position to the analogue sulfanylacetamide group of compound D (Figure 29), suggesting the importance of the presence of a combination of H-bond acceptor and donor features located in this region. The 3-cyano group is inserted in a sub-cavity between Val853.32, Leu863.33, Thr893.36, Ser2797.42, and His2807.43. No clear interaction with binding site is given by this group, even though the presence of some space between this function and the polar groups of Ser2797.42 and His2807.43 could allow the presence of a water molecule providing a sort of “bridge interaction” between ligand and binding site residues, as observed, for example, in the case of a crystal structure of $A_{2A}$ AR in complex with ZM241385 (54). The substituted aromatic group at the 4-position is located in a sub-cavity given by TM1, TM2, TM3, and TM7 residues, in close proximity to Tyr101.35, Ala642.61, Ile672.64, Ser682.65, and Ile2767.39. Further residues in proximity of this group are Val853.32 and Phe173.
Molecular modeling studies

Figure 30: Docking of compound 37 at A$_2$B AR: the first binding mode (A) was observed and reported in literature mainly for a series of A$_1$ receptor agonists (300), while the second (B) was already observed also at the A$_2$A receptor (299).
The interaction is mainly hydrophobic, and the presence of a hydrophobic group in para-position of the phenyl ring (BAY60-6583, Figure 31 and compound 37, Figure 30) suggests the presence of a large hydrophobic feature located within TM1, TM2, and TM7 domains, similar to the e2-position of nucleosides agonists. This group seems critical in particular for pyridine derivatives. Finally, the 5-cyano group points towards the extracellular environment and is located in proximity to the couple of residues Glu174-Lys269.

Concerning in particular docking at A2B AR for compound 37 (Figure 30) receptor interaction is given in particular through Asn254 (TM6) and Phe173 (EL2), even if further contacts with residues belonging to TM2, TM6, and TM7 that probably play a role for receptor activation are observable (300) (299).
Evaluation of the antinociceptive effect and stability studies

6. Evaluation of the antinociceptive effect and stability studies

6.1 Evaluation of the antinociceptive effect

Experimental evidence (325) supports the use of caffeine and of other A3/A2A antagonists as well A3 AR agonists in the reduction of hyperalgesia or in antinociception. With this in mind, and to clarify these paradoxical data, the pain reliever effect of compound 1 (dual A3 inverse agonist/A2A antagonist), derivative 3 (carboxylic acid analogue of 1, as possible metabolite) and 15, which behaves as partial A3 AR agonist (Figure 32), were evaluated in a rat model of chemotherapy-dependent neuropathy induced by oxaliplatin.

Anticancer therapy based on the repeated administration of oxaliplatin is limited by the development of a neuropathic syndrome difficult to treat. Oxaliplatin neurotoxicity is based on complex nervous mechanisms, the comprehension of the role of single neurotransmitters and the knowledge of the signal flow among cells is matter of importance to improve therapeutic chances. Neuropathic pain evolves from the physiological role of nociceptive pain to pathological aspects depending on the complex response of the nervous system to a lesion or disease of its somatosensory component. Electrical, molecular and cellular activity participate in sensitizing nervous circuits of periphery, dorsal horn as well as anterior cingulate gyrus, prefrontal cortex, amygdala, and periaqueductal gray leading to pain-mediating signals (326). The maladaptive plasticity of the central nervous system (CNS) assumes increasing evidence in the pathophysiology of chemotherapy-induced neuropathies (327) (328), a peculiar iatrogenic damage of the nervous tissue that results in therapy dose reduction or discontinuation and negatively influences quality of life on cancer survivors (329). Oxaliplatin is effective in various solid tumors (330) (331). Colorectal cancer is the main target, introduced for the management of the advanced stages oxaliplatin is currently the regimen of choice for the adjuvant treatment of patients with curative resection of node-positive colon cancer (332) (333). The dose-limiting toxicity of this compound is the development of peripheral neuropathy (334) related to the total cumulative dose and to the treatment doseintensity (335). Although acute symptoms typically resolve within a week, cumulative oxaliplatin causes chronic neuropathy in approximately 50% of patients receiving doses higher than 1000 mg/m². Signs and symptoms of chronic neuropathy include pain, paresthesia, hypoesthesia, dysesthesia, and changes in proprioception that persist between cycles (336). The pathophysiology of oxaliplatin neurotoxicity involves multiple aspects. Molecular damages to peripheral nerves and dorsal root ganglia (327) result in decreased cellular metabolism and axoplasmatic transport (337). Despite the oxaliplatin limited ability to cross the blood brain barrier (338) (339), the CNS appears strongly involved since oxidative damage (338), peroxisome derangement and intracellular signaling alteration have been shown at spinal and supraspinal...
Evaluation of the antinociceptive effect and stability studies

level (327) (339). In the spinal dorsal horn of oxaliplatin-treated mice, Renn and co-workers (328) observed an increased activity of wide dynamic range neurons.

On the other hand, oxaliplatin induces a significant glial cell density increase differently according to cell type, anatomical region and treatment time-points revealing a prominent role of astrocytes (327). In recent years, there have been further significant developments that enhance an understanding of the role of adenosine in nociception. Thus, novel methods useful for taking advantage of A₁ AR antinociceptive effect have been developed (144), and the role of adenosine A₂A in nociception has been further elaborated (160). Furthermore, a contribution of endogenous adenosine and AR modulation to the actions of several pharmacological agents, as well as non-pharmacological procedures, used to manage pain has been described. As previously assumed (see Introduction) A₁ AR and A₃A AR, as well as A₂B AR are fully involved in neuropathic pain modulation.

Pro-nociceptive effects mediated by A₂B AR activation were discovered only in the last 10 years. And recently, the focus of attention has moved to the antinociceptive effect exerted by A₁ AR agonists at the spinal cord.
Evaluation of the antinociceptive effect and stability studies

level thus hypothesizing a therapeutic potential for the treatment of neuropathic pain (258). Also A2A AR seems to be involved in pain response. However, the finding regarding the A2A AR signaling in pain have been controversial, with studies supporting both pro- and anti-nociceptive roles. Moreover, it is still controversial the role in antinociception mediated by one of the most known non selective AR antagonists, caffeine. In fact, with respect to pain, caffeine (100 mg) is an adjuvant analgesic in humans when given in combination with common analgesics (acetylsalicylic acid, acetaminophen/paracetamol, ibuprofen). Furthermore, a few is known about the effect of caffeine (340) in neuropathic pain. Recent reports, assess a role of the potential of caffeine in this disease to its antagonistic properties at A1 and A2A ARs. The hyperalgesic effect mediated by A1 AR blockade may be compensated for by an antinociceptive effect of antagonism at A2A subtype. Thus, it is particularly important to deepen the role of the balanced A1/A2A antagonism of caffeine in nociception.

This oxaliplatin-induced allodynia assay was performed by the group of Prof. Carla Ghelardini at the NEUROFARBA Department – Pharmacology and Toxicology section, of the University of Florence.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>vehicle + vehicle</td>
<td>18.4 ± 0.2</td>
<td>18.6 ± 1.2</td>
</tr>
<tr>
<td>oxaliplatin + vehicle</td>
<td>10.9 ± 1.2*</td>
<td>11.7 ± 0.6*</td>
</tr>
<tr>
<td>oxaliplatin + 1 (0.03 mg Kg⁻¹ p.o.)</td>
<td>11.3 ± 0.3*</td>
<td>12.8 ± 0.6</td>
</tr>
<tr>
<td>oxaliplatin + 1 (0.1 mg Kg⁻¹ p.o.)</td>
<td>10.3 ± 0.4*</td>
<td>14.5 ± 0.6^</td>
</tr>
<tr>
<td>oxaliplatin + 1 (0.3 mg Kg⁻¹ p.o.)</td>
<td>10.3 ± 0.3*</td>
<td>17.0 ± 0.7^^</td>
</tr>
<tr>
<td>oxaliplatin + 3 (0.1 mg Kg⁻¹ p.o.)</td>
<td>10.6 ± 0.5*</td>
<td>12.8 ± 1.2</td>
</tr>
<tr>
<td>oxaliplatin + 3 (0.3 mg Kg⁻¹ p.o.)</td>
<td>11.0 ± 0.4*</td>
<td>14.2 ± 1.5</td>
</tr>
<tr>
<td>oxaliplatin + 15 (0.3 mg Kg⁻¹ p.o.)</td>
<td>11.5 ± 0.5*</td>
<td>12.2 ± 0.5</td>
</tr>
<tr>
<td>oxaliplatin + 15 (1.0 mg Kg⁻¹ p.o.)</td>
<td>10.6 ± 0.2*</td>
<td>16.6 ± 1.1^</td>
</tr>
</tbody>
</table>

Table 15: Oxaliplatin (2.4 mg kg⁻¹, i.p.) was dissolved in glucose 5% and administered daily for 2 weeks. After 14 days from the first injection of oxaliplatin, P301 (suspended in 1% carboxymethylcellulose) was per os (p.o.) administered. Control animals were treated with vehicles. Pain-related behavior (i.e. lifting and licking of the hind paw) were observed and the time (seconds) of the first sign was recorded. *P<0.01 vs vehicle + vehicle treated animals; ^P<0.05 and ^^P<0.01 vs oxaliplatin + vehicle treated mice. Each value represents the mean of 10 mice.
After repeated treatment with oxaliplatin (2.4 mg kg\(^{-1}\) daily intraperitoneally for 2 weeks), animals developed a characteristic thermal allodynia to cold stimuli evaluable by the cold plate test (licking latency 10.9 ± 1.2 s in comparison to 18.4 ± 0.2 s of control animals). While the partial agonist 15 showed only a modest effect 30 min after treatment (Table 15), a single treatment with 1 per os was able to dose-dependently decreased oxaliplatin-induced pain. Starting from 0.1 mg kg\(^{-1}\) the compound significantly reduced the cold hypersensitivity for 45 min after administration. The 1 high dose tested (0.3 mg kg\(^{-1}\)) was able to fully revert allodynia peaking 30 min after treatment. The behavior of this two compound in this pharmacological assay can be seen as paradoxal: activation of A\(_1\) AR is known to exert an antinociceptive effect in a large variety of pain models in literature while A\(_1\) AR blocking can be associated to pro-nociceptive action. In contrast, the expected pain reliever effect of compound 15, the A\(_1\) AR partial agonist, was not evidenced in this model. Moreover, the A\(_1\) inverse agonist/A\(_2A\) antagonist 1 exerts antinociceptive effect that appears to be modulated by A\(_2A\) AR that could outweigh the pro-nociceptive effect mediated by the A\(_1\) subtype. Also the carboxylic acid 3 (Figure 25) was evaluated in this model in order to clarify if the data on antinociceptive actions of compound 1 could be related to its metabolic hydrolysis to 3. In this model, 3, whose pharmacological profile was not yet evaluated, shows to be less active than the corresponding methyl ester 1 in this oxaliplatin-induced allodynia assay at 0.1 mg/Kg p.o. and 0.3 mg/Kg p.o., despite a higher peak of effect at 30 minutes after somministration. These results do not help the understanding of the pathophysiological role of A\(_1\) and A\(_2A\) ARs in neuropathic pain. Thus, in the future others A\(_1\) AR agonists, dual A\(_1\)/A\(_2A\) AR antagonist or inverse agonist, and A\(_1\) AR inverse agonist already obtained, will be tested in this model. In order to evaluate the potential role of the carboxylic acid 3 as metabolite of the carboxylic ester 1 in the antinociceptive action of this latter, stability studies on human and rat plasma were performed.

6.2 Stability studies

The stability study on compound 1 was performed by the group of Dr. Gianluca Bartolucci at the NEUROFARBA Department – Pharmaceutical Science section, of the University of Florence. The degradation profiles were obtained by using LC-MS/MS analysis monitoring the variation of the analyte 1 concentration, by using BAY60-6583 as internal standard, at different incubation times. Comparison of results obtained in a phosphate buffer solution (PBS) and plasma permits to distinguish between chemical stability and bioavailability of the analyte. Both Ketoprofene ethyl ester and Enalapril degradation experiments were carried out to check the hydrolytic activity of human or rat plasma batch employed, respectively. In order to summarize all experimental data, the half-life (T1/2) of compound 1 was reported in Table 16.

In the graphs are shown the degradation profiles of 1 in human plasma and PBS (figure 33) and in rat plasma (Figure 34) using Ketoprofen ethyl ester and Enalapril as control enzyme activity of the human and rat plasma, respectively.
Evaluation of the antinociceptive effect and stability studies

The data obtained in this study confirm the stability of 1 during the whole oxaliplatin-induced neuropathy assay in rats showing a high hydrolyzing time in these conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human Plasma T 1/2 (min)</th>
<th>Rat Plasma T 1/2 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEE</td>
<td>93</td>
<td>-</td>
</tr>
<tr>
<td>Enalapril</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>1</td>
<td>&gt;240</td>
<td>170</td>
</tr>
</tbody>
</table>

Table 16

Degradation profiles of 1 indicate a similar trend in rat and human plasma, leading to validate the pharmacological model here used.

Figure 33
Evaluation of the antinociceptive effect and stability studies

Figure 34

\[ y = -0.0021x + 0.8562 \]
\[ R^2 = 0.9386 \]

\[ y = -0.0079x + 1 \]
\[ R^2 = 1 \]
7. Studies on oligodendrocytes precursor cells

Current available multiple sclerosis (MS) therapies target immune modulation with some efficacy, however, concomitantly with adverse side effects (341). Oligodendrocytes are the only myelin-producing cells in the CNS (342). Damaged oligodendrocytes no longer generate myelin and remyelination requires generation of new mature oligodendrocytes from the differentiation of oligodendrocyte progenitor cells (OPCs) (Figure 35) (343). These cellular resources are especially active after demyelinating episodes in early phases of MS. Indeed, OPCs actively proliferate, migrate to and repopulate the lesioned areas. Ultimately, efficient remyelination is accomplished when new oligodendrocytes reinvest nude neuronal axons, restoring the normal properties of impulse conduction. As the disease progresses, this fundamental process fails. Multiple causes seem to contribute to such transient decline, including the failure of OPCs to differentiate and enwrap the vulnerable neuronal axons. For example, the observation that OPCs are present in MS lesions but fail to differentiate into mature oligodendrocytes (344) suggests that the remyelination process is blocked at a premyelinating stage in demyelinating lesions. There are no effective remyelination therapies for MS patients. Thus, OPCs are a viable treatment target for MS clinical therapy (345) (346).

Purinergic signaling (exerted by adenosine and ATP) has recently emerged as a most pervasive mechanism for intercellular communication in the nervous system, affecting communication between many types of neurons and all types of glial cells (347). Not only neurons but also glial cells in fact release and respond to purinergic molecules, under both physiological and pathological conditions (348). Concerning oligodendrocytes, it has been demonstrated that purines exert multiple effects including increased motility, proliferation and differentiation of cultured OPCs (349). Among purines, adenosine is a neuromodulator of the CNS. It is known that OPCs express all these receptors, as detected by RT-PCR in cultured OPCs, including A$_{2B}$AR (350). To date, a functional role has been attributed only to A$_1$ and A$_{2A}$ AR (349). It has recently clarified the role of A$_{2A}$ AR on oligodendrogenesis, demonstrating that their selective activation decreases outward rectifying, sustained, inward K$^+$ currents and inhibits in vitro OPC differentiation towards mature, myelinating, oligodendrocytes, without affecting cell division (351). Conversely, adenosine, through the activation of A$_1$ AR, seems to be a primary activity-dependent signal inhibiting the proliferation and promoting differentiation of premyelinating progenitors into myelinating oligodendrocytes (350) and stimulating OPC migration (352). The functional role of the A$_{2A}$ AR in oligodendrocytes has not yet been clarified because, as already assumed in this thesis, A$_{2A}$ AR is the least studied and still remains the most enigmatic AR subtype. However, there is a growing interest in A$_{2A}$ AR in recent years, as it has been shown to play a role in inflammation and cancer and is even considered a promising new pharmacotherapeutic target (320). Of particular note is that in the brain during different pathological conditions, such cerebral ischemia,
Studies on OPCs

eextracellular adenosine is elevated to levels sufficient for A2B AR (349). White matter damage is a clinically important aspect of several central nervous system diseases, including stroke. Cerebral white matter primarily consists of axonal bundles unsheathed with myelin secreted by mature oligodendrocytes, which play an important role in neurotransmission between different areas of gray matter. During the acute phase of stroke, damage to oligodendrocytes leads to white matter dysfunction through the loss of myelin. For effective remyelination to take place, OPCs play critical roles by proliferating and differentiating into mature oligodendrocytes, which help to decrease the burden of axonal injury. Although it was demonstrated that the selective A2A AR antagonism prevented myelin disorganization induced 24 h after occlusion of medial cerebral artery (MCAo) in rat (353), the role of A2B AR both on ischemia-induced neuronal and glial damage was not investigated during cerebral ischemia.

The aim of this study was to clarify the oligodendrogenetic potential of A2B AR, in particular to investigate, in cultured OPCs at different times of maturation (from t0 to t10, Figure 35), whether A2B AR modulates ionic currents and cell proliferation and maturation by using selective A2B AR agonists (commercially available and newly synthetized), alone or in combination with A2B AR selective antagonists. All these experiments were carried out in cultured rat oligodendrocytes by using patch clamp recordings and immunocytochemical analysis (Figure I) and performed by Dr. Anna Maria Pugliese, belonging to the Prof. Felicita Pedata group at the NEUROFARBA Department – Pharmacology and Toxicology section, of the University of Florence.

Figure 35: Immunocytochemical analysis of OPCs at different times of maturation and ouward K+ current variation during maturation
Studies on OPCs

To examine the effects of $A_{2B}$ AR stimulation on the in vitro oligodendrocyte progression towards mature phenotypes, an immunocytochemical labeling of OPC cultures was performed. OPC were allowed to differentiate for different days in differentiation medium (DM) (t6 and t10) in order to characterize the time course of the expression of specific markers of cell differentiation. In parallel experiments, OPC cultures will be differentiated in the presence of different concentrations of the $A_{2B}$ AR agonists 37 and **BAY 60-6583** (Tocris, Italy) (Figure 15) alone or in combination with the $A_{2B}$ AR antagonist **PSB-603** (Figure 16) in order to verify whether $A_{2B}$ AR receptor activation could influence OPC differentiation.

Preliminary experiments demonstrated that selective stimulation of $A_{2B}$ AR in cultured OPCs reduces the amplitude of outward currents, as recorded in patch clamp experiments (Figure 36). The selective stimulation was guaranteed by silencing $A_1$, $A_{2A}$ and $A_3$ ARs in cultures. These currents were abolished when $K^+$ was replaced by equimolar $Cs^+$ concentration into the intracellular pipette solution indicating that ramp-evoked outward currents are $K^+$ currents. Compound 37, applied at the concentrations of 50 nM, reduces the amplitude of outward currents elicited by a ramp voltage protocol (Figure 36 A and B). Similar effect to that produced by 37 was recorded in the presence of **BAY 60-6583** which was applied at concentrations of 1 and 10 µM. Thus, applying $A_{2B}$ AR antagonist PSB-603 the effect of both agonists was reverted, confirming the involvement of $A_{2B}$ AR (Figure 37 A and B).

Figure 36: (A) Outward currents elicited by 37 in a ramp voltage protocol; (B) net outward $K^+$ current sensitive to 37
Thus, in this assay was demonstrated that A$_{2B}$ AR is involved in OPC maturation. In particular, activation of A$_{2B}$ AR inhibits OPC differentiation in oligodendrocytes by blocking outward K$^+$ currents, essential for the differentiation process. Furthermore, compound 37 exert its effect at lower concentrations than BAY60-6583, the reference agonist reported in literature. Finally, two other compounds acting as full agonist, 66 and 67, will be tested in this in vitro model, despite their scarce selectivity at A$_{2B}$ AR, since in this assay the other AR subtypes will be silenced.
Conclusions and perspectives

8. Conclusions and perspectives

The research reported in this Ph.D. thesis, though it has to be deepened, furthers our understanding about the ARs. New AR ligands were developed preferentially belonging to the aminopyridine-3,5-dicarbonitrile series whose structure-activity relationships have been till now poorly investigated. In fact, most of the compounds reported in the literature are included in patent documents. In this work, many derivatives were synthesized and biologically evaluated in binding and functional assays in order to assess affinity and selectivity towards a specific AR subtype, but also to evaluate their pharmacological profile. The data obtained confirmed the peculiarity of this series whose derivatives are not only characterized by a wide range of affinities but interestingly endowed with different degree of efficacies at the different ARs.

Based also on molecular modelling studies two set of compounds, named DCP1 and DCP2B were designed preferentially targeting the A1 and A2B AR, respectively.

- Most of the compounds belonging to the DCP1 set (compounds 1-25, Tables 5, 7-8) are endowed with high affinity and good selectivity at the hA1 AR. Some selected derivatives showed also an inverse agonist profile with the only exception of compound 15 which emerged as partial agonist bearing a peculiar 2-phenylthiazole group at R6. Thus, further modification at the level of the thiazole moiety will be planned in order to evaluate the importance of this substituent for obtaining hA1 AR agonists.

  Moreover, when evaluated in an oxaliplatin-induced neuropathic pain model in mice, compound 1 (dual A1 inverse agonist/A2A antagonist) resulted to revert allodynia at very low doses after oral administration (Table 15). This effect is similar to that exerted by both caffeine and other A1/A2A antagonists as well as A1 AR agonists in reverting hyperalgesia and in antinociception.

  Stability studies in mouse and human plasma on 1 (Figure 33, 34) confirmed no significant chemical modification of the compound for the whole duration of the assay. Thus, to clarify this paradoxical data, other molecules belonging to this set, endowed with similar degree of affinities and efficacies with respect to 1, will be tested in this in vivo assay, also to evaluate their potentiality for neuropathic pain treatment.

The results obtained on the DCP1 set, especially in the in vivo assay, suggested that the amino-3,5-dicyanopyridine scaffold could be of interest for obtaining non-nucleoside AR agonists as to prevent two serious problems hampering the development of classical adenosine-like agonists: the low oral bioavailability and the short half-life due to the presence of the typical ribose (or ribose-mimicking) moiety.
Conclusions and perspectives

- DCP2B set is composed of compounds preferentially targeting the hA\textsubscript{2B} receptor ([26-67, Tables 9-12). Most of them showed to behave as partial agonist, with the best results concerning activity and selectivity of compound 37, which is three fold more potent than the reference agonist BAY60-6583. This result can be considered a real breakthrough since BAY60-6583 is absolutely the sole potent and selective hA\textsubscript{2B} AR agonist reported in literature.

Moreover, it is noteworthy that derivatives 66 and 67, showing high activity at the hA\textsubscript{2B}AR but scarce selectivity, emerged as the only two amino-3,5-dicyanopyridines reported till now which are endowed with a full agonist profile. With this in mind, a study to clarify the structural requirements which are important in determining the A\textsubscript{2B} pharmacological profile of these compounds is currently on going.

Thus, identification of new selective hA\textsubscript{2B} agonists could be of help in deepening the medicinal chemistry and the pharmacology related to this still unknown AR subtype. In fact, compound 37 was used as pharmacological tool in an oligodendrocyte precursor cell (OPC) differentiation assay in order to clarify whether the A\textsubscript{2B} receptor represents a promising target to modulate endogenous remyelination process in multiple sclerosis patients. Compound 37 inhibits OPC differentiation into oligodendrocytes at lower concentration than those used for BAY60-6583 demonstrating the role of A\textsubscript{2B}AR in this important physiological process (Figure 36, 37).

Moreover, docking studies were performed on selected DCP1 and DCP2B derivatives at A\textsubscript{1} and A\textsubscript{2B} homology receptor models, respectively, showing the best binding poses for compounds 15 (Figure 27) and 37 (Figure 30) and the most important residues involved in the binding at these two AR subtypes. It seems to clarify the role of R\textsubscript{4} and R\textsubscript{6} substituents, containing atoms or groups able to engage hydrogen-bonding interactions with the receptors, in modulating affinity and efficacy of the amino-3,5-diyanopyridine derivatives.

Moreover, since some affinity was also achieved at the hA\textsubscript{2A} and hA\textsubscript{3} AR with some of the compounds detailed in this thesis, it may be possible that this heterocycle core could be also manipulated to enhance affinity and selectivity for these subtypes. In fact, neither A\textsubscript{2A} nor A\textsubscript{3} agonists belonging to the amino-3,5-dicyanopyridine series has been ever designed.

- Preliminary studies on the newly synthesized 7-amino-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile system, derived from molecular complications of the amino-3,5-dicyanopyridine core, were reported. Biological results on a small set of compounds (70-73, Table 14), that bind with good affinity the hA\textsubscript{1} AR, indicated that this scaffold suitably decorated could generate new AR ligands. No functional data are currently available, thus preventing any conclusion about the influence of this structural modification on the pharmacological profile of the new compounds. Introduction of suitable substituents on the bicyclic ring in order to modulate affinity and selectivity at the AR subtypes is on going.
The microwave-assisted syntheses were performed using an Initiator EXP Microwave Biotage instrument (frequency of irradiation: 2.45 GHz). Silica gel plates (Merck silica gel 60 F254, 250 μm) were used for analytical chromatography; while, preparative silica gel plates (Merck silica gel 60 F254, 2 mm) and silica gel 60 (Merck, 70-230 mesh) were employed for preparative thin layer (TLC) and column chromatography. All melting points were determined on a Gallenkamp melting point apparatus. All final compounds revealed a purity not less than 95%. The IR spectra were recorded with a Perkin-Elmer Spectrum RX I spectrometer or Shimadzu IR-prestige 21 in Nujol mulls and are expressed in cm$^{-1}$. The $^1$H NMR spectra were obtained with a Bruker Avance 400 MHz instrument. The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent. The following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, and Ar = aromatic protons. The following abbreviation are used for solvents and reactive products: ACN = Acetonitrile, DMF = Dimethylformamide, TEA = Triethylamine, NMP = N-Methyl-2-pyrrolidone, EtOAc = Ethyl acetate, CHCl$_3$ = Chloroform, DCM = Dichloromethane, Et$_2$O = Diethyl ether, EtOH = Ethanol, HCl = Hydrochloric acid, MeOH = Methanol, D$_2$O = Deuterium oxide, AcOH = Acetic acid, Py = Pyridine, DIPEA = NN-Diisopropylethylamine, DMSO = Dimethyl sulfoxide, CDCl$_3$ = Deuterated chloroform, THF = Tetrahydrofuran, rt = room temperature, eq = equatorial protons, ax = axial protons, exc D$_2$O = protons exchanging with deuterium oxide.
General procedure for the synthesis of 74-77, 88-94, 112-117 and 140

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Compound</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>2-furyl</td>
<td>93</td>
<td>4-CH3-(C₆H₄)</td>
</tr>
<tr>
<td>75</td>
<td>3-furyl</td>
<td>94</td>
<td>4-SCH₂-(C₆H₄)</td>
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<tr>
<td>76</td>
<td>2-thienyl</td>
<td>112</td>
<td>4-(OCH₂C₆H₅)-(C₆H₄)</td>
</tr>
<tr>
<td>77</td>
<td>3-pyridyl</td>
<td>113</td>
<td>4-(OCH₂C₆H₅)-(C₆H₄)</td>
</tr>
<tr>
<td>88</td>
<td>3-thienyl</td>
<td>114</td>
<td>4-(OCH₂CH(CH₃)₂)-(C₆H₄)</td>
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<tr>
<td>89</td>
<td>4-pyridyl</td>
<td>115</td>
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<td>90</td>
<td>CH₃C₆H₅</td>
<td>116</td>
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<tr>
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<td>117</td>
<td>4-(OCH₂C(CH₃)=CH₂)-(C₆H₄)</td>
</tr>
<tr>
<td>92</td>
<td>4-F-(C₆H₄)</td>
<td>140</td>
<td>4-OH-(C₆H₄)</td>
</tr>
</tbody>
</table>

Procedures

**Method A:** To a stirred solution of the suitable aldehyde (10.00 mmol), 1,8-diazabicycloundec-7-ene (5% mol) and malononitrile (20.00 mmol) in 10% EtOHaq (20ml), thiophenol (10.00 mmol) was added after 20 min. Thus, the reaction mixture was stirred at 55°C for 3 to 18 h. The suspension was then cooled at rt and a solid precipitated which was collected by filtration, washed with Et₂O (5-10 ml) and then dried in oven at 60°C overnight.

**Method B:** To a solution in water (50 ml) of the suitable aldehyde (10.00 mmol) and malononitrile (20.00 mmol), tetrabutylammonium fluoride (10% mol) is added. After 20 minutes, thiophenol (10.00 mmol) is added and the mixture is allowed to stir at 80°C for 2 hours. After completing of the reaction the mixture is allowed to cool at rt and the water is removed. The residue obtained is dissolved in AcOEt and dried over anhydrous sodium sulfate. The solution is then filtered, the solvent removed under vacuum and the residue obtained is triturated with a mixture Et₂O : EtOH (10:1) and filtered in order to obtain the pure product.

**Method C:** A suspension of Al₂O₃ (10% p/v) in water (2 ml) was added to a solution of the suitable aldehyde (10.00 mmol) and malononitrile (20.00 mmol) in water (18 ml), and the mixture was allowed to stir at rt for 10 min. After addition of thiophenol (10.00 mmol) the suspension was heated at reflux for 6 h and then cooled at rt. The water was removed under reduced pressure and the residue was triturated with a mixture of Et₂O/EtOH (5:1). The solid obtained was filtered and extracted with hot EtOAc (4 x 100 ml). The dried organic phases (Na₂SO₄) were evaporated in order to obtain the pure compound.
Examples:

2-Amino-4-(furan-2-yl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (74)

Method B; Yield: 18%; mp: 191-193°C (lit. 178-180°C) (MeOH); ^1H NMR (400 MHz, DMSO-d$_6$): 8.13 (s, 1H, Fur), 7.79 (br s, 2H, NH$_2$, exc. D$_2$O), 7.60 (d, 2H, Ar), 7.50 (m, 3H, Ar+Fur), 7.43 (t, 1H, Ar), 6.86 (s, 1H, Fur). IR (cm$^{-1}$): 3303, 3212, 2215.

2-Amino-4-(furan-3-yl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (75)

Method B; Yield: 22%; mp: 204-206°C (MeOH); ^1H NMR (400 MHz, DMSO-d$_6$): 8.22-8.29 (m, 1H, Fur), 7.93-7.94 (m, 1H, Fur ), 7.81 (br s, 2H, NH$_2$, exc D2O), 7.59- 7.60 (m, 2H, Ar), 7.50-7.51 (m, 3H, Ar), 6.90-6.91 (m, 1H, Fur). IR (cm$^{-1}$): 3328, 3218, 2220.

2-Amino-6-(phenylthio)-4-(thiophen-2-yl)pyridine-3,5-dicarbonitrile (76)

Method B; Yield: 32%; mp: 216-217°C (lit. 208-210°C) (MeOH); ^1H NMR (400 MHz, DMSO-d$_6$): 7.97 (d, 1H, Th, J = 4 Hz), 7.86 (br s, 2H, NH$_2$, exc D2O), 7.58-7.61 (m, 3H, Ar + Th), 7.50-7.51 (m, 3H, Ar), 7.30 (dd, 1H, Th, J1= 3.7 Hz, J2 = 4.8 Hz); IR (cm$^{-1}$): 3352, 3216, 2210.

2'-Amino-6'-(phenylthio)-[3,4'-bipyridine]-3',5'-dicarbonitrile (77)

Method C; Yield: 31%; mp: >300°C (EtOH/DMF); ^1H NMR (400 MHz, DMSO-d$_6$): 8.77 (m, 1H, Py), 8.05 (d, 1H Py, J= 8.08 Hz), 7.85 (br s, 2H, NH$_2$, exc D$_2$O), 7.60-7.65 (m, 4H, Ar +1H Py), 7.50-7.52 (m, 3H, 2H Ar + 1H Py), IR (cm$^{-1}$): 3374, 3307, 2212, 2221.
Experimental section

2-Amino-6-(phenylthio)-4-(thiophen-3-yl)pyridine-3,5-dicarbonitrile (88)

Method B; Yield: 14%; mp: 236-238°C (lit. 235-236°C) (MeOH); ^1H NMR (400 MHz, DMSO-d$_6$): 8.06-8.07 (m, 1H, Th), 7.78-7.80 (m, 3H, NH$_2$ + Th, 2H exc D$_2$O), 7.59-7.61 (m, 2H, Ar), 7.49-7.51 (m, 3H, Ar), 7.40 (d, 1H, Th, J= 4.9 Hz), IR (cm$^{-1}$): 3333, 3217, 2210.

2-Amino-6-(phenylthio)-[4,4'-bipyridine]-3,5-dicarbonitrile (89)

Method C; Yield: 32%; mp: 204-206°C (lit. 208-209°C) (EtOH); ^1H NMR (400 MHz, DMSO-d$_6$): 8.26 (m, 1H, Fur), 7.93 (m, 1H, Fur), 7.81 (br s, 2H, NH$_2$, exc D$_2$O), 7.60 (m, 2H, Ar), 6.91 (m, 1H, Fur), 7.51 (m, 3H, Ar); IR (cm$^{-1}$): 3328, 3218, 2220.

2-Amino-4-benzyl-6-(phenylthio)pyridine-3,5-dicarbonitrile (90)

Method C; Yield: 35%; mp: 247-249°C (MeOH); ^1H NMR (400 MHz, DMSO-d$_6$): 7.81 (br s, 2H, NH$_2$ exc D$_2$O), 7.58 (m, 2H, Ar), 7.5 (m, 3H, Ar), 7.37 (m, 2H, Ar), 7.28 (m, 3H, Ar), 4.12 (s, 2H, CH$_2$); IR (cm$^{-1}$): 3329, 3225, 2198.

2-Amino-4-(4-chlorophenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (91) (354)

Method C; Yield: 37%; mp: 236-237°C (EtOH); ^1H NMR (400 MHz, DMSO-d$_6$): 7.75 (br s, 2H, NH$_2$ exc D$_2$O), 7.49-7.65 (m, 6H, Ar), 7.40-7.44 (m, 3H, Ar).

2-Amino-4-(4-fluorophenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (92) (355)

Method C; Yield: 36%; mp: 220-222°C (lit. 221-223°C); ^1H NMR (400 MHz, DMSO-d$_6$): 7.85 (s, 2H, NH$_2$, exc D$_2$O), 7.63 (m, 4H, Ar), 7.54 – 7.48 (m, 3H, Ar), 7.44 (t, J = 8.8 Hz, 2H, Ar)
**Experimental section**

2-Amino-6-(phenylthio)-4-(p-tolyl)pyridine-3,5-dicarbonitrile (93) (355)

\[
\text{Method C; Yield: 32%; mp: 220-221}^\circ\text{C (lit. 208-211}^\circ\text{C); }^1\text{H NMR (400 MHz, DMSO-}d_6\text{): 7.87 (br s, 2H, NH}_2\text{, exc D}_2\text{O), 7.55-7.65 (m, 3H, Ar), 7.37-7.48 (m, 6H, Ar), 2.41 (s, 3H, CH}_3\text{)}
\]

2-Amino-4-(4-(methylthio)phenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (94) (354)

\[
\text{Method C; Yield: 28%; mp: 244-246}^\circ\text{C (lit.254-256}^\circ\text{C); }^1\text{H NMR (400 MHz, DMSO-}d_6\text{): 7.81 (br s, 2H, NH}_2\text{, exc D}_2\text{O), 7.64 – 7.57 (m, 2H, Ar), 7.50 (m, 5H, Ar), 7.43 (m, 2H, Ar), 2.56 (s, 2H)}
\]

2-Amino-4-(4-(cyclopropylmethoxy)phenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (112)

\[
\text{Method A and C; Yield: 33%; mp: 261-263}^\circ\text{C (EtOH); }^1\text{H NMR (400 MHz, DMSO-}d_6\text{): 7.70 (br s, 2H, NH}_2\text{, exc D}_2\text{O), 7.60-7.62 (m, 2H, Ar), 7.48-7.51 (m, 5H, Ar), 7.10 (d, 2H, Ar, J = 8.1 Hz), 3.92 (d, 2H, CH}_2\text{, J = 7.8 Hz), 1.25-1.28 (m, 1H, CH), 0.58-0.62 (m, 2H, 2CH eq), 0.36-0.38 (m, 2H, 2CH ox); IR (cm}^{-1}\text{): 3330, 3220, 2224}
\]

2-Amino-4-(4-(cyclobutylmethoxy)phenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (113)

\[
\text{Method A; Yield: 18%; mp: 180-182}^\circ\text{C (MeOH); }^1\text{H NMR (400 MHz, DMSO-}d_6\text{): 7.76 (br s, 2H, NH}_2\text{, exc D}_2\text{O), 7.61-7.60 (m, 2H, Ar), 7.51-7.49 (m, 3H, Ar), 7.12 (d, 2H, Ar, J = 8.60 Hz), 4.05 (d, 2H, OCH}_2\text{, J = 6.5 Hz), 2.77-2.75 (m, 1H, CH), 2.11-2.09 (m, 2H, CH}_3\text{), 1.93-1.89 (m, 4H, 2CH}_2\text{); IR (cm}^{-1}\text{): 3348, 3280, 2216}
\]
Experimental section

2-Amino-4-(4-isobutoxyphenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (114)

Method A; Yield: 26%; mp: 180-182°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-\( \text{d}_6 \)): 7.74 (s, 2H, \(NH_2\), exc D\(_2\)O), 7.61-7.59 (m, 2H, Ar), 7.50-7.49 (m, 5H, Ar), 7.11 (d, 2H, Ar, J= 8.76 Hz), 3.84 (d, 2H, OCH\(_2\), J= 6.4 Hz), 2.09-2.02 (m, 1H, CH), 1.02 (s, 3H, CH\(_3\)), 1.00 (s, 3H, CH\(_3\)); IR (cm\(^{-1}\)): 3302, 3232, 2214

2-Amino-4-(4-ethoxyphenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (115)

Method A; Yield: 37%; mp: 252-254°C (2-propanol); \(^1\)H NMR (400 MHz, DMSO-\( \text{d}_6 \)): 7.6 (s, 2H, \(NH_2\), exc D\(_2\)O), 7.62-7.60 (m, 2H, Ar), 7.58-7.48 (m, 5H, Ar), 7.11 (d, 2H, Ar, J= 8.8 Hz), 4.13 (q, 2H, OCH\(_2\), J= 6.8 Hz), 1.37 (t, 3H, CH\(_3\), J= 7.2 Hz); IR (cm\(^{-1}\)): 3344, 3215, 2216

4-(4-(Allyloxy)phenyl)-2-amino-6-(phenylthio)pyridine-3,5-dicarbonitrile (116)

Method A; Yield: 28%; mp: 234-236°C (EtOAc/CHx); \(^1\)H NMR (400 MHz, DMSO-\( \text{d}_6 \)): 7.77 (s, 2H, \(NH_2\), exc D\(_2\)O), 7.61-7.60 (m, 2H, Ar), 7.59-7.58 (m, 3H, Ar), 7.51 (d, 2H, Ar, J= 8.8 Hz), 7.14 (d, 2H, Ar, J= 8.8), 6.13-6.03 (m, 1H, CH), 5.45 (d, 1H, CH\(_{\text{allyl}}\)), 5.30 (d, 1H, CH\(_{\text{allyl}}\)), 4.67 (d, 2H, OCH\(_2\), J= 5.6 Hz); IR (cm\(^{-1}\)): 3358, 3215, 2214, 1624

2-Amino-4-(4-((2-methylallyl)oxy)phenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (117)

Method A; Yield: 25%; mp: 198-200°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-\( \text{d}_6 \)): 7.76 (s, 2H, \(NH_2\), exc D\(_2\)O), 7.60-7.61 (m, 2H, Ar), 7.60-7.59 (m, 3H, Ar), 7.55 (d, 2H, Ar, J= 8.4 Hz), 7.14 (d, 2H, Ar, J= 8.4 Hz), 5.11 (d, 1H, CH), 5.00 (d, 1H, CH), 4.58 (s, 2H, CH\(_2\)), 1.81 (s, 3H, CH\(_3\)); IR (cm\(^{-1}\)): 3481, 3369, 2212
2-Amino-4-(4-hydroxyphenyl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (140) (297)

Method A; Yield: 29%; mp: > 300°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-
\(d_6\)): 10.08 (brs, 1H, OH, exc D\(_2\)O), 7.72 (brs, 2H, NH\(_2\), exc D\(_2\)O), 7.60-7.41
(m, 5H, Ar), 7.40 (d, 2H, Ar, J= 8.4 Hz), 6.92 (d, 2H, Ar, J= 8.4 Hz); IR (cm\(^{-1}\)): 3491, 3368, 2216

Synthesis of N-(4-(2,2-dicyanovinyl)phenyl)acetamide (138) (356)

Malononitrile (17.3 mmol) and piperidine (2 gtt) were added to a solution of 4-acetamidobenzaldehyde (17.3
mmol) in EtOH (20 ml). The mixture was heated at 80°C for 2h until reaction completion, then cooled to rt
affording an orange solid which was filtered and washed with Et\(_2\)O (5 ml) and petroleum ether (2 ml). Yield:
63%; mp: 234-236°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-
\(d_6\)): 10.51 (s, 1H, NH, exc D\(_2\)O), 8.37 (s, 1H, CH), 7.94
(d, 2H, Ar, J= 8.4 Hz), 7.80 (d, 2H, Ar, J= 8.8 Hz), 2.11 (s, 3H, CH\(_3\)); IR (cm\(^{-1}\)): 2224; 1693

Synthesis of N-(4-(2-amino-3,5-dicyano-6-(phenylthio)pyridin-4-yl)phenyl)acetamide (137) (356)

A solution of compound 138 (5.4 mmol), malononitrile (5.4 mmol), thiophenol (5.4 mmol) and TEA (0.54
mmol) in EtOH (30 ml) was heated at 100°C for 2h. Then, cooling at rt afforded a yellow solid which was
filtered and washed with Et\(_2\)O (5 ml). Yield: 44%; mp: >300°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-
\(d_6\)): 10.24 (s, 1H, NH, exc D\(_2\)O), 7.76 (s, 2H, NH\(_2\), exc D\(_2\)O), 7.74 (m, 2H, Ar), 7.61-7.60 (m, 5H, Ar,), 7.50 (d, 2H, Ar, J= 8 Hz), 2.10 (s, 3H, CH\(_3\)); IR (cm\(^{-1}\)): 3481; 3344; 3298; 2212; 1681
Experimental section

General procedure for the synthesis of 78-81, 95-101, 118-123, 139 and 141

**Procedure:**

To a stirred solution of compounds 74-77, 88-94, 112-117, 137 and 140 (10 mmol) in dry DMF (1 ml) maintained at rt and under nitrogen atmosphere, an excess of sodium sulfite (33 mmol) was added. The reaction mixture was then heated at 80°C for 2 h. When the reaction was completed, 1N HCl (25 ml) was added followed by addition of 6N HCl to obtain a huge precipitate which is filtered and washed with water (20 ml) and Et₂O (5 ml).

**Examples**

2-Amino-4-(furan-2-yl)-6-mercaptopyridine-3,5-dicarbonitrile (78) (357)

Yield: 96%; mp: 260-261°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 12.94 (br s, 1H, SH, exc D₂O), 8.12 (d, 1H, Fur, J= 1.4 Hz), 7.85 (br s, 2H, NH₂, exc D₂O), 7.43 (d, 1H, J= 3.6 Hz, Fur), 6.84 (dd, J= 3.6, 1.7 Hz, 1H, Fur); IR (cm⁻¹): 3306, 3196, 2214.
2-Amino-4-(furan-3-yl)-6-mercaptopypyridine-3,5-dicarbonitrile (79)

Yield: 92%; mp: 260-262°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 12.97 (br s, 1H, SH, exc D₂O), 8.25-8.27 (m, 1H, Fur), 8.00 (br s, 2H, NH₂, exc D₂O), 7.90 (t, J = 1.6 Hz, 1H, Fur), 6.84-6.85 (m, 1H, Fur); IR (cm⁻¹): 3307, 3192, 2216.

2-Amino-6-mercaptop-4-(thiophen-2-yl)pyridine-3,5-dicarbonitrile (80) (358)

Yield: 90%; mp: 270-272°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 13.01 (br s, 1H, SH, exc D₂O), 7.80 (br s, 2H, NH₂, exc D₂O), 7.95 (d, J = 5 Hz, 1H, CH thiophene), 7.55 (d, J = 3.6 Hz, 1H, CH thiophene), 7.26 (t, J = 4.2 Hz, 1H, CH thiophene); IR (cm⁻¹): 3317, 3206, 2216.

2'-Amino-6'-mercaptop-[3,4'-bipyridine]-3',5'-dicarbonitrile (81) (359)

Yield: 86%; mp: >300°C (EtOH/DMF); ¹H NMR (400 MHz, DMSO-d₆): 13.20 (br s, 1H, SH, exc D₂O), 8.76-8.79 (m, 2H, Py), 8.27 (br s, 2H, NH₂, exc D₂O), 8.06 (d, J = 7.84 Hz, 1H, Py), 7.66 (t, J = 6.60 Hz, 1H, Py); IR (cm⁻¹): 3319, 3124, 2212.

2-Amino-6-mercaptop-4-(thiophen-3-yl)pyridine-3,5-dicarbonitrile (95)

Yield: 89%; mp: 237-239°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 12.98 (br s, 1H, SH, exc D₂O), 8.03-8.04 (m, 1H, CH thiophene), 7.85 (br s, 2H, NH₂, exc D₂O), 7.74-7.76 (m, 1H, CH thiophene), 7.34-7.36 (m, 1H, CH thiophene); IR (cm⁻¹): 3315, 3204, 2208.

2-Amino-6-mercaptop-[4,4'-bipyridine]-3,5-dicarbonitrile (96)

Yield: 89%; mp: >300°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 13.2 (br s, 1H, SH, exc D₂O), 8.8 (d, J = 1.2 Hz, 2H, Py), 7.56 (d, J = 1.2 Hz, 2H, Py); IR (cm⁻¹): 3310, 3193, 2225.
2-Amino-4-benzyl-6-mercaptopyridine-3,5-dicarbonitrile (97)

Yield: 80%; mp: 231-233°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 12.96 (br s, 1H, SH exc D$_2$O), 7.82 (br s, 2H, NH$_2$ exc D$_2$O), 7.36 (m, 2H, Ar), 7.28 (m, 3H, Ar), 4.02 (s, 2H, CH$_2$); IR (cm$^{-1}$): 3319, 3280, 2229.

2-Amino-4-(4-chlorophenyl)-6-mercaptopyridine-3,5-dicarbonitrile (98) (354)

Yield: 80%; mp: 202-204°C (MeOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 13.12 (br s, 1H, SH, exc D$_2$O), 8.01 (br s, 2H, NH$_2$, exc D$_2$O), 7.65 (d, J = 8 Hz, 2H, Ar), 7.58 (d, J = 8 Hz, 2H, Ar), IR (cm$^{-1}$): 3320, 3192, 2220.

2-Amino-4-(4-fluorophenyl)-6-mercaptopyridine-3,5-dicarbonitrile (99) (355)

Yield: 86%; mp: 234-236°C (MeOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 13.03 (br s, 1H, SH, exc D$_2$O), 8.01 (br s, 2H, NH$_2$, exc D$_2$O), 7.6 (m, 2H, Ar), 7.38 (m, 2H, Ar); IR (cm$^{-1}$): 3319, 3182, 2224

2-Amino-6-mercapto-4-(p-tolyl)pyridine-3,5-dicarbonitrile (100) (355)

Yield: 83%; mp: 228-230°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 13.03 (br s, 1H, SH, exc D$_2$O), 7.97 (br s, 2H, NH$_2$, exc D$_2$O), 7.39 (q, 4H, Ar, J = 8.2 Hz), 2.40 (s, 3H, CH$_3$); IR (cm$^{-1}$): 3322, 3190, 2215
Experimental section

2-Amino-6-mercapto-4-(4-(methylthio)phenyl)pyridine-3,5-dicarbonitrile (101) (354)

Yield: 82%; mp: 188-190°C (EtOH); \(^{1}H\) NMR (400 MHz, DMSO-\(d_6\)): 13.1 (br s, 1H, SH, exc D\(_2\)O), 7.45 (d, 2H, Ar = 8 Hz), 7.4 (d, 2H, Ar = 8 Hz), 2.51 (s, 3H, CH\(_3\)); IR (cm\(^{-1}\)): 3311, 3195, 2222

2-Amino-4-(4-(cyclopropylmethoxy)phenyl)-6-mercaptopyridine-3,5-dicarbonitrile (118) (293)

Yield: 80%; mp: 168-170°C (EtOAc); \(^{1}H\) NMR (400 MHz, DMSO-\(d_6\)): 12.9 (br s, 1H, SH, exc D\(_2\)O), 7.8 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.45 (d, 2H, Ar = 8 Hz), 7.08 (d, 2H, Ar = 8 Hz), 3.91 (d, 2H, CH\(_2\), J = 7.8 Hz), 1.23-1.27 (m, 1H, CH), 0.57-0.62 (m, 2H, 2CH \(eq\)), 0.33-0.37 (m, 2H, 2CH \(ax\)); IR (cm\(^{-1}\)): 3309, 3190, 2219

2-Amino-4-(4-(cyclobutylmethoxy)phenyl)-6-mercaptopyridine-3,5-dicarbonitrile (119)

Yield: 84%; mp: 256-259°C (EtOH); \(^{1}H\) NMR (400 MHz, DMSO-\(d_6\)): 12.95 (s, 1H, SH, exc D\(_2\)O), 7.77 (s, 2H, NH\(_2\), exc D\(_2\)O), 7.45 (d, 2H, Ar = 8.8 Hz), 7.09 (d, 2H, Ar = 8.4 Hz), 4.04 (d, 2H, OCH\(_2\), J = 6 Hz), 2.87-2.73 (m, 1H, CH), 2.10-1.92 (m, 2H, CH\(_3\)), 1.9-1.89 (m, 4H, 2CH\(_2\)); IR (cm\(^{-1}\)): 3474, 3335, 2214

2-Amino-4-(4-isobutoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (120)

Yield: 86%; \(^{1}H\) NMR (400 MHz, DMSO-\(d_6\)): 13.02 (s, 1H, SH, exc D\(_2\)O), 7.77 (s, 2H, NH\(_2\), exc D\(_2\)O), 7.45 (d, 2H, Ar = 8.8 Hz), 7.09 (d, 2H, Ar = 8.4 Hz), 3.83 (d, 2H, CH\(_2\), J = 6.4 Hz), 2.06-2.03 (m, 1H, CH), 1.02 (s, 3H, CH\(_3\)), 0.99 (s, 3H, CH\(_3\)
2-Amino-4-(4-ethoxyphenyl)-6-mercaptopyridine-3,5-dicarbonitrile (121)

Yield: 76%; mp: 290-292°C (EtOH); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 12.99 (s, 1H, SH, exc D\textsubscript{2}O), 8.11 (s, 2H, NH\textsubscript{2}, exc D\textsubscript{2}O), 7.54 (d, 2H, Ar, J= 8.8 Hz), 7.14 (d, 2H, Ar, J= 8.8 Hz), 4.14 (q, 2H, CH\textsubscript{2}), 1.38 (t, 3H, CH\textsubscript{3}), 1.38 (t, 3H, CH\textsubscript{3}); IR (cm\textsuperscript{-1}): 3336, 3117, 2212

4-(4-(Allyloxy)phenyl)-2-amino-6-mercaptopyridine-3,5-dicarbonitrile (122)

Yield: 84%; mp: 208-211°C (EtOAc); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 12.98 (s, 1H, SH, exc D\textsubscript{2}O), 7.90 (s, 2H, NH\textsubscript{2}, exc D\textsubscript{2}O), 7.47 (d, 2H, Ar, J= 8.6 Hz), 7.12 (d, J= 8.6 Hz, 2H, Ar); 6.11-6.04 (m, 1H, CH\textsubscript{allyl}), 5.47-5.38 (dd, J\textsubscript{gem} = 1.2 Hz, J\textsubscript{trans} = 17.2 Hz, 1H, CH), 5.30 (d, J= 10.4 Hz, 1H, CH), 4.66 (d, J= 5.6 Hz, 2H, OCH\textsubscript{2}); IR (cm\textsuperscript{-1}): 3476, 3319, 3209, 2212

2-Amino-6-mercapto-4-(4-((2-methylallyloxy)phenyl)pyridine-3,5-dicarbonitrile (123)

Yield: 72%; (EtOH); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 12.98 (s, 1H, SH, exc D\textsubscript{2}O), 8.12-8.10 (s, 1H, CH\textsubscript{3}), 7.53 (d, 2H, Ar, J= 7.4 Hz), 7.08 (d, 2H, Ar, J= 8.8), 5.09 (s, 1H, CH), 4.90 (s, 1H, CH), 4.58 (s, 2H, OCH\textsubscript{2}), 1.80 (s, 3H, CH\textsubscript{3})

N-(4-(2-amino-3,5-dicyano-6-mercaptopyridin-4-yl)phenyl)acetamide (139) (356)

Yield: 40%; mp: 275-277°C (d)(EtOH/EtOAc); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 12.94 (s, 1H, SH, exc D\textsubscript{2}O), 10.37 (s, 1H, NH, exc D\textsubscript{2}O), 8.19 (s, 2H, NH\textsubscript{2}, exc D\textsubscript{2}O), 7.73 (d, 2H, Ar, J= 8.0 Hz), 7.44 (d, 2H, Ar, J= 8.0 Hz), 2.08 (s, 3H, CH\textsubscript{3}); IR (cm\textsuperscript{-1}): 3317, 3215, 2214, 1539
**Experimental section**

2-Amino-4-(4-hydroxyphenyl)-6-mercaptopypyridine-3,5-dicarbonitrile (141) (297):

![Chemical structure image]

Yield: 69%; mp: >300°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-d<sub>6</sub>): 12.94 (br s, 1H, SH, exc D<sub>2</sub>O), 10.09 (br s, 1H, OH, exc D<sub>2</sub>O), 7.96-7.89 (br s, NH<sub>2</sub>, exc D<sub>2</sub>O), 7.38 (d, 2H, Ar, J= 8.8 Hz), 6.90 (d, 2H, Ar, J= 8.4)

**General procedure for the synthesis of p-alkyloxybenzaldehydes (106-111)**

![Chemical structure image]

**Procedures:**

**Method A:** To a solution of 4-hydroxybenzaldehyde (12.3 mmol) in dried acetone (20 ml), potassium carbonate (18.5 mmol) was added, followed by the suitable alkyl halide (18.5 mmol). The mixture was heated at reflux until starting material disappears (6-48h) monitored by TLC. Then, the mixture was cooled to rt and the insoluble material was filtered and washed with acetone (3 x 20 ml). The resulting filtrates were collected and evaporated under vacuum, affording the desired compounds as viscous oils.

**Method B:** To a mixture of 4-hydroxybenzaldehyde (8.2 mmol), cyclopropyl methanol (9.02 mmol) and triphenylphosphine (10.66 mmol) in THF dry maintained at 0°C, diethyl azodicarboxylate (DEAD, 10.66 mmol) was carefully added within 10/15 min, keeping temperature under 0°C. The mixture was kept at 0°C for further 10 min, then warmed to rt and stirred for 24h. The reaction mixture was then evaporated carefully until affording a raw semisolid which was purified by silica gel flash chromatography using DCM 9 : MeOH 1 as eluent affording compounds as a viscous oil.

**Method C:** Potassium carbonate (18.5 mmol), the suitable alkyl halide (18.5 mmol) and potassium iodide (0.123 mmol) were sequentially added to a solution of 4-hydroxybenzaldehyde (12.3 mmol) in DMF dry (15 ml). The mixture was refluxed until reaction completion (TLC monitoring). The mixture was then cooled to rt and water (50 ml) was added. The resulting suspension was extracted with AcOEt (4 x 30 ml) and the organic phases were collected, dried (Na<sub>2</sub>SO<sub>4</sub>), and the solvent removed under vacuum, affording compounds as a viscous oil.
Examples

4-(Cyclopropylmethoxy)benzaldehyde (106) (360)

Method A and B; Yield: 77%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 9.86 (s, 1H, CHO), 7.85 (d, 2H, Ar, J=8.1 Hz), 7.10 (d, 2H, Ar, J=7.7 Hz), 3.93 (d, 2H, CH\(_2\), J=7.8 Hz), 1.22-1.28 (m, 1H, CH), 0.56-0.61 (m, 2H, 2CH eq), 0.33-0.36 (m, 2H, CH ax)

4-(Cyclobutylmethoxy)benzaldehyde (107) (361)

Method A; Yield: 67%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 9.86 (s, 1H, CHO), 7.85 (d, 2H, Ar, J= 8.8 Hz), 7.11 (d, 2H, Ar, J= 8.4 Hz), 4.06 (d, 2H, OCH\(_2\), J= 6.8 Hz), 2.77-2.71 (m, 1H, CH), 2.08-2.07 (m, 2H, CH\(_2\)), 1.90-1.82 (m, 4H, 2CH\(_2\))

4-Isobutoxybenzaldehyde (108) (362)

Method C; Yield: 66%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 9.86 (s, 1H, CHO), 7.85 (d, 2H, Ar, J= 8.8 Hz), 7.10 (d, 2H, Ar, J=8.8 Hz), 3.84 (d, 2H, OCH\(_2\), J= 6.4 Hz), 2.06-1.98 (m, 1H, CH), 1.00 (s, 3H, CH\(_3\)), 0.96 (s, 3H, CH\(_3\))

4-Ethoxybenzaldehyde (109) (363)

Method A; Yield: 83%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 9.86 (s, 1H, CHO), 7.85 (d, 2H, Ar, J= 8.4 Hz), 7.10 (d, 2H, Ar, J=8.8 Hz), 4.13 (q, 2H, OCH\(_2\), J= 6.8 Hz), 1.35 (t, 3H, CH\(_3\), J= 7.2 Hz)

4-(Allyloxy)benzaldehyde (110) (364)

Method A; Yield: 97%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 9.87 (s, 1H, CHO), 7.87 (d, 2H, Ar, J= 8.6 Hz), 7.15 (d, 2H, Ar, J= 8.7 Hz), 6.10-6.02 (m, 1H, CH\(_{allyl}\)), 5.45-5.40 (dd, 1H, CH\(_2\), J\(_{gem}\)= 1.6 Hz, J\(_{cis}\)=18 Hz), 5.31-5.24 (dd, 1H, CH\(_2\), J\(_{gem}\)= 10.4 Hz, J\(_{trans}\)= 22 Hz), 4.70 (d, 2H, OCH\(_2\), J= 5.2 Hz)
Experimental section

4-((2-Methylallyl)oxy)benzaldehyde (111) (365)

\[
\begin{align*}
\text{Method A; Yield: 85%; } ^1\text{H NMR (400 MHz, DMSO-}d_6\text{): } & 9.87 (s, 1H, CHO), 7.86 (d, 2H, Ar, } J= 8.4 \text{ Hz), 7.13 (d, 2H, Ar, } J= 8.4 \text{ Hz), 5.09 (d, 1H, CH, } J= 0.8 \text{ Hz), 4.98 (d, 1H, CH, } J= 1.2 \text{ Hz), 4.57 (s, 2H, CH}_2, 1.77 (s, 3H, CH}_3
\end{align*}
\]

Synthesis of 2-(1-ethoxyethylidene)malononitrile (103) (366)

A suspension of malononitrile (0.45 mmol) and triethylorthoacetate (0.5 mmol) was refluxed for 2 h. At reaction completion, the liquid phase was removed by distillation at reduced pressure, affording a solid which was recovered with Et\textsubscript{2}O (5 ml), filtered and recrystallized from EtOH. Yield: 82%; mp: 145-147°C (EtOH); \(^1\text{H NMR (400 MHz, DMSO-}d_6\text{): } 4.41 (q, 2H, CH}_2, J = 4 \text{ Hz), 2.42 (s, 3H, CH}_3, 1.31 (t, 3H, CH}_3, J = 4 \text{ Hz); IR (cm}^{-1} ): 2245, 2238.

Synthesis of 2-amino-6-mercapto-4-methylpyridine-3,5-dicarbonitrile (104) (367)

Thiocyanacetamide (22.3 mmol) was portiowise added to a solution of compound 102 (23.00 mmol) and NaOCH\textsubscript{3} (24.79 mmol) in MeOH (25 ml). The reaction mixture was refluxed for 4 h, then, the solvent was removed under vacuum and the resulting yellow solid was recovered with Et\textsubscript{2}O and filtered. Yield: 62%; mp: 226-228°C (EtOH); \(^1\text{H NMR (400 MHz, DMSO-}d_6\text{): } 12.94 (br s, 1H, SH, exc D}_2\text{O), 7.10 (br s, 2H, NH}_2, exc D}_2\text{O), 2.30 (s, 3H, CH}_3); IR (cm}^{-1} ): 3338, 3253, 2248.
**Experimental section**

**Synthesis of (1H-imidazol-2-yl)methanol (105)** (297)

![Diagram of (1H-imidazol-2-yl)methanol](image)

Imidazole-2-carboxyaldehyde (20.8 mmol) was added to a suspension of sodium borohydride (41.6 mmol) in absolute EtOH (30 ml) under nitrogen atmosphere which was then stirred at rt for 2h. After reaction completion, the excess of sodium borohydride was quenched with a 20% aqueous sodium hydroxide solution and the resulting suspension was filtered. The filtrate was extracted with EtOAc (5 x 15 ml) and the collected organic phases were dried (Na₂SO₄), and evaporated under reduced pressure to yield a white solid that was used for the next step without any further purification. Yield: 46%; ¹H NMR (400 MHz, DMSO-d₆): 11.89 (s, 1H, NH, exc D₂O), 6.90 (s, 2H, 2CH), (s, 1H, OH, exc D₂O), 4.44 (s, 2H, CH₂).

**Synthesis of 2-(bromomethyl)-1H-imidazole hydrobromide (102)** (297)

![Diagram of 2-(bromomethyl)-1H-imidazole hydrobromide](image)

A solution of 104 (3.1 mmol) in 33% HBr in acetic acid (8 ml) was heated at 100°C for 4h. Then, the solvent was removed by distillation at reduced pressure and the red-brown residue was triturated with petroleum ether (10 ml), filtrate and quickly used for the next step without any further purification. Yield: 70%; ¹H NMR (400 MHz, DMSO-d₆): 14.00 (s, 1H, NH, exc D₂O), 7.71 (s, 2H, 2CH), 4.84 (s, 2H, CH₂).

**Synthesis of 1H-pyrazole-3-carboxylic acid (125)** (308)

![Diagram of 1H-pyrazole-3-carboxylic acid](image)

Potassium permanganate (128.7 mmol) was added in small portions to a solution of 3-methyl-1H-pyrazole (58.5 mmol) in water (225 ml), and the mixture was heated at reflux for 4 h. After cooling to rt, the suspension was filtered and the filtrate was concentrated under vacuum to small volume. 6M HCl (80 ml) was added and the mixture was kept cool for 1h to afford a white solid which was collected by filtration and dried at 60°C. Yield: 62%; mp: 193-195°C (Et₂O); ¹H NMR (400 MHz, DMSO-d₆): 13.01 (br s, 1H, COOH, exc D₂O), 7.72 (s, 1H, CH), 6.68 (s, 1H, CH).
Experimental section

Synthesis of ethyl 1H-pyrazole-3-carboxylate (126) (368)

Thionyl chloride (20.6 mmol) was added to a solution of compound 125 (18.7 mmol) in absolute EtOH (20 ml). Then the mixture was refluxed until starting material disappeared (TLC monitoring). The excess of liquid reactant was removed under reduced pressure, and water (25 ml) was added affording a white solid which was collected by filtration and used for the next step without any further purification. Yield: 76%; $^1$H NMR (400 MHz, DMSO-$d_6$): 13.48 (s, 1H, NH, exc D$_2$O), 7.81 (s, 1H, CH), 6.76 (s, 1H, CH), 4.27 (q, $J = 7.1$ Hz, 2H, CH$_2$), 1.29 (t, $J = 7.1$ Hz, 3H, CH$_3$).

Synthesis of (1H-pyrazol-3-yl)methanol (127) (308)

Lithium aluminum hydride (35.7 mmol) was portionwise added to a suspension of compound 126 (17.8 mmol) in anhydrous THF (40 ml), maintained in an ice bath keeping temperature under 5°C. The mixture was allowed to warm at rt, stirred for 2 h and then, while keeping in an ice bath, MeOH (1 ml) was carefully added, followed by a solution of aqueous MeOH (4:1). The resulting solid was filtered and the filtrate was extracted with EtOAc (3 x 25 ml). The collected organic phases were washed with brine (25 ml), dried (Na$_2$SO$_4$) and filtered. Evaporation of solvent afforded a yellow oil which was used without any further purification for the next step. Yield: 66%; $^1$H NMR (400 MHz, DMSO-$d_6$): 12.58 (s, 1H, NH, exc D$_2$O), 7.51 (s, 1H, CH), 6.15 (s, 1H, CH), 5.07 (s, 1H, OH, exc D$_2$O), 4.44 (s, 7H, CH$_7$).

Synthesis of 3-(chloromethyl)-1H-pyrazole (124) (308)

An excess of thionyl chloride (15.1 mmol) was added to a suspension of compound 127 (10.1 mmol) in anhydrous DCM (20 ml), and the mixture was refluxed for 3 h. Then, removal of liquid component under reduced pressure afforded a yellowish solid which is dried in desiccator at rt. Yield: 86%; $^1$H NMR (400 MHz, DMSO-$d_6$): 7.66 (s, 1H, CH), 6.32 (s, 1H, CH), 4.72 (s, 2H, CH$_2$).
Experimental section

**Synthesis of (1H-1,2,4-triazol-3-yl)methanol hydrochloride (129)** (309)

![HCl](image)

A solution of methyl 1H-1,2,4-triazole-3-carboxylate (7.87 mmol) in anhydrous THF (25 ml) was slowly added to a suspension of lithium aluminum hydride (19.76 mmol) in anhydrous THF (10 ml) and the mixture was refluxed for 12 h. At reaction completion MeOH (1 ml) was carefully added followed by 50 % aqueous MeOH (5 ml) affording a solid that was filtered and washed with MeOH (20 ml). The filtrate was then evaporated and the resulting residue was triturated with absolute EtOH (40 ml) and conc. HCl (1 ml). The mixture was concentrated under vacuum and the resulting semisolid was worked up with a mixture of MeOH and Et₂O (1:1, 10 ml), filtered and dried in desiccator at rt. Yield: 72%; mp: 138-140°C; ¹H NMR (400 MHz, DMSO-d₆): 13.85 (br s, 1H, NH, exc D₂O), 8.90 (s, 1H, CH), 5.69 (t, 1H, OH, exc D₂O), 4.70 (s, 2H, CH₂).

**Synthesis of 3-(chloromethyl)-1H-1,2,4-triazole (128)** (309)

![Cl](image)

A solution of compound 129 (2.21 mmol) in thionyl chloride (10 ml) was refluxed for 6 h. Then, the excess of chlorinating agent was removed under vacuum affording a residue that was triturated with a mixture Et₂O/EtOH (5:1, 10 ml), filtered and washed with petroleum ether (5 ml). Yield: 40%; mp: 102-104°C; ¹H NMR (400 MHz, DMSO-d₆): 14.03 (br s, NH, exc D₂O), 8.54 (s, 1H, CH), 4.77 (s, 2H, CH₂).

**Synthesis of 4-(chloromethyl)thiazol-2-amine (130)** (310)

![Cl](image)

To a solution of 1,3-dichloroacetone (26.8 mmol) in anhydrous acetone (100 ml), thiourea (26.27 mmol) was added in small portions. The mixture was allowed to stir at rt for 12 h. The resulting white solid was filtered, washed with acetone (30 ml) and Et₂O (10 ml), suspended in EtOH (100 ml) and stirred for 3 h at rt. The solid was then removed by filtration and the filtrate was evaporated affording a white solid. Yield: 37%; mp: 117-119°C (Acetone); ¹H NMR (400 MHz, DMSO-d₆): 9.13 (br s, 2H, NH₂), 6.96 (s, 1H, CH), 4.67 (s, 2H, CH₂).
**Synthesis of 4-(chloromethyl)-2-phenylthiazole (131)** (369)

![Chemical structure of 4-(chloromethyl)-2-phenylthiazole (131)](image)

1,3-Dichloroacetone (18.22 mmol) was added to a solution of thiobenzamide (3.64 mmol) in dry acetone (45 ml) and the mixture was refluxed for 8 h. After cooling at rt, the white crystalline precipitated was filtered and washed with fresh acetone (10 ml). The solid obtained was then dissolved in sulfuric acid (1.2 ml) and the resulting solution was stirred for 30 min at rt. Then 20 ml of iced-water was carefully added affording white crystals, that were quickly filtered and stored at 4°C. Yield: 65%; mp: 53-55°C; $^1$H NMR (400 MHz, CDCl$_3$): 8.05 – 7.89 (m, 2H, Ar), 7.55 – 7.39 (m, 3H, Ar), 7.33 (s, 1H, CH), 4.77 (s, 2H, CH$_2$).

**Synthesis of 3-(chloromethyl)benzamide (132)** (311)

![Chemical structure of 3-(chloromethyl)benzamide (132)](image)

Oxalyl chloride (14.90 mmol) and dry DMF (2 gtt) were added to a solution of 3-(chloromethyl)benzoic acid (6.00 mmol) in anhydrous DCM (30 ml). The reaction mixture was allowed to stir at rt overnight. The solvent was removed under vacuum and the oily residue was immediately dissolved in a saturated ammonia solution in dioxane (100 ml). The reaction mixture was stirred at rt overnight and the resulting white crystals were collected by filtration and washed with Et$_2$O (2 ml). Yield: 60%; mp: 130-131°C (Et$_2$O); $^1$H NMR (400 MHz, DMSO-d$_6$): 8.0 (br s, 1H, NH, exc D$_2$O), 7.95 (s, 1H, Ar), 7.83-7.85 (m, 1H, Ar), 7.83-7.85 (m, 1H, Ar), 7.45-7.49 (m, 1H, Ar), 7.41 (br s, 1H, NH, exc D$_2$O), 4.81 (s, 2H, CH$_2$); IR (cm$^{-1}$): 3350; 3182; 1651.

**Synthesis of benzyl 2-chloroacetate (133)** (370)

![Chemical structure of benzyl 2-chloroacetate (133)](image)

Benzylalcohol (46.24 mmol) and p-toluenesulfonic acid (0.63 mmol) were added to a solution of chloroacetic acid (55.35 mmol) in toluene (25 ml). After heating at 140°C for 2 h, the reaction mixture was cooled to rt and washed with a 5% sodium hydrogen carbonate (2 x 25 ml) and water (25 ml). The collected organic phases were dried (Na$_2$SO$_4$) and evaporated affording a yellowish oil that was used without any further purification.
Experimental section

for the next step. Yield: 78%; $^1$H NMR (400 MHz, CDCl$_3$): 7.59 – 7.30 (m, 5H, Ar), 5.25 (s, 2H, OCH$_2$), 4.12 (s, 2H, CH$_2$Cl)

**Synthesis of 2-chloro-N-hydroxyacetamide (134)** (371)

\[
\text{Cl} - \overset{\text{N}}{\text{O}} - \text{H}
\]

50 % Aqueous solution of hydroxylamine (12.00 mmol) was added to ethyl chloroacetate (12.00 mmol) in a 50 ml round bottomed flask and stirred for 5 minutes at rt. Then, the mixture was cooled at 4°C for 15 min, re-warmed to rt and stirred for 3 h. The precipitate was collected by filtration and used for the next step without any further purification. Yield: 53%; $^1$H NMR (400 MHz, DMSO): 7.41 (br s, 2H, OH + NH, exc D$_2$O), 3.94 (s, 2H, CH$_2$).

**Synthesis of ethyl 2-chloroacetimidate hydrochloride (136)** (372)

\[
\text{Cl} - \overset{\text{O}}{\text{NH}} - \overset{\text{HCl}}{\text{H}}
\]

Anhydrous HCl was bubbled for 1 h at 0°C in a solution of chloroacetonitrile (66.22 mmol) in absolute EtOH (5 ml) and Et$_2$O anhydrous (30 ml), then the reaction mixture was allowed to stir at rt overnight under nitrogen atmosphere. Concentration of the solution under vacuum afforded white crystals that were collected by filtration and used for the following step without any further purification. Yield: 86%; $^1$H NMR (400 MHz, DMSO): 12.4 (br s, 1H, NH, exc D$_2$O), 4.38 (s, 2H, CH$_2$), 4.17 (q, 2H, J = 7.1 Hz, CH$_2$), 1.22 (t, 3H, J = 7.1 Hz, CH$_3$).

**Synthesis of 2-(chloromethyl)-4,5-dihydro-1H-imidazole (135)** (373)

Ethyl 2-chloroacetimidate hydrochloride 136 (9.49 mmol) was added in small portions to a solution of ethylenediamine (14.24 mmol) in absolute EtOH (25 ml) maintained at 0°C. The mixture was stirred at 0°C for 1h, then a solution of anhydrous HCl (82.3 mmol) in absolute EtOH (25 ml) was carefully added in small portions, keeping temperature to 0°C. The mixture was stirred at 0°C for 1 h and then was warmed at rt and
stirred overnight. Concentration at small volume of the mixture afforded a white solid which was filtered and washed with EtOH (5 ml) and Et₂O (5 ml). Yield: 82%; ¹H NMR (400 MHz, DMSO): 10.90-10.59 (br s, 2H, NH₂⁺, exc D₂O), 4.67 (s, 2H, CH₂), 3.89 (s, 4H, 2CH₂).

General procedure for the synthesis of final compounds A, 1-16, 26-35, 37-67 and 142

Sodium hydrogencarbonate (2.00 mmol) and the suitable alomethyl-derivative (1.00 mmol) were added to a solution of the mercapto compound 78-81, 95-101, 104, 118-123, 139 and 141 (1.00 mmol) in DMF dry (1 ml). The reaction mixture was stirred at rt until disappearance of the starting material (TLC monitoring). At reaction completion, water was added (25 ml) to precipitate a solid which was collected by filtration and triturated with Et₂O (5 ml). The crude product was then recrystallized or purified by column chromatography or preparative TLC using DCM 9 : MeOH 1, EtOAc 5 : CHx 5 : MeOH 1, CHx 7 : EtOAc 3 or EtOAc 1 : CHx 1 as eluents.

Examples

2-Amino-4-(furan-2-yl)-6-((2-hydroxyethyl)thio)pyridine-3,5-dicarbonitrile (A) (298)

yield: 63%; mp: 171-173°C (EtOAc/CHx); ¹H NMR (400 MHz, DMSO-d₆): 8.11-8.12 (m, 1H, Fur), 7.99 (br s, 2H, NH₂, exc D₂O), 7.39 (d, 1H, Fur, J = 3.6 Hz), 6.83 (dd, 1H, Fur, J₁ = 1.72, J₂ = 3.6 Hz), 4.99 (t, 1H, OH, exc D₂O, J = 5.4 Hz), 3.65 (q, 2H, CH₂, J = 12 Hz), 3.33 (t, 2H, CH₂, J = 6.3 Hz); IR (cm⁻¹): 3320, 3219, 2212.

Methyl 3-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoate (1)

yield: 62%; mp: 208-210°C (MeOH/2-methoxyethanol); ¹H NMR (400 MHz, DMSO-d₆): 8.09-8.08 (m, 1H, Fur), 8.00 (s, 2H, NH₂, exc D₂O), 7.83-7.85 (m, 3H, Ar), 7.47 (t, 1H, Ar, J = 7.72 Hz), 7.38 (d, 1H,
Experimental section

129

Fur, $J = 3.64 \text{ Hz}$, 6.83 (dd, 1H, fur, $J_1 = 1.72 \text{ Hz}, J_2 = 3.64 \text{ Hz}$), 4.58 (s, 2H, CH$_2$), 3.86 (s, 3H, CH$_3$); IR (cm$^{-1}$): 2214, 1728.

3-(((6-Amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzamide (2)

Yield: 43%; mp: 249-251°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 8.10 (d, $J = 1.1 \text{ Hz}, 1\text{H, Fur}$), 8.07 (br s, 2H, NH$_2$, exc D$_2$O) 7.98 (s, 1H, Ar), 7.96 (br s, 1H, NH amide, exc D$_2$O), 7.76 (d, $J = 7.8 \text{ Hz}, 1\text{H, Ar}$), 7.68 (d, $J = 7.7 \text{ Hz}, 1\text{H, Ar}$), 7.49 – 7.29 (m, 3H, Ar, NH amide, Fur, overlapping, partially exc D$_2$O), 6.83 (dd, $J = 3.6, 1.8 \text{ Hz}, 1\text{H, Fur}$), 4.53 (s, 2H, CH$_2$). IR (cm$^{-1}$): 3424, 3360, 3331, 3213, 2210, 1643

3-(((6-Amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)benzoic acid (3)

Yield: 35%; mp: 255-257°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 13.01 (s, 1H, COOH, exc D$_2$O), 8.10 (d, $J = 1.0 \text{ Hz}, 1\text{H, Fur}$), 8.05 (s, 1H, Ar), 8.13 (br s, 2H, NH$_2$, exc D$_2$O), 7.86 – 7.76 (m, 2H, Ar), 7.44 (t, $J = 7.7 \text{ Hz}, 1\text{H, Ar}$), 7.39 (d, $J = 3.6 \text{ Hz}, 1\text{H, Fur}$), 6.83 (dd, $J = 3.6, 1.7 \text{ Hz}, 1\text{H, Fur}$), 4.58 (s, 2H, CH$_2$). IR (cm$^{-1}$): 3329, 3327, 2214, 1541

2-Amino-4-(furan-2-yl)-6-((3-(hydroxymethyl)benzyl)thio)pyridine-3,5-dicarbonitrile (4)

Yield: 43%; mp: 193-195°C (EtOAc); $^1$H NMR (400 MHz, DMSO-d$_6$): 8.24 (s, 1H, Fur), 8.10 (br s, 2H, NH$_2$, exc D$_2$O), 7.90 (t, $J = 1.6 \text{ Hz}, 1\text{H, Fur}$), 7.42 (s, 1H, Ar), 7.37 (d, $J = 7.5 \text{ Hz}, 1\text{H, Ar}$), 7.27 (t, $J = 7.5 \text{ Hz}, 1\text{H, Ar}$), 7.21 (d, $J = 7.5 \text{ Hz}, 1\text{H, Ar}$), 6.87 (s, 1H, Fur), 5.19 (t, $J = 5.7 \text{ Hz}, 1\text{H, OH}$, exc D$_2$O), 4.55 – 4.43 (m, 4H, 2CH$_2$ partially overlapping). IR (cm$^{-1}$): 3398, 3314, 3215, 2214

2-Amino-6-((3-cyanobenzyl)thio)-4-(furan-2-yl)pyridine-3,5-dicarbonitrile (5)

Yield: 68%; mp: 193-195°C (EtOH); $^1$H NMR (400 MHz, DMSO): 8.23 (s, 1H, Fur), 8.15 (br s, 2H, NH$_2$, exc D$_2$O), 8.08 (s, 1H, Fur), 7.90 (dd, $J = 3.6, 1.8 \text{ Hz}, 1\text{H, Ar}$), 7.88 (d, $J = 8.0 \text{ Hz}, 1\text{H, Ar}$), 7.72 (d, $J = 7.7 \text{ Hz}, 1\text{H, Ar}$), 7.53
(t, J = 7.8 Hz, 1H, Ar), 6.86 (s, 1H, Fur), 4.50 (s, 2H, CH₂). IR (cm⁻¹): 3410, 3321, 3228, 2227, 2212

**Methyl 5-(((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)methyl)furan-2-carboxylate (6)**

Yield: 64%; mp: 260-262°C (Acetone); ¹H NMR (400 MHz, DMSO-d₆): 8.25 (s, 1H, Fur), 7.91 (s, 1H, Fur), 7.25 (d, J = 3.5 Hz, 1H, SCH₂Fur), 6.88 (s, 1H, Fur), 6.79 (d, J = 3.4 Hz, 1H, SCH₂Fur), 4.62 (s, 2H, CH₂), 3.80 (s, 3H, CH₃). IR (cm⁻¹): 3402, 3334, 3240, 2222, 2210

**Methyl 3-(((6-amino-3,5-dicyano-4-(furan-3-yl)pyridin-2-yl)thio)methyl)benzoate (7)**

Yield: 61%; mp: 229-231°C (MeOH/2-methoxyethanol); ¹H NMR (400 MHz, DMSO-d₆): 8.24 (s, 1H, Fur), 8.09 (s, 1H, Fur), 8.34 – 7.77 (br s, 2H, NH₂, exc D₂O), 7.90 (t, J = 1.7 Hz, 1H, Ar), 7.85 (s, 1H, Ar), 7.83 (s, 1H, Ar), 7.47 (t, J = 7.7 Hz, 1H, Ar), 6.87 (d, J = 1.8 Hz, 1H, Fur), 4.58 (s, 2H, CH₂), 3.86 (s, 3H, CH₃). IR (cm⁻¹): 3373, 3113, 2210, 1527

**Methyl 3-(((6-amino-3,5-dicyano-4-(thiophen-2-yl)pyridin-2-yl)thio)methyl)benzoate (8)**

Yield: 76%; mp: 199-201°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 8.10 (s, 1H, Ar), 7.94 (d, J = 5.0 Hz, 1H, CH thiophene), 8.55 – 7.72 (m, 2H, NH₂, exc D₂O), 7.86 (s, 1H, Ar), 7.84 (s, 1H, Ar), 7.56 (d, J = 2.7 Hz, 1H, CH thiophene), 7.47 (t, J = 7.7 Hz, 1H, Ar), 7.27 (dd, J = 4.9, 3.8 Hz, 1H, CH thiophene), 4.59 (s, 2H, CH₂), 3.86 (s, 3H, CH₃). IR (cm⁻¹): 3462, 3329, 3217, 2210, 1547

**Methyl 3-(((6'-amino-3',5'-dicyano-[3,4'-bipyridin]-2'-yl)thio)methyl)benzoate (9)**

Yield: 21%; mp: 222-224°C (2-methoxyethanol/EtOH); ¹H NMR (400 MHz, DMSO-d₆): 8.83 – 8.67 (m, 2H, Ar), 8.23 (br s, 2H, NH₂, exc D₂O), 8.11 (s, 1H, Ar), 8.03 (d, J = 7.9 Hz, 1H, Ar), 7.86 (t, J = 7.8 Hz, 2H, Ar), 7.61 (dd, J = 7.7, 4.9 Hz, 1H, Ar), 7.48 (t, J = 7.7 Hz, 1H, Ar), 4.61 (s, 2H, CH₂), 3.86 (s, 3H, CH₃); ¹³C NMR (100 MHz, DMSO-d₆): 166.44, 159.88, 155.82, 151.73, 149.01, 139.08, 136.86, 134.89, 130.65, 130.36, 129.29, 128.51, 124.01, 115.48, 93.84, 86.77, 52.65, 33.05; IR (cm⁻¹): 3373, 3314, 3152, 2210, 1728
2-Amino-6-((cyanomethyl)thio)-4-(furan-2-yl)pyridine-3,5-dicarbonitrile (10)

Yield: 43%; mp: 297-299°C (2-methoxyethanol); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.16 (br s, 2H, NH\(_2\)), 8.17 – 8.11 (m, 1H, Fur), 7.51 – 7.39 (m, 1H, Fur), 6.86 (dd, \(J = 3.7, 1.8\) Hz, 1H, Fur), 4.33 (s, 2H, CH\(_2\)). IR (cm\(^{-1}\)): 3404, 3319, 3228, 1791, 1723, 1594, 1584, 1545, 1507, 1458, 1444, 1437, 1321, 1269, 1207, 1175, 1155, 1141, 1127, 1094, 1032, 1004, 967, 927, 875, 826, 775, 731, 695, 654, 610, 579, 540.

Benzyl 2-((6-amino-3,5-dicyano-4-(furan-2-yl)pyridin-2-yl)thio)acetate (11)

Yield: 30%; mp: 222-224°C (EtOAc/EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.12 (s, 1H, Fur), 7.99 (s, 2H, NH\(_2\)), 7.42 (d, \(J = 3.6\) Hz, 1H, Fur), 7.41 – 7.20 (m, 5H, Ar), 6.85 (dd, \(J = 3.6, 1.7\) Hz, 1H, Fur), 5.19 (s, 2H, CH\(_2\)Bn), 4.28 (s, 2H, SCH\(_2\)). \(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)): 168.41, 166.98, 160.51, 145.44, 144.25, 136.24, 128.85, 128.54, 128.31, 116.99, 116.08, 116.01, 113.35, 89.77, 82.38, 67.10, 32.44; IR (cm\(^{-1}\)): 3441, 3314, 3209, 2214, 1732, 1604, 1594, 1584, 1545, 1507, 1458, 1444, 1437, 1321, 1269, 1207, 1175, 1155, 1141, 1127, 1094, 1032, 1004, 967, 927, 875, 826, 775, 731, 695, 654, 610, 579, 540.

2-Amino-4-(furan-2-yl)-6-((2-oxo-2-phenylethyl)thio)pyridine-3,5-dicarbonitrile (12)

Yield: 47%; mp: 251-253°C (EtOAc); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.28 (d, \(J = 5.7\) Hz, 1H, Fur), 8.08 (t, \(J = 6.3\) Hz, 2H, Ar), 7.94 (br s, 2H, NH\(_2\)), 7.93 (d, \(J = 5.5\) Hz, 1H, Fur), 7.71 (dd, \(J = 13.4, 6.6\) Hz, 1H, Ar), 7.65 – 7.52 (m, 2H, Ar), 6.91 (d, \(J = 4.8\) Hz, 1H, Fur), 5.01 (d, \(J = 5.8\) Hz, 2H, CH\(_2\)). IR (cm\(^{-1}\)): 3485, 3350, 2206, 1618.

2-Amino-4-(furan-2-yl)-6-((pyridin-3-ylmethyl)thio)pyridine-3,5-dicarbonitrile (13)

Yield: 80%; mp: 228-230°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.77 (d, \(J = 2.1\) Hz, 1H, Fur), 8.45 (dd, \(J = 4.8, 1.5\) Hz, 1H, Ar), 8.89 – 7.52 (br s, 2H, NH\(_2\)), 8.10 (d, \(J = 1.7\) Hz, 1H, Fur), 7.94 (d, \(J = 7.9\) Hz, 1H, Ar), 7.38 (d, \(J = 3.6\) Hz, 1H, Ar), 7.34 (dd, \(J = 7.8, 4.8\) Hz, 1H, Ar), 6.83 (dd, \(J = 3.6, 1.7\) Hz, 1H, Fur), 4.49 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3385, 3086, 2206.
2-Amino-4-(furan-2-yl)-6-((pyridin-2-ylmethyl)thio)pyridine-3,5-dicarbonitrile (14)

Yield: 44%; mp: 212-214°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.48 (d, J = 4.7 Hz, 1H, Ar), 8.11 (br s, 2H, NH\(_2\), exc D\(_2\)O), 8.00 (d, J = 1.5 Hz, 1H, Fur), 7.75 (td, J = 7.7, 1.7 Hz, 1H, Ar), 7.60 (d, J = 7.8 Hz, 1H, Ar), 7.37 (d, J = 3.6 Hz, 1H, Fur), 7.34 – 7.24 (m, 1H, Ar), 6.80 (dd, J = 3.6, 1.7 Hz, 1H, Fur), 4.56 (s, 2H, CH\(_2\)); \(^13\)C NMR (100 MHz, DMSO-d\(_6\)): 167.73, 160.59, 150.03, 145.54, 144.30, 137.29, 124.18, 123.00, 116.85, 116.14, 113.29, 89.68, 82.11, 35.95; IR (cm\(^{-1}\)): 3389, 3311, 3148, 2206

2-Amino-4-(furan-2-yl)-6-(((2-phenylthiazol-4-yl)methyl)thio)pyridine-3,5-dicarbonitrile (15)

Yield: 75%; mp: 202-204°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.12 (br s, 2H, NH\(_2\), exc D\(_2\)O), 8.10 (s, 1H, Fur), 7.93 (dd, J = 6.4, 2.7 Hz, 2H, Ar), 7.86 (s, 1H, CH thiazole), 7.64 – 7.42 (m, 3H, Ar), 7.39 (d, J = 3.6 Hz, 1H, Fur), 6.83 (d, J = 1.9 Hz, 1H, Fur), 4.64 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3373, 3200, 3178, 3103, 2212

2-Amino-6-(((2-aminothiazol-4-yl)methyl)thio)-4-(furan-2-yl)pyridine-3,5-dicarbonitrile (16)

Yield: 79%; mp: 243-245°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.10 (s, 1H, Fur), 8.06 (br s, 2H, NH\(_2\) Py, exc D\(_2\)O), 7.39 (d, J = 3.5 Hz, 1H, Fur), 6.98 (br s, 2H, NH\(_2\) thiazole, exc D\(_2\)O), 6.84 (s, 1H, CH thiazole), 6.62 (s, 1H, Fur), 4.30 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3452, 3352, 3317, 3217, 3115, 2218

2-(((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(furan-3-yl)pyridine-3,5-dicarbonitrile (26)

Yield: 53%; mp: 271-273°C dec. (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 11.83 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.26 (s, 1H, CH, Fur), 8.02 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.92 (s, 1H, CH, Fur), 7.07 (br s, 1H, CH imidazole), 6.88 (s, 1H, CH, Fur), 6.86 (br s, 1H, CH imidazole), 4.5 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3328, 3220, 2211
2-{{(1H-Imidazol-2-yl)methyl}thio}-6-amino-4-(thiophen-3-yl)pyridine-3,5-dicarbonitrile (27)

Yield: 73%; 233-235°C (ACN); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 11.83 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.05 (d, 1H, CH thiophene, \(J = 4\) Hz), 8.04 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.78 (t, 1H, CH thiophene, \(J = 4\) Hz), 7.39 (d, 1H, CH thiophene, \(J = 4\) Hz), 7.07 (br s, 1H, CH imidazole), 6.85 (br s, 1H, CH imidazole), 4.50 (s, 2H, CH\(_2\)); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)): 166.79, 160.36, 153.48, 142.99, 133.83, 129.32, 128.16, 127.92, 115.88, 93.29, 86.01, 27.24; IR (cm\(^{-1}\)): 3400, 3321, 2218

2'-{{(1H-Imidazol-2-yl)methyl}thio}-6'-amino-[3,4'-bipyridine]-3',5'-dicarbonitrile (28)

Yield: 64%; mp: 238-240°C (ACN); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 12.18 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.78 (d, 1H, CH Py, \(J = 4\) Hz), 8.75 (s, 1H, CH Py), 8.25 (br s, 2H, NH\(_2\), exc D\(_2\)O), 8.03 (d, 1H, CH Py, \(J = 8\) Hz), 7.62 (t, 1H, CH Py, \(J = 8\) Hz), 7.02 (br s, 2H, CH imidazole), 4.54 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3379, 3308, 2212, 1658

2-{{(1H-Imidazol-2-yl)methyl}thio}-6-amino-[4,4'-bipyridine]-3,5-dicarbonitrile (29)

Yield: 64%; mp: > 300°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 11.87 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.81 (d, 2H, CH Py, \(J = 8\) Hz), 8.2 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.59 (d, 2H, CH Py, \(J = 8\) Hz), 7.09 (s, 1H, CH imidazole), 6.86 (s, 1H, CH imidazole), 4.52 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3312, 3175, 2210

2-{{(1H-Imidazol-2-yl)methyl}thio}-6-amino-4-benzylpyridine-3,5-dicarbonitrile (30)

Yield: 70%; mp: 246-248°C dec (EtOH); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 11.82 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.14 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.34 (d, 2H, Ar, \(J = 8\) Hz), 7.24 (m, 3H, Ar), 6.95 (sl, 2H, CH imidazole), 4.47 (s, 2H, SCH\(_2\)), 4.05 (s, 2H, CH\(_2\)); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)): 166.73, 160.33, 159.10, 142.89, 136.61, 129.38, 128.78, 127.64, 115.61, 86.95, 27.15; IR (cm\(^{-1}\)): 3431, 3357, 2377
Experimental section

2-((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(4-chlorophenyl)pyridine-3,5-dicarbonitrile (31)

Yield: 42%; mp: 229-231°C dec (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 11.87 (br s, 1H, NH imidazole, exc D\(_2\), O), 8.2 (br s, 2H, NH\(_2\), exc D\(_2\), O), 7.65 (d, 2H, Ar, J = 8 Hz), 7.59 (d, 2H, Ar, J = 8 Hz), 7.08 (s, 1H, CH imidazole), 6.85 (s, 1H, CH imidazole), 4.5 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3336, 3244, 2220

2-((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(4-fluorophenyl)pyridine-3,5-dicarbonitrile (32)

Yield: 80%; mp: 223-225°C dec (EtOAc); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 11.9 (br s, 1H, NH imidazole, exc D\(_2\), O), 8.2 (br s, 2H, NH\(_2\), exc D\(_2\), O), 7.6 (m, 2H, Ar), 7.45 (t, 2H, Ar, J = 8.8 Hz), 7.0 (br s, 2H, CH imidazole), 4.5 (s, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3314, 3145, 2229

2-((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(p-tolyl)pyridine-3,5-dicarbonitrile (33)

Yield: 67%; mp: 259-261°C dec (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 11.85 (br s, 1H, NH imidazole, exc D\(_2\), O), 8.14 (br s, 2H, NH\(_2\), exc D\(_2\), O), 7.47 (d, 2H, Ar, J = 8 Hz), 7.38 (d, 2H, Ar, J = 8 Hz), 7.07 (br s, 1H, CH imidazole), 6.86 (s, 1H, CH imidazole), 4.5 (s, 2H, CH\(_2\)), 2.39 (s, 3H, CH\(_3\)); IR (cm\(^{-1}\)): 3318, 3129, 2213

2-((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(4-(methylthio)phenyl)pyridine-3,5-dicarbonitrile (34)

Yield: 95%; mp: 257-259°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 11.87 (br s, 1H, NH imidazole, exc D\(_2\), O), 8.12 (br s, 2H, NH\(_2\), exc D\(_2\), O), 7.47 (d, 2H, Ar, J = 8 Hz), 7.42 (d, 2H, Ar, J = 8 Hz), 7.08 (s, 1H, CH imidazole), 6.85 (s, 1H, CH imidazole), 4.5 (s, 2H, CH\(_2\)), 2.51 (s, 3H, CH\(_3\)); \(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)): 166.65, 160.26, 158.33, 143.00, 142.31, 130.19, 129.43, 125.69, 115.81, 93.54, 86.35, 27.23, 14.47; IR (cm\(^{-1}\)): 3420, 3390, 2223
2-(((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-methylpyridine-3,5-dicarbonitrile (35)

Yield: 66%; mp: 267-269°C (EtOH); $^1$H NMR (400 MHz, DMSO): 11.87 (br s, 1H, NH, exc D$_2$O), 8.12 (br s, 2H, NH$_2$, exc D$_2$O), 7.02 (br s, 2H, 2CH imidazole), 4.51 (s, 2H, CH$_2$), 2.43 (s, 3H, CH$_3$); IR (cm$^{-1}$): 3455, 3363, 2473

2-(((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-{(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (37)

Yield: 63%; mp: 226-228°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 11.86 (br s, 1H, NH imidazole, exc D$_2$O), 8.15 (br s, 2H, NH$_2$, exc D$_2$O), 7.46 (d, 2H, Ar, J = 8.2 Hz), 7.9 (d, 2H, Ar, J = 8 Hz); 7.0 (br s, 2H, CH imidazole), 4.5 (s, 2H, CH$_2$, SCH$_2$), 3.9 (d, 2H, CH$_2$, J = 8 Hz), 1.26 (m, 1H, CH cyclopropyl), 0.63-0.53 (m, 4H, CH$_2$ eq), 0.46 – 0.23 (m, 2H, CH$_2$ ax); $^{13}$C NMR (100 MHz, DMSO-d$_6$): 166.59, 160.74, 160.32, 158.58, 155.20, 143.02, 130.64, 126.03, 115.96, 115.91, 114.98, 93.64, 86.38, 72.75, 29.71, 10.53, 3.62; IR (cm$^{-1}$): 3430, 3387, 3205, 2229

2-(((1H-Imidazol-5-yl)methyl)thio)-6-amino-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (38)

Yield: 32%; mp: 186-188°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 11.97 (s, 1H, NH imidazole, exc D$_2$O), 8.04 (s, 2H, NH$_2$, exc D$_2$O), 7.59 (s, 1H, CH imidazole), 7.46 (d, J = 8.5 Hz, 2H, Ar), 7.18 (s, 1H, CH imidazole), 7.08 (d, J = 8.5 Hz, 2H, Ar), 4.40 (s, 2H, SCH$_2$), 3.90 (d, J = 6.9 Hz, 2H, OCH$_2$), 1.26 (dd, J = 15.5, 11.2 Hz, 1H, CH cyclopropane), 0.73 – 0.47 (m, 2H, CH$q_e$), 0.47 – 0.24 (m, 2H, ax); $^{13}$C NMR (100 MHz, DMSO-d$_6$): 167.24, 160.67, 160.17, 158.49, 134.33, 130.63, 126.15, 116.02, 114.94, 93.81, 86.04, 72.72, 10.52, 3.60; IR (cm$^{-1}$): 3318, 3205, 3080, 2212
2-Amino-4-(4-(cyclopropylmethoxy)phenyl)-6-(((4,5-dihydro-1H-imidazol-2-yl)methyl)thio)pyridine-3,5-dicarbonitrile (39)

Yield: 37%; mp: 260-262°C (2-propanol/EtOAc); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 7.40 (d, \(J = 8.5\) Hz, 2H, Ar), 7.19 (s, 2H,NH\(_2\), exc D\(_2\)O), 7.12 (d, \(J = 8.1\) Hz, 2H, Ar), 6.44 (s, 1H, NH, exc D\(_2\)O), 5.82 (s, 2H, SCH\(_2\)), 3.92 (d, \(J = 7.1\) Hz, 2H, CH\(_3\)), 3.77 (t, \(J = 9.3\) Hz, 2H, CH\(_2\) imidazoline), 3.23 (t, \(J = 10.4\) Hz, 2H, CH\(_2\) imidazoline), 1.36 – 1.21 (m, 1H, CH), 0.72 – 0.52 (m, 2H, CH\(_2\) eq), 0.50 – 0.29 (m, 2H, CH\(_2\) ax)

2-(((1H-Benz[d]imidazol-2-yl)methyl)thio)-6-amino-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (40)

Yield: 64%; mp: >300°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 12.3 (br s, 1H, NH, exc D\(_2\)O), 7.9-8.4 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.57 (d, 1H, \(J = 7.8\), Ar), 7.46-7.48 (m, 3H, Ar), 7.15-7.20 (m, 2H, Ar), 7.09 (d, 2H, \(J = 8.72\), Ar), 4.71 (s, 2H, CH\(_2\)), 3.91 (d, 2H, CH\(_2\), \(J = 7.08\)), 1.22-1.30 (m, 1H, CH), 0.57-0.62 (m, 2H, 2CH eq), 0.33-0.37 (m, 2H, 2CH ax); IR (cm\(^{-1}\)): 3396; 3331; 2210

2-(((1H-Pyrazol-5-yl)methyl)thio)-6-amino-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (41)

Yield: 37%; mp: 133-136°C (EtOH/CHx); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 12.7 (br s, 1H, NH, exc D\(_2\)O), 8.0 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.67 (br s, 1H, CH pyrazole), 7.47 (d, 2H, \(J = 8.28\), Ar), 7.09 (d, 2H, \(J = 8.48\), Ar), 6.32 (s, 1H, CH pyrazole), 4.49 (s, 2H, CH\(_2\)), 3.91 (d, 2H, CH\(_2\), \(J = 6.88\)), 1.22-1.26 (m, 1H, CH), 0.58-0.62 (m, 2H, 2CH eq), 0.36-0.40 (m, 2H, 2CH ax)
Experimental section

2-(((1H-1,2,4-Triazol-1-yl)methyl)thio)-6-amino-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (42)

Yield: 40%; mp: 235-237°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆):
8.99 (s, 1H), 8.75 – 7.78 (m, 2H, NH₂, exc D₂O), 8.04 (s, 1H, CH triazole), 7.48 (d, 2H, Ar, J = 8.7 Hz), 7.08 (d, 2H, Ar, J = 8.8 Hz), 6.03 (s, 2H, CH₂), 3.90 (d, 2H, CH₂, J = 7.1 Hz), 1.34 – 1.17 (m, 1H, CH cyclopropyl), 0.64 – 0.54 (m, 2H, CH₂ eq), 0.38-0.32 (m, 2H CH₂ ax); IR (cm⁻¹): 3311, 2212

2-(((1H-1,2,4-Triazol-5-yl)methyl)thio)-6-amino-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (43)

Yield: 72%; mp: 240-242°C (EtOH/Et₂O); ¹H NMR (400 MHz, DMSO-d₆): 13.9 (br s, 1H, NH, exc D₂O), 8.3 (br s, 1H, CH triazole), 8.1 (s, 2H, NH₂, exc D₂O), 7.48 (d, 2H, J = 7.80, Ar), 7.09 (d, 2H, J = 8.48, Ar), 4.59 (s, 2H, SCH₂), 3.91 (d, 2H, J = 7.08, OCH₂), 1.23-1.29 (m, 1H, CH), 0.57-0.62 (m, 2H, 2CH₂ eq), 0.34-0.37 (m, 2H, 2CH₂ ax); IR (cm⁻¹): 3441; 3323; 3207; 2212

2-(((1-Tetrazol-5-yl)methyl)thio)-6-amino-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (44)

Yield: 64%; mp: 225-227°C (EtOH/Et₂O); ¹H NMR (400 MHz, DMSO-d₆): 16.1 (br s, 1H, NH, exc D₂O), 8.0 (br s, 2H, NH₂, exc D₂O), 7.48 (d, 2H, J = 8.64, Ar), 7.09 (d, 2H, J = 8.64, Ar), 4.78 (s, 2H, SCH₂), 3.91 (d, 2H, OCH₂, J = 7.04), 1.23-1.29 (m, 1H, CH), 0.58-0.61 (m, 2H, 2CH₂ eq), 0.34-0.37 (m, 2H, 2CH₂ ax); IR (cm⁻¹): 3442; 3323; 3224; 2225; 2208
2-Amino-6-(((2-aminothiazol-4-yl)methyl)thio)-4-(4-(cyclopropylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (45)

Yield: 87%; mp: 238-240°C (EtOH/Et₂O); ¹H NMR (400 MHz, DMSO-d₆): 8.02 (br s, 2H, NH₂, exc D₂O), 7.46 (d, 2H, J= 8.68, Ar), 7.08 (d, 2H, J= 8.72, Ar), 6.98 (br s, 2H, NH₂, exc D₂O), 6.63 (s, 1H, CH thiazole), 4.31 (s, 2H, SCH₂), 3.90 (d, 2H, J= 7.04, OCH₂), 1.23-1.29 (m, 1H, CH), 0.57-0.62 (m, 2H, 2CH eq), 0.34-0.37 (m, 2H, 2CH αx); IR (cm⁻¹): 3423; 3327; 3184-3170; 2216

2-Amino-4-(4-(cyclopropylmethoxy)phenyl)-6-((pyridin-3-ylmethyl)thio)pyridine-3,5-dicarbonitrile (46)

Yield: 80%; 190-192°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 8.7 (s, 1H, Ar), 8.45 (s, 1H, Ar), 8.11 (br s, 2H, NH₂, exc D₂O), 7.94 (d, 1H, J= 5.68, Ar), 7.42 (d, 2H, J= 6.8 Hz, Ar), 7.35-7.37 (m, 1H, Ar), 7.05 (d, 2H, Ar, J= 6.9 Hz), 4.48 (s, 2H, SCH₂), 3.86 (d, 2H, J= 7.0 Hz, OCH₂), 1.19-1.22 (m, 1H, CH), 0.57-0.59 (m, 2H, 2CH eq), 0.30-0.34 (m, 2H, 2CH αx); IR (cm⁻¹): 3369; 2212

2-((6-Amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl)thio)-N-methylacetamide (47)

Yield: 71%; mp: 274-276°C (Acetone); ¹H NMR (400 MHz, DMSO-d₆): 8.18 – 7.85 (2 br s, 3H, NH₂ + NH, exc D₂O), 7.48 (d, J = 8.7 Hz, 2H, Ar), 7.10 (d, J = 8.7 Hz, 2H, Ar), 3.89 (s, 2H, SCH₂), 2.62 (d, J = 4.5 Hz, 2H, OCH₂), 1.27 (m, 1H, CH), 0.61 (q, J = 6.3 Hz, 2H, CH₂ eq), 0.36 (q, J = 4.5 Hz, 2H, CH₂ αx); ¹³C NMR (100 MHz, DMSO-d₆): 167.78, 166.54, 160.75, 160.16, 158.59, 130.64, 126.03, 115.90, 115.00, 93.69, 86.40, 72.75, 33.72, 26.50, 10.55, 3.60 IR (cm⁻¹): 3390; 3323; 3223; 2208
2-((6-Amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl)thio)-N-(hydroxymethyl)-acetamide (48)

Yield: 79%; mp: 202-204°C (MeOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 8.69 (t, 1H, NH, J= 6.28 Hz, exc D$_2$O); 7.8-8.2 (br s, 2H, NH$_2$, exc D$_2$O), 7.46-7.50 (m, 2H, Ar), 7.08-7.12 (m, 2H, Ar), 5.72 (t, 1H, OH, J = 6.42 Hz, exc D$_2$O), 4.53 (t, 2H, NCH$_2$O, J= 5.96), 3.90-3.92 (1 d + 1 s, 4H, 2CH$_2$ overlapping), 1.21-1.31 (m, 1H, CH), 0.58-0.62 (m, 2H, 2CH eq), 0.34-0.38 (m, 2H, 2CH ax); IR (cm$^{-1}$): 3394; 3319 bl; 3224; 2208; 1639

2-((6-Amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl)thio)-N-hydroxyacetamide (49)

Yield: 60%; mp: 176-178°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 10.58 (br s, 1H, OH, exc D$_2$O), 9.06 (br s, 1H, NH, exc D$_2$O), 8.01 (br s, 2H, NH$_2$, exc D$_2$O), 7.48 (d, J = 8.6 Hz, 2H, Ar), 7.10 (d, J = 8.6 Hz, 2H, Ar), 3.91 (d, J = 6.9 Hz, 2H, SCH$_2$), 3.81 (s, 2H, CH$_3$), 1.32 – 1.19 (m, 1H, CH), 0.60 (m, 2H, CH$_2$, eq), 0.42 – 0.29 (m, 2H, CH$_2$ ax); IR (cm$^{-1}$): 3649, 3331, 3223, 2208

Methyl 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl)thio)acetate (50)

Yield: 41%; mp: 205-207°C (EtOH); $^1$H NMR (400 MHz, DMSO-d$_6$): 7.9 (br s, 2H, NH$_2$, exc D$_2$O), 7.48 (d, 2H, J= 8.8, Ar), 7.1 (d, 2H, J= 8.84, Ar), 4.20 (s, 2H, SCH$_2$), 3.91 (d, 2H, J= 7.04, OCH$_2$), 3.69 (s, 3H, OCH$_3$), 1.24-1.28 (m, 1H, CH), 0.58-0.62 (m, 2H, 2CH eq), 0.35-0.38 (m, 2H, 2CH ax); IR (cm$^{-1}$): 3446; 3342; 3224; 2210; 1739
Methyl 3-(((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl)thio)methyl)benzoate (51)

Yield: 58%; mp: 158-160°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 8.10 (s, 1H, Ar), 7.90-8.35 (br s, 2H, NH, exc D\(_2\)O), 7.84-7.87 (m, 2H, Ar), 7.44-7.49 (m, 3H, Ar), 7.07 (d, 2H, J = 8.8, Ar), 4.59 (s, 2H, SCH\(_2\)), 3.90 (d, 2H, 2CH eq, J = 7.04), 3.86 (s, 3H, OCH\(_3\)), 1.23-1.27 (m, 1H, CH), 0.58-0.60 (m, 2H, 2CH eq), 0.34-0.36 (m, 2H, 2CH ax); IR (cm\(^{-1}\)): 3396; 3331; 3230; 2210; 1707

3-(((6-Amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl)thio)methyl)benzamide (52)

Yield: 47%; mp: 243-245°C (EtOAc/2-methoxyethanol); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 8.05 (br s, 2H, NH, exc D\(_2\)O), 8.00 (s, 1H, Ar), 7.96 (s, 1H, NH amide, exc D\(_2\)O), 7.77 (d, 1H, J = 7.80 Hz, Ar), 7.69 (d, 1H, J = 7.73 Hz, Ar), 7.47-7.38 (m, 4H, 2Ar + NH amide), 7.07 (d, 2H, J = 8.8, Ar), 4.54 (s, 2H, SCH\(_2\)), 3.90 (d, 2H, J = 7.04, OCH\(_2\)), 1.22-1.28 (m, 1H, CH), 0.58-0.61 (m, 2H, 2CH eq), 0.33-0.37 (m, 2H, 2CH ax); IR (cm\(^{-1}\)): 3441; 3387; 3356; 3169; 2212; 1635

2-(((1H-Imidazol-2-y1)methyl)thio)-6-amino-4-(4-(cyclobutylmethoxy)phenyl)pyridine-3,5-dicarbonitrile (53)

Yield: 20%; 214-216°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 11.87 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.10 (br s, 2H, NH, exc D\(_2\)O), 7.46 (d, 2H, J = 7.76 Hz, Ar), 7.09 (d, 2H, J = 7.76 Hz, Ar), 7.08 (s, 1H, CH imidazole), 6.96 (s, 1H, CH imidazole), 4.49 (s, 2H, SCH\(_2\)), 4.03 (d, 2H, OCH\(_3\), J = 5.96 Hz), 2.7-2.6 (m, 1H, CH cyclobutyl), 2.08-2.07 (m, 2H, CH\(_2\) cyclobutyl), 1.91-1.88 (m, 4H, 2CH\(_2\) cyclobutyl); IR (cm\(^{-1}\)): 3385; 3320; 3217; 2210
Experimental section

2-((6-Amino-3,5-dicyano-4-(4-cyclobutylmethoxy)phenyl)pyridin-2-yl)thio)acetamide (54)

Yield: 21%; mp: 244-246°C (EtOAc/CHx); \(^1\)H NMR (400 MHz, DMSO-d<sub>6</sub>): 7.99 (br s, NH<sub>2</sub>, exc D<sub>2</sub>O), 7.50 (br s, 1H, NH<sub>2</sub> amide, exc D<sub>2</sub>O), 7.49 (d, 2H, J= 8.6 Hz, Ar), 7.26 (s, 1H, NH<sub>2</sub> amide, exc D<sub>2</sub>O), 7.10 (d, 2H, J= 8.6 Hz, Ar), 4.04 (d, 2H, OCH<sub>2</sub>, J= 6.6 Hz), 4.03 (s, 2H, SCH<sub>2</sub>), 2.78- 2.67 (m, 1H, CH cyclobutyl), 1.99-1.95 (m, 4H, 2CH<sub>2</sub> cyclobutyl); IR (cm<sup>-1</sup>): 3664, 3361, 3252, 2214, 1635

2-(((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(4-isobutoxyphenyl)pyridine-3,5-dicarbonitrile (55)

Yield: 31%; mp: 230-232°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d<sub>6</sub>): 11.84 (br s, 1H, NH imidazole, exc D<sub>2</sub>O), 8.14 (s, 2H, NH<sub>2</sub>, exc D<sub>2</sub>O), 7.47 (d, 2H, J= 8.7 Hz, Ar), 7.10 (d, 2H, J= 8.6 Hz, Ar), 7.08 (s, 1H, CH imidazole), 6.85 (s, 1H, CH imidazole), 4.50 (s, 2H, SCH<sub>2</sub>), 3.84 (d, 2H, OCH<sub>2</sub>, J= 6.3 Hz), 2.09-2.02 (m, 1H, CH isopropyl), 1.00 (2 d, 6H, 2 CH<sub>3</sub> isopropyl, J= 6.7 Hz); IR (cm<sup>-1</sup>): 3387, 3315, 3215, 2208

2-((6-Amino-3,5-dicyano-4-(4-isobutoxyphenyl)pyridin-2-yl)thio)acetamide (56)

Yield: 21%; mp: 233-235°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d<sub>6</sub>): 7.99 (br s, 2H, NH<sub>2</sub>, exc D<sub>2</sub>O), 7.49 (s, 1H, NH<sub>2</sub> amide, exc D<sub>2</sub>O), 7.48 (d, 2H, J= 8.4 Hz, Ar), 7.25 (s, NH<sub>2</sub> amide, exc D<sub>2</sub>O), 7.11 (d, 2H, J= 8.8 Hz, Ar), 3.88 (s, 2H, SCH<sub>2</sub>), 3.84 (d, 2H, OCH<sub>2</sub>, J= 6.4 Hz), 2.08-2.02 (m, 1H, CH isopropyl), 1.00 (d, 6H, J= 6.8 Hz, 2CH<sub>3</sub> isopropyl); IR (cm<sup>-1</sup>): 3537; 3375; 3196; 2208; 1639

2-(((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-(4-ethoxyphenyl)pyridine-3,5-dicarbonitrile (57)

Yield: 41%; mp: 242-244°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d<sub>6</sub>): 11.84 (br s, 1H, NH imidazole, exc D<sub>2</sub>O), 8.12 (s, 2H, NH<sub>2</sub>, exc D<sub>2</sub>O), 7.47 (d, 2H, J= 8.6 Hz, Ar), 7.09 (d, 2H, J= 8.7 Hz, Ar), 7.08 (s, 1H, CH imidazole), 6.85 (s, 1H, CH imidazole), 4.49 (s, 2H, SCH<sub>2</sub>), 4.2 (q, 2H, J= 6.8 Hz, OCH<sub>3</sub>), 1.36 (t, 3H, J= 6.8 Hz, CH<sub>3</sub>); \(^13\)C NMR (100 MHz, DMSO-d<sub>6</sub>): 166.60,
Experimental section

160.65, 160.32, 158.56, 143.01, 130.67, 126.06, 115.94, 115.89, 114.94, 93.66, 86.40, 63.83, 27.23, 15.06;
IR (cm⁻¹): 3506, 3398, 3159, 2212

2-((6-Amino-3,5-dicyano-4-(4-ethoxyphenyl)pyridin-2-yl)thio)acetamide (58)

Yield: 35%; mp: 235-237°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 7.98 (br s, 2H, NH₂, exc D₂O), 7.50 (s, 1H, NH₂ amide, exc D₂O), 7.48 (d, 2H, J= 8.8 Hz, Ar), 7.23 (s, 1H, NH₂ amide, exc D₂O), 7.10 (d, 2H, J= 8.8 Hz, Ar), 4.12 (q, 2H, OCH₂, J= 7.2 Hz), 3.88 (s, 2H, SCH₂), 1.36 (t, 3H, J= 7.2 Hz, CH₃); IR (cm⁻¹): 3396, 3180, 2210, 1637

2-((6-Amino-3,5-dicyano-4-(4-ethoxyphenyl)pyridin-2-yl)thio)-N-methylacetamide (59)

Yield: 32%; mp: 262-264°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆):
7.96 (br s, 1H, NH, exc D₂O), 7.95 (br s, 2H, NH₂, exc D₂O), 7.48 (d, 2H, J= 8.5 Hz, Ar), 7.09 (d, 2H, J= 8.4 Hz, Ar), 4.12 (q, 2H, J= 7.2 Hz, OCH₂), 3.88 (s, 2H, CH₂), 1.36 (t, 3H, J= 6.8 Hz, CH₃); ¹³C NMR (100 MHz, DMSO): 167.78, 166.54, 160.15, 158.57, 130.67, 126.05, 115.93, 114.94, 63.83, 33.71, 26.56, 15.07; IR (cm⁻¹): 3394, 3223, 2210, 1637

2-(((1H-Pyrazol-5-yl)methyl)thio)-6-amino-4-(4-ethoxyphenyl)pyridine-3,5-dicarbonitrile (60)

Yield: 29%; mp: 195-197°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 12.72 (br s, 1H, NH pyrazole, exc D₂O), 8.05 (s, 2H, NH₂, exc D₂O), 7.65 (s, 1H, CH pyrazole), 7.47 (d, 2H, J= 8.3 Hz, Ar), 7.09 (d, 2H, J= 8.4 Hz, Ar), 6.31 (s, 1H, CH pyrazole), 4.48 (s, 2H, SCH₂), 4.11 (q, 2H, J= 6.8 Hz, OCH₂), 1.36 (t, 3H, J= 6.8 Hz, CH₃); IR (cm⁻¹): 3456, 3319, 3215, 2218

2-(((1H-Imidazol-2-yl)methyl)thio)-4-(4-(allyloxy)phenyl)-6-aminopyridine-3,5-dicarbonitrile (61)

Yield: 21%; mp: 216-218°C (EtOH/EtOAc); ¹H NMR (400 MHz, DMSO-d₆): 11.86 (s, 1H, NH imidazole, exc D₂O), 8.09 (s, 2H, NH₂, exc D₂O), 7.48 (d, 2H, J= 8.5 Hz, Ar), 7.12 (d, 2H, J= 8.6 Hz, Ar), 142
7.07 (s, 1H, CH \textit{imidazole}), 6.85 (s, 1H, CH \textit{imidazole}), 6.11-6.04 (m, 1H \textit{vinyl}, CH), 5.40 (d, 1H, CH \textit{vinyl}), 5.30 (d, 1H, CH \textit{vinyl}), 4.62 (d, 2H, J= 5.2 Hz, OCH\textsubscript{2}), 4.48 (s, 2H, SCH\textsubscript{2})

2-((4-{(4-allyloxy)phenyl}-6-amino-3,5-dicyanopyridin-2-yl)thio)acetamide (62)

Yield: 67%; mp: 204-206°C (EtOH); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 8.00 (s, 2H, NH\textsubscript{2}, exc D\textsubscript{2}O), 7.50 (d, J= 8.6 Hz, 3H, Ar + NH amide, partially exc D\textsubscript{2}O), 7.26 (s, 1H, NH amide, exc D\textsubscript{2}O), 7.14 (d, J= 8.7 Hz, 2H, Ar), 6.08 (ddd, J= 22.4, 10.5, 5.3 Hz, 1H, CH \textit{allyl}), 5.45 (d, J= 17.3 Hz, 1H, CH\textsubscript{2} \textit{allyl}), 5.30 (d, J= 10.5 Hz, 1H, CH\textsubscript{2} \textit{allyl}), 4.67 (d, J= 5.1 Hz, 2H, OCH\textsubscript{2}), 3.89 (s, 2H, SCH\textsubscript{2}); IR (cm\textsuperscript{-1}): 3325, 3215, 3184, 2212

2-((1H-Pyrazol-5-yl)methyl)thio)-4-{(4-allyloxy)phenyl}-6-aminopyridine-3,5-dicarbonitrile (63)

Yield: 65%; mp: 129-131°C (EtOH); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 12.75 (s, 1H, NH pyrazole, exc D\textsubscript{2}O), 8.06 (s, 2H, NH\textsubscript{2}, exc D\textsubscript{2}O), 7.59 (s, 1H, CH pyrazole), 7.49 (d, J= 8.7 Hz, 2H, Ar), 7.13 (d, J= 8.7 Hz, 2H, Ar), 6.32 (s, 1H, CH pyrazole), 6.18 – 5.95 (m, 1H, CH \textit{allyl}), 5.45 (d, J= 17.3 Hz, 1H, CH\textsubscript{2} \textit{allyl}), 5.30 (d, J= 10.4 Hz, 1H, CH\textsubscript{2} \textit{allyl}), 4.67 (d, J= 5.2 Hz, 2H, OCH\textsubscript{2}), 4.49 (s, 2H, SCH\textsubscript{2}); \textsuperscript{13}C NMR (100 MHz, DMSO): 160.25, 160.23, 158.49, 133.86, 130.67, 128.81, 126.41, 125.65, 118.35, 115.95, 115.18, 111.08, 109.95, 105.12, 93.71, 86.31, 68.84; IR (cm\textsuperscript{-1}): 3335, 3179, 2210

2-((1H-Imidazol-2-yl)methyl)thio)-6-amino-4-((2-methylallyl)oxy)phenyl)pyridine-3,5-dicarbonitrile (64)

Yield: 36%; mp: 224-226 (EtOH/EtOAc); \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}): 11.85 (br s, 1H \textit{imidazole}, exc D\textsubscript{2}O), 8.08 (s, 2H, NH\textsubscript{2}, exc D\textsubscript{2}O), 7.48 (d, 2H, J= 8.4 Hz, Ar), 7.12 (d, 2H, J= 8.4 Hz, Ar), 7.07 (s, 1H, CH \textit{imidazole}), 6.85 (s, 1H, CH \textit{imidazole}), 5.09 (s, 1H, CH\textsubscript{2} \textit{propene}), 4.99 (d, 1H, CH\textsubscript{2} \textit{propene}), 4.56 (s, 2H, SCH\textsubscript{2}), 4.49 (s, 2H, OCH\textsubscript{2}), 1.79 (s, 3H, CH\textsubscript{3}); \textsuperscript{13}C NMR (100 MHz, DMSO-d\textsubscript{6}): 167.19, 161.03, 160.95, 159.11, 143.71, 141.67, 131.75, 131.28, 126.93, 116.58, 115.93, 115.80, 113.79, 94.22, 87.00, 72.14, 27.75, 20.32; IR (cm\textsuperscript{-1}): 3404, 3377, 3336, 2210
Experimental section

2-((6-Amino-3,5-dicyano-4-(4-((2-methylallyl)oxy)phenyl)pyridin-2-yl)thio)acetamide (65)

Yield: 27%; mp: 224-226°C (EtOH); 1H NMR (400 MHz, DMSO-d$_6$):
7.99 (s, NH$_2$, exc D$_2$O), 7.51 (s, 1H, NH$_2$ amide, exc D$_2$O), 7.50 (d, 2H, J= 8.6 Hz, Ar), 7.25 (s, 1H, NH$_2$ amide, exc D$_2$O), 7.13 (d, 2H, J= 8.6 Hz, Ar), 5.10 (s, 1H, CH$_2$ propene), 4.99 (s, 1H, CH$_2$ propene), 4.57 (s, 2H, OCH$_2$), 3.88 (s, 2H, SCH$_2$), 1.08 (s, 3H, CH$_3$); IR (cm$^{-1}$): 3481, 3334, 3234, 2212, 1681

N-((4-((1H-imidazol-2-yl)methyl)thio)-6-amino-3,5-dicyanopyridin-4-yl)phenylacetamide (66) (295)

Yield: 61%; mp: 266-268°C (EtOH/CHx); 1H NMR (400 MHz, DMSO-d$_6$): 11.84 (br s, 1H, NH imidazole, exc D$_2$O), 10.22 (s, 1H, NH amide, exc D$_2$O), 8.11 (s, NH$_2$, exc D$_2$O), 7.73 (d, 2H, J= 8.3 Hz, Ar), 7.46 (d, 2H, J= 8.4 Hz, Ar), 7.05 (s, 1H, CH imidazole), 6.86 (s, 1H, CH imidazole), 4.49 (s, 2H, CH$_2$), 2.09 (s, 3H, CH$_3$); IR (cm$^{-1}$): 3387, 3211, 2214, 1683

2-((4-(4-Acetamidophenyl)-6-amino-3,5-dicyanopyridin-2-yl)thio)acetamide (67)

Yield: 19%; 279-281°C (MeOH); 1H NMR (400 MHz, DMSO-d$_6$): 10.21 (br s, 1H, NHCOCH$_3$, exc D$_2$O), 8.00 (s, 2H, NH$_2$, exc D$_2$O), 7.74 (d, 2H, J= 7.6 Hz, Ar), 7.48 (d, 2H, J= 8.4 Hz, Ar), 7.47 (s, 1H, NH$_2$ amide, exc D$_2$O), 7.24 (s, 1H, NH$_2$ amide, exc D$_2$O), 3.88 (s, 2H, CH$_2$), 2.09 (s, 3H, CH$_3$); 13C NMR (100 MHz, DMSO-d$_6$): 169.38, 169.27, 166.67, 160.10, 158.52, 141.70, 129.72, 128.44, 119.12, 115.82, 93.65, 86.29, 33.78, 24.57; IR (cm$^{-1}$): 3419, 3367, 3208, 1647

2-((6-Amino-3,5-dicyano-4-(4-hydroxyphenyl)pyridin-2-yl)thio)acetamide (142)

Yield: 24%; (EtOH); 1H NMR (400 MHz, DMSO-d$_6$): 10.08 (br s, 1H, OH, exc D$_2$O), 7.97 (br s, 2H, NH$_2$, exc D$_2$O), 7.50 (br s, 1H, NH$_2$ amide, exc D$_2$O), 7.38 (d, 2H, Ar, J= 8.4 Hz), 7.24 (s, 1H, NH$_2$ amide, exc D$_2$O), 6.92 (d, 2H, Ar, J= 8.4 Hz), 3.88 (s, 2H, CH$_2$); IR (cm$^{-1}$): 3331, 3176, 2214, 1658
**Experimental section**

**Synthesis of 2-chloro-4-(furan-2-yl)-6-((2-hydroxyethyl)thio)pyridine-3,5-dicarbonitrile (85)**

![Chemical structure](image)

Isoamyl nitrite (9.05 mmol) was added to a suspension of equimolar amount of copper (II) chloride in ACN dry (5 ml) under nitrogen atmosphere and the reaction mixture was stirred 20 min at rt. Then, A (1.51 mmol) was added and the reaction was allowed to proceed for a weekend. 1N HCl was then added and the resulting mixture was extracted with DCM (4 x 25 ml). The collected organic phases were washed with brine (2 x 20 ml) and dried (Na₂SO₄). The solvent was removed under vacuum and the yellow residue triturated with Et₂O, filtered and recrystallized by EtOH. Yield: 67 %; mp: 197-199°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 8.27 (s, 1H, Fur), 7.66 (d, J = 3.6 Hz, 1H, Fur), 6.95 (d, J = 3.6 Hz, 1H, Fur), 3.70 (t, J = 6.3 Hz, 2H, CH₂), 3.41(t, J = 6.3 Hz, 2H, CH₂); IR (cm⁻¹): 2230

**General procedure for the synthesis of compounds (21-24)**

![Chemical structure](image)

**Procedure**

An excess of the suitable primary amine (2.00 mmol) was added to a solution of 85 (1.00 mmol) in DMF dry (0.5 ml) and the mixture was stirred at rt for 5-16 h. At reaction completion, water (40 ml) was added in order to afford a solid which was filtered and washed with Et₂O (5 ml).

**Examples**

**2-(Cyclopropylamino)-4-(furan-2-yl)-6-((2-hydroxyethyl)thio)pyridine-3,5-dicarbonitrile (21)**

Yield: 91%; mp: 214-216°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 8.29 (br s, 1H, NH, exc D₂O), 8.10 (s, 1H, Fur), 7.37 (d, J = 3.4 Hz, 1H, Fur), 6.84 (s, 1H, Fur), 5.01 (t, J = 5.4 Hz, 1H, OH, exc D₂O), 3.70 (dd, J = 12.1, 6.2 Hz, 2H, O-CH₂), 3.38
Experimental section

2-(Cyclopentylamino)-4-(furan-2-yl)-6-((2-hydroxyethyl)thio)pyridine-3,5-dicarbonitrile (22)

Yield: 89%; mp: 185-187°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 8.10 (s, 1H, Fur), 7.83 (d, J = 6.8 Hz, 1H, NH, exc D₂O), 7.36 (d, J = 3.5 Hz, 1H, Fur), 6.82 (s, 1H, Fur), 5.03 (t, J = 5.3 Hz, 1H, OH, exc D₂O), 4.60 – 4.37 (m, 1H, CH cyclopentyl), 3.67 (dd, J = 12.1, 6.2 Hz, 2H, OCH₂), 3.31 (t, J = 6.2 Hz, 2H, SCH₂) 1.96 (dd, J = 10.6, 6.4 Hz, 2H, CH₂ cyclopentyl), 1.82 – 1.46 (m, 6H, 3CH₂ cyclopentyl); ¹³C NMR (100 MHz, DMSO-d₆): 168.59, 157.83, 145.56, 144.17, 116.73, 116.31, 116.07, 113.23, 89.74, 83.21, 59.98, 53.70, 33.33, 32.26, 24.29; IR (cm⁻¹): 3466, 3348, 2210

4-(Furan-2-yl)-2-((2-hydroxyethyl)thio)-6-((4-iodophenyl)amino)pyridine-3,5-dicarbonitrile (23)

Yield: 99%; mp: 218-220°C (Acetone); ¹H NMR (400 MHz, DMSO-d₆): 9.93 (br s, 1H, NH, exc D₂O), 8.15 (s, 1H, Fur), 7.72 (d, J = 8.5 Hz, 2H, Ar), 7.45 (d, J = 3.5 Hz, 1H, Fur), 7.38 (d, J = 8.6 Hz, 2H, Ar), 6.96 – 6.78 (m, 1H, Fur), 4.96 (t, J = 5.8 Hz, 1H, OH, exc D₂O), 3.58 – 3.46 (m, 2H, OCH₂), 3.16 (t, J = 6.1 Hz, 2H, SCH₂); ¹³C NMR (100 MHz, DMSO-d₆): 178.96, 168.75, 156.80, 145.36, 144.60, 138.22, 137.53, 126.43, 117.20, 115.92, 115.83, 113.36, 92.69, 89.73, 59.82, 33.60 ; IR (cm⁻¹): 3394, 3296, 2216

2-(Benzylamino)-4-(furan-2-yl)-6-((2-hydroxyethyl)thio)pyridine-3,5-dicarbonitrile (24)

Yield: 86%; mp: 171-173°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 8.80 (s, 1H, NH, exc D₂O), 8.11 (s, 1H, Fur), 7.40 (d, J = 3.5 Hz, 1H, Fur), 7.38 – 7.29 (m, 4H, Ar), 7.25 (s, 1H, Ar), 6.85 (s, 1H, Fur), 4.98 (t, J = 5.4 Hz, 1H, OH, exc D₂O), 4.70 (d, J = 5.4 Hz, 2H, NHCH₂), 3.48 (dd, J = 11.6, 5.9 Hz, 2H, OCH₂), 3.16 (t, J = 6.1 Hz, 2H, SCH₂); ¹³C NMR (100 MHz, DMSO-d₆): 168.81, 158.22, 145.51, 144.02, 139.43, 128.80, 127.35, 116.83, 116.22, 116.08, 113.29, 90.03, 83.13, 59.74, 45.18, 33.42; IR (cm⁻¹): 3344, 2208
Experimental section

Synthesis of N-(3,5-dicyano-4-(furan-2-yl)-6-(phenylthio)pyridin-2-yl)acetamide (86)

A solution of compound 74 (0.94 mmol) and dry pyridine (0.23 mmol) in acetic anhydride (21.16 mmol, 2 ml) was refluxed for 28h. After removal of the solvent under vacuum, a brown solid was obtained which was recovered with Et₂O and purified by silica gel column chromatography, eluting system CHX/EtOAc 7:3. Yield: 41%; mp: 213-215°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 10.82 (s, 1H, NH, exc D₂O), 8.23 (d, J = 1.2 Hz, 1H, Fur), 7.69 – 7.62 (m, 2H, Ar), 7.54 (m, J = 15.1, 4.6 Hz, 3H, Ar), 6.92 (d, J = 3.7, 1.8 Hz, 1H, Fur), 1.92 (s, 3H, CH₃); IR (cm⁻¹): 3358, 3147, 2216, 1705

Synthesis of N-(3,5-dicyano-4-(furan-2-yl)-6-mercaptopyridin-2-yl)acetamide (87)

Sodium sulfide (1.19 mmol) was added to a solution of compound 86 (0.36 mmol) in dry DMF (0.5 ml), and the reaction mixture was heated at 80°C for 3h. Then, 0.1N HCl (25 ml) was added followed by addition of 6N HCl up to that a huge precipitate is formed. The solid was collected by filtration, washed with water (20 ml) and Et₂O (5 ml), and used for the next reaction without any further purification. Yield: 98%; ¹H NMR (400 MHz, DMSO-d₆): 12.99 (s, 1H, SH, exc D₂O), 8.19 (s, 1H, Fur), 7.50 (d, J = 3.4 Hz, 1H, Fur), 6.89 (s, 1H, Fur), 2.23 (s, 3H, CH₃)
Synthesis of N-(3,5-dicyano-4-(furan-2-yl)-6-((2-hydroxyethyl)thio)pyridin-2-yl)acetamide (25)

Sodium hydrogencarbonate (0.53 mmol) and 2-bromoethanol (0.39 mmol) were sequentially added to a stirred solution of compound 87 (0.35 mmol) in dry DMF (1 ml). The reaction mixture was kept at rt for 3h and then water was added affording a light brown solid which was filtered and washed with water (10 ml) and Et₂O (5 ml). The crude product was purified by preparative TLC using EtOAc as eluting system and then recrystallized. Yield: 20%; mp: 164-166°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 11.13 (s, 1H, NH, exc D₂O), 8.21 (s, 1H, Fur), 7.53 (d, J = 3.6 Hz, 1H, Fur), 6.90 (dd, J = 3.6, 1.7 Hz, 1H, Fur), 5.07 (s, 1H, OH, exc D₂O), 3.70 (t, J = 6.2 Hz, 2H, OCH₂), 3.42 (t, J = 6.2 Hz, 2H, SCH₂), 2.21 (s, 3H, CH₃); ¹³C NMR (100 MHz, DMSO-d₆): 169.37, 168.55, 155.12, 144.96, 144.30, 118.22, 115.22, 115.09, 113.76, 113.27, 98.59, 94.79, 59.69, 33.87, 24.06; IR (cm⁻¹): 3321, 2218, 1718

Synthesis of 3,6-diamino-5-cyano-4-(4-(cyclopropylmethoxy)phenyl)thieno[2,3-b]pyridine-2-carboxamide (68)

A solution of BAY-606583 (0.71 mmol) and potassium hydroxide (1.42 mmol) in absolute EtOH (10 ml) was refluxed for 3h. After completion of the reaction, iced-water (30 ml) was added and the mixture was neutralized with 5N HCl, then the green solid was filtered and washed with water (10 ml) and Et₂O (5 ml). Yield: 85%; mp: 260-262°C (1,4-Dioxane); ¹H NMR (400 MHz, DMSO-d₆): 7.42 (d, 2H, J= 8 Hz, Ar); 7.28 (br s, 2H, NH₂, exc D₂O); 7.12 (d, 2H, J= 8.4 Hz, Ar); 6.97 (br s, 2H, NH₂, exc D₂O); 5.70 (s, 2H, NH₂ amide, exc D₂O); 3.91 (d, 2H, J= 6.8 Hz, OCH₂); 1.28-1.27 (m, 1H, CH), 0.66-0.51 (m, 2H, CH₂ eq), 0.44-0.27 (m, 2H, CH₂ ax); ¹³C NMR (100 MHz, DMSO-d₆): 167.23, 163.83, 160.08, 158.93, 152.75, 146.75, 130.05, 125.74, 116.43, 115.29, 114.78, 93.36, 90.93, 72.71, 10.56; IR (cm⁻¹): 3466, 3452, 3346, 3315, 3136, 2214, 1629.
Synthesis of 3,6-diamino-5-cyano-4-(4-(cyclobutylmethoxy)phenyl)thieno[2,3-b]pyridine-2-carboxamide (69)

Equimolar amount of potassium carbonate and the suitable alkyl halide (4.61 mmol) were sequentially added to a solution of compound 142 (3.07 mmol) in dry acetone (20 ml). The mixture was heated at reflux for 98h. Then, the mixture was cooled to rt, filtered and the insoluble material was extracted with acetone (3 x 20 ml). The collected organic phases were evaporated under vacuum affording a solid which was recovered with water (40ml) and the mixture was extracted with DCM (4 x 25 ml), washed with brine (2 x 25 ml) and dried (Na₂SO₄). The solid obtained after removal of the solvent was purified by preparative TLC, eluting system EtOAc/CHX 6:4. Yield: 18%; mp: 260-262°C (Dioxane); ^1H NMR (400 MHz, CDCl₃): 7.37 (d, 2H, J= 8.4 Hz, Ar); 7.10 (d, 2H, J= 8.4 Hz, Ar); 5.90 (s, 2H, NH₂ amide, exc D₂O); 5.49 (s, 2H, NH₂, exc D₂O); 5.22 (s, 2H, NH₂, exc D₂O); 4.03 (d, 2H, J= 6.4 Hz, OCH₃); 2.88-2.82 (m, 1H, CH); 2.20-2.17 (m, 2H, CH₂); 2.05-1.90 (m, 4H, 2CH₂); 13C NMR (100 MHz, DMSO-d₆): 167.26, 163.82, 160.25, 158.92, 152.78, 146.78, 130.03, 125.72, 116.41, 115.31, 114.75, 93.31, 90.91, 72.02, 34.39, 18.56; IR (cm⁻¹): 3462, 3323, 3216, 3190, 2220, 1639

Synthesis of 2-amino-4-(furan-2-yl)-6-methoxypyridine-3,5-dicarbonitrile (83) (374)

A solution of sodium methoxide (30.45 mmol) in MeOH (20 ml) was rapidly added to a solution of 2-furaldehyde (20.82 mmol), malononitrile (20.82 mmol) and tetrabutylammonium fluoride (2.08 mmol) in MeOH (10 ml) at rt. Then, further malononitrile (20.82 mmol) was added and the mixture was allowed to reflux for 3h. After completion, the reaction mixture was filtered over celite pad and the filtrate evaporated under vacuum. The crude residue was purified by silica gel column chromatography using DCM as eluent. Yield: 7%; mp: 205-207°C (EtOH); ^1H NMR (400 MHz, DMSO-d₆): 8.09 (s, 1H, Fur), 8.05 (br s, 2H, NH₂, exc D₂O), 7.38 (s, 1H, Fur), 6.83 (s, 1H, Fur), 3.97 (s, 3H, CH₃); IR (cm⁻¹): 3406, 3345, 3244, 2222
Experimental section

Synthesis of 2-amino-4-(furan-2-yl)-6-hydroxypyridine-3,5-dicarbonitrile (84)

Compound 83 (1.41 mmol) was suspended in a mixture of glacial acetic acid (4 ml) and concentrated HCl (1 ml) and heated at 100°C for 2 h. Then, the reaction mixture was cooled at rt affording a precipitate which was collected by filtration, washed with water (20 ml) and Et₂O (5 ml) and dried in oven at 60°C. Yield: 81%; mp: 186-188°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 11.85 (br s, 1H, OH, exc D₂O), 8.08 (d, J = 1.1 Hz, 1H, Fur), 7.72 (br s, 2H, NH₂, exc D₂O), 7.36 (d, J = 3.6 Hz, 1H, Fur), 6.82 (dd, J = 3.6, 1.7 Hz, 1H, Fur); IR (cm⁻¹): 3319, 3199, 2226, 2212

Synthesis of 2-amino-4-(furan-2-yl)-6-(2-hydroxyethoxy)pyridine-3,5-dicarbonitrile (20)

A sealed tube containing a mixture of compound 84 (0.53 mmol), 2-bromoethanol (0.58 mmol) and sodium hydrogencarbonate (0.79 mmol) in dry DMF (1 ml) was irradiated at 80°C in a microwave reactor for 3 h. After completion, water (30 ml) was added and the mixture was extracted with DCM (3 x 25 ml). The collected organic layers were washed with brine (2 x 20 ml), dried (Na₂SO₄) and evaporated under reduced pressure to yield a pale green solid that was purified by preparative TLC using DCM/MeOH 9:1 as eluent. Yield: 56%; mp: 207-209°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 8.09 (s, 1H, Fur), 8.03 (br s, 2H, NH₂, exc D₂O), 7.38 (d, J = 3.5 Hz, 1H, Fur), 6.93 – 6.69 (m, 2H, Fur), 4.91 (t, J = 5.3 Hz, 1H, OH, exc D₂O), 4.54 – 4.17 (m, 2H, OCH₂), 3.73 (dd, J = 10.0, 5.2 Hz, 2H, CH₂OH; ¹³C NMR (100 MHz, DMSO-d₆): 166.70, 162.20, 146.50, 145.86, 116.47, 116.36, 115.79, 113.19, 79.48, 69.52, 64.57, 59.40
Synthesis of 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (82) (301)

![Diagram of 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile](image)

Malononitrile (15.13 mmol) was added in small portions to a solution of trimethylorthobenzoate (7.53 mmol) in dry pyridine (0.6 ml). The mixture was heated at 100°C for 6 h and then cooled in ice bath. Concentrated HCl (2.7 ml) was carefully added and the mixture was heated again at 100°C for 2h. After cooling to rt, the resulting precipitate was collected by filtration, suspended in a saturated aqueous solution of sodium hydrogencarbonate, re-filtered and finally washed with water (10 ml). Yield: 61%; mp: >300°C (MeOH); \(^1^H\) NMR (400 MHz, DMSO-d6): 7.57-7.61 (m, 5H, Ar), 8.27 (br s, 1H, NH, exc D\(_2\)O), 8.68 (br s, 1H, NH, exc D\(_2\)O); IR (cm\(^{-1}\)): 3356, 3179, 2225, 1660, 1564

General procedure for synthesis of 6-amino substituted compounds 19,36

![Diagram of General procedure for synthesis of 6-amino substituted compounds](image)

Procedure

Equimolar amount of the suitable primary amine and triethylamine (2.00 mmol) were added to a solution of compound 82 (1.00 mmol) in a mixture THF/EtOH (2:1, 10.5 ml) and the reaction was refluxed for 1.5-6 h. Then, water (20 ml) was added affording a precipitate which was collected by filtration and washed with Et\(_2\)O (5 ml).

Examples

2-Amino-6-((2-hydroxyethyl)amino)-4-phenylpyridine-3,5-dicarbonitrile (19)

![Diagram of 2-Amino-6-((2-hydroxyethyl)amino)-4-phenylpyridine-3,5-dicarbonitrile](image)

Yield: 22%; mp: 230-231°C (EtOH); \(^1^H\) NMR (400 MHz, DMSO-d6): 7.55-7.53 (m, 3H, Ar), 7.47-7.45 (m, 2H, Ar), 7.37 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.20 (t, J = 5.1 Hz, 1H, NH, exc D\(_2\)O), 4.74 (t, J = 5.2 Hz, 1H, OH, exc D\(_2\)O), 3.54 (q, J = 10.8 Hz, 2H, NHCH\(_2\)), 3.47 (t, J = 5.8 Hz, 2H, OCH\(_3\)); IR (cm\(^{-1}\)): 3472, 3345, 3223, 2204.
2-(((1H-Imidazol-2-yl)methyl)amino)-6-amino-4-phenylpyridine-3,5-dicarbonitrile (36)

Yield: 57%; mp: 208-210°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 11.64 (br s, 1H, NH imidazole, exc D\(_2\)O), 8.38 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.88 (t, J = 5.5 Hz, 1H, NH, exc D\(_2\)O), 7.61-7.45 (m, 5H, Ar), 7.04 (s, 1H, CH imidazole), 4.56 (d, J = 5.5 Hz, 2H, CH\(_2\)); IR (cm\(^{-1}\)): 3360, 3322, 3186, 2207.

Synthesis of 2-amino-4-(furan-2-yl)-6-((2-hydroxyethyl)amino)pyridine-3,5-dicarbonitrile (17)

A solution of compound 74 (0.47 mmol) and 2-aminoethanol (1.88 mmol) in DMF (2 ml) was heated at 100°C for 4h. After cooling at rt, water (10 ml) was added and the resulting precipitate was filtered and washed with water (10 ml) and Et\(_2\)O (5 ml). Yield: 92%; mp: 230-232°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 8.03 (d, J = 0.96 Hz, 1H, Fur), 7.33 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.24 (d, J = 0.56 Hz, 1H, Fur), 7.19 (t, J = 5.36 Hz, 1H, NH, exc D\(_2\)O), 6.78 (dd, J = 3.5, 1.7 Hz, 1H, Fur), 4.76 (t, 1H, OH, exc D\(_2\)O), 3.54 (q, J = 11 Hz, 2H, NHCH\(_2\)), 3.47 (q, J = 11 Hz, 2H, OCH\(_2\)); IR (cm\(^{-1}\)): 3334, 2204.

Synthesis of 2-amino-6-((2-hydroxyethyl)amino)-4-(thiophen-2-yl)pyridine-3,5-dicarbonitrile (18)

A solution of compound 76 (0.60 mmol) and 2-aminoethanol (2.40 mmol) in DMF (2 ml) was heated at 100°C for 2h. After cooling at rt, water (10 ml) was added and the resulting precipitate was filtered, washed with water (10 ml) and Et\(_2\)O (5 ml) and recrystallized from EtOH. Yield: 88%; mp: 220-222°C (EtOH); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): 7.87 (d, J = 4.7 Hz, 1H, CH thiophene), 7.47 (d, J = 1.6 Hz, 1H, CH thiophene), 7.42 (br s, 2H, NH\(_2\), exc D\(_2\)O), 7.26-7.22 (m, 2H, NH + CH thiophene, partially exc D\(_2\)O), 4.74 (t, J = 5.1 Hz, 1H, OH, exc D\(_2\)O), 3.54 (q, J = 10.9 Hz, 2H, NHCH\(_2\)), 3.48 (q, J = 10.9, 2H, OCH\(_2\)); IR (cm\(^{-1}\)): 3491, 3451, 3338, 2202.
**Synthesis of ethyl 2-cyano-3,3-bis(methylthio)acrylate (143)** (375)

Potassium carbonate (100 mmol) was added to a solution of ethyl cyanoacetate (100 mmol) in DMF dry (95 ml) and the mixture was allowed to stir at rt for 3h under nitrogen atmosphere. Then, carbon disulfide (300 mmol) was slowly dropped in the reaction mixture and the suspension stirred again for 2h before adding methyl iodide (200 mmol) and stirring at rt overnight. Then, addition of water (400 ml) while strong stirring yielded a solid that was collected by filtration and dried in desiccator. Yield: 88%; $^1$H NMR (400 MHz, DMSO-$d_6$): 4.21 (q, $J$ = 7.1 Hz, 2H CH$_2$), 2.73 (s, 3H, SCH$_3$), 2.62 (s, 3H, SCH$_3$), 1.25 (t, $J$ = 7.1 Hz, 3H CH$_3$); IR (cm$^{-1}$): 3262, 2207, 1697

**Synthesis of 4-(methylthio)-2-oxo-6-phenyl-2H-pyran-3-carbonitrile (144)** (317)

Compound 143 (4.87 mmol) and acetophenone (6.33 mmol) were sequentially added to a suspension of potassium hydroxide (5.84 mmol) in DMSO dry (20 ml). The reaction mixture was allowed to stir at rt for 5h. When the reaction was completed, iced water (70 ml) was added and the mixture was stirred for 2h to afford a yellow solid that was collected by filtration, washed with water (100 ml) and dried in oven at 60°C overnight. Yield: 56%; $^1$H NMR (400 MHz, DMSO-$d_6$): 8.06 (d, $J$ = 7.3 Hz, 2H, Ar), 7.74 – 7.49 (m, 3H, Ar), 7.26 (s, 1H, Ar), 2.85 (s, 3H, SCH$_3$).
**Synthesis of 2-(5-phenyl-1H-pyrazol-3-yl)acetonitrile (145)** (376)

![Chemical structure of 145](image_url)

A solution of compound 144 (1.43 mmol) and hydrazine monohydrate (2.16 mmol) in pyridine (25 ml) was heated at reflux for 2.5h. The solvent was then removed under vacuum and the residue was recovered with CHCl₃ (50 ml). The organic phase was washed with water (2 x 25 ml), dried (Na₂SO₄) and the solvent evaporated under reduced pressure. The brown residue was triturated with petroleum ether (5 ml) and EtOH (1 drop) and recovered by filtration. Yield: 71%; $^1$H NMR (400 MHz, DMSO-d$_6$): 13.31 (br s, 1H, exc D$_2$O, NH), 7.74 (d, $J$ = 7.5 Hz, 2H, Ar), 7.46 (t, $J$ = 7.3 Hz, 2H, Ar), 7.41 – 7.32 (m, 1H, Ar), 6.68 (s, 1H, CH pyrazole), 4.00 (s, 2H, CH$_2$). IR (cm$^{-1}$): 3135, 3113, 2250.

**Synthesis of tert-butyl 3-(cyanomethyl)-5-phenyl-1H-pyrazole-1-carboxylate (146)**

![Chemical structure of 146](image_url)

Dry TEA (5.97 mmol), dimethylaminopyridine (0.35 mmol) and BOC-anhydride (2.63 mmol) were added to a stirred solution of compound 145 (1.76 mmol) in dry ACN at 0°C. After reagent addition, the mixture was allowed to warm to rt and stirred for 1h. Then, brine (10 ml) was added and the mixture extracted with EtOAc (3 x 5 ml). The collected organic phases were dried (Na₂SO₄) and the solvent removed under vacuum, leading to a red residue which was triturated with Et₂O and filtered to yield a light brown solid, used for the next step without any further purification. Yield: 86%; $^1$H NMR (400 MHz, DMSO-d$_6$): 7.90 (d, $J$ = 7.0 Hz, 2H, Ar), 7.53 – 7.37 (m, 3H, Ar), 7.08 (s, 1H, CH pyrazole), 4.39 (s, 2H, CH$_2$), 1.63 (s, 9H, CH$_3$ t-Bu).
Experimental section

Synthesis of tert-butyl 3-(1-cyano-2,2-bis(methylthio)vinyl)-5-phenyl-1H-pyrazole-1-carboxylate (147)

Compound 146 (3.52 mmol) was dissolved in dry DMSO (20 ml) at 0°C and carbon disulfide (7.94 mmol), methyl iodide (14.12 mmol) were added and followed by the portion wise addition of sodium hydride (7.06 mmol, 60% in mineral oil) while keeping the temperature under 10°C. After 30min at 5°C, the mixture was warmed to rt and stirred for 3h. Then, the mixture was transferred into a 250 ml round bottomed flask and a saturated solution of ammonium chloride (20 ml) was added, followed by addition of cold water (150 ml). The mixture was stirred until an orange gluey solid is formed, then the aqueous layer was removed by decantation. The residue was suspended in 2-propanol and after few minutes of stirring an off-white solid precipitated which was collected by filtration. Yield: 61%; 1H NMR (400 MHz, DMSO-d$_6$): 7.91 (d, $J = 6.9$ Hz, 2H, Ar), 7.55-7.37 (m, 3H, Ar), 7.31 (s, 1H, CH pyrazole), 2.65 (s, 3H, SCH$_3$), 2.39 (s, 3H, SCH$_3$), 1.60 (s, 9H, CH$_3$ t-Bu).

Synthesis of 3,3-bis(methylthio)-2-(5-phenyl-1H-pyrazol-3-yl)acrylonitrile (148)

Compound 147 (0.77 mmol) was allowed to stir in of a 10% solution of trifluoroacetic acid in DCM dry (25 ml) for 1.5h. Then, the solvents were removed under reduced pressure affording a brown solid that was triturated with a saturated solution of sodium bicarbonate (25 ml), collected by filtration and dried in oven at 60°C. Yield: 93%; 1H NMR (400 MHz, DMSO-d$_6$): 13.70 (br s, 1H, NH), 7.79 (d, $J = 7.5$ Hz, 2H, Ar), 7.47 (t, $J = 7.5$ Hz, 2H, Ar), 7.38 (t, $J = 7.4$ Hz, 1H, Ar), 7.04 (s, 1H, CH pyrazole), 2.59 (s, 3H, SCH$_3$), 2.50 (s, 3H, overlapping with DMSO-d$_6$, SCH$_3$).
Synthesis of 7-amino-5-(methylthio)-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile (149)

A solution of compound 148 (2.78 mmol), cyanamide (8.35 mmol) and paratoluensulfonic acid (2.78 mmol) in NMP (3 ml) was irradiated with microwave at 160°C for 1h (4 x 15 min cycle). At the end of each irradiation cycle, cyanamide (8.35 mmol) was added. The reaction mixture was diluted with water (200 ml) and stirred for 5h to afford a solid that was collected by filtration and extracted with acetone (50 ml). The solvent was evaporated under reduced pressure affording a yellow solid that was recovered with petroleum ether (10 ml). Yield: 89%; mp: 104-106°C (MeOH); 1H NMR (400 MHz, DMSO-d$_6$): 8.95 (br s, 1H, NH$_2$, exc D$_2$O), 8.52 (br s, 1H, NH$_2$, exc D$_2$O), 8.10 (d, $J$ = 6.9 Hz, 2H, Ar), 7.48 (m, 3H, Ar), 6.96 (s, 1H, CH pyrazole), 2.63 (s, 3H, SCH$_3$). IR (cm$^{-1}$): 3319, 2204.

Synthesis of 7-amino-5-(methylsulfonyl)-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile (150)

3-Chloroperoxybenzoic acid (4.53 mmol) was added to a solution of compound 149 (1.81 mmol) in DCM dry (40 ml) at 0°C. The mixture was allowed to stir at 0 – 5°C for 2h and then at rt for further 5h. The solvent was then removed under vacuum at rt and a 5% sodium bicarbonate solution (25 ml) was added to the residue. The mixture was stirred for 20 min and the resulting solid was filtered and washed with water (20 ml) and petroleum ether (10 ml) affording a brown powder which was dried in oven at 60°C. Yield: 86%; (MeOH); 1H NMR (400 MHz, DMSO-d$_6$): 9.35 (br s, 1H, NH$_2$, exc D$_2$O), 8.90 (br s, 1H, NH$_2$, exc D$_2$O), 8.18 (dd, $J$ = 11.3, 4.4 Hz, 2H, Ar), 7.61 – 7.41 (m, 4H, Ar + pyrazole), 3.36 (s, 3H, SO$_2$CH$_3$).
**General procedure for the synthesis of compound (70-73)**

![Chemical structure of compound]

**Procedure**

A solution of compound 150 (1.00 mmol), the suitable primary amine (7.1 mmol) and N,N-diisopropylethylamine (4.1 mmol) in anhydrous dioxane (10 ml) was heated at 90°C for 24-48 h. After completion, the reaction mixture was allowed to cool at rt and water (40 ml) was added. Extraction was performed with EtOAc (3 x 20 ml) and the collected organic phases were washed with brine (2 x 20 ml), dried (Na₂SO₄), and evaporated under vacuum to yield the final compounds which were purified by silica gel column chromatography or by recrystallization.

**Examples**

7-Amino-5-(benzlamino)-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile (70)

![Chemical structure of compound 70]

Yield: 84%; mp: 245-247°C (EtOH); ¹H NMR (400 MHz, DMSO-d₆): 8.43 (br s, 1H, NH₂, exc D₂O), 8.02 (dt, J = 3.3, 1.9 Hz, 2H, Ar), 7.98 (br s, 1H, NH₂, exc D₂O), 7.79 (t, J = 6.1 Hz, 1H, NH, exc D₂O), 7.51 – 7.39 (m, 3H, Ar), 7.39 – 7.28 (m, 4H, Ar), 7.28 – 7.18 (m, 1H, Ar), 6.55 (s, 1H, CH); ¹³C NMR (100 MHz, DMSO-d₆): 157.90, 155.73, 148.69, 144.17, 140.68, 132.47, 129.61, 129.15, 128.66, 127.80, 127.10, 126.93, 118.03, 90.60, 60.25, 44.42; IR (cm⁻¹): 3222, 3136, 2251

7-Amino-5-((3-methoxybenzyl)amino)-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile (71)

![Chemical structure of compound 71]

Yield: 79%; mp: 198-200°C (MeOH); ¹H NMR (400 MHz, DMSO-d₆): 8.43 (br s, 1H, NH₂, exc D₂O), 8.07 – 7.99 (m, 2H, Ar), 7.96 (br s, 1H, NH₂, exc D₂O), 7.75 (t, J = 6.2 Hz, 1H, NH, exc D₂O), 7.53 – 7.37 (m, 3H, Ar), 7.29 – 7.19 (m, 1H, Ar), 6.94 (d, J = 7.4 Hz, 2H, Ar), 6.80 (dd, J = 9.1, 1.6 Hz, 1H, Ar), 6.55 (s, 1H, CH), 4.59 (d, J = 6.1 Hz, 2H, CH₂), 3.74 (s, 3H, CH₃); ¹³C NMR (100 MHz, DMSO-d₆): 159.68, 157.86, 155.73, 148.66, 144.14, 142.31, 132.46, 129.76, 129.60, 129.16, 126.93, 119.96, 118.02, 113.59, 112.44, 90.62, 55.40, 44.37; IR (cm⁻¹): 3222, 3136, 2251
Experimental section

7-Amino-5-((furan-2-ylmethyl)amino)-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile (72)

Column chromatography eluent CHx 1 : EtOAc 1; Yield: 55%; mp: 206-208°C (EtOAc); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 8.49 (br s, 1H, NH\(_2\), exc D\(_2\)O), 8.19 – 7.86 (m, 3H, Ar + NH\(_2\), partially exc D\(_2\)O), 7.67 (t, \(J = 5.9\) Hz, 1H, NH, exc D\(_2\)O), 7.57 (d, \(J = 0.9\) Hz, 1H, Fur), 7.52 – 7.36 (m, 3H, Ar), 6.56 (s, 1H, CH), 6.40 (dd, \(J = 3.1, 1.8\) Hz, 1H, Fur), 6.32 (d, \(J = 3.0\) Hz, 1H, Fur), 4.59 (d, \(J = 5.8\) Hz, 2H, CH\(_2\)); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): 157.64, 155.73, 153.45, 148.63, 144.05, 132.43, 129.61, 129.15, 126.92, 117.84, 110.91, 107.46, 90.14, 38.09; IR (cm\(^{-1}\)): 3304, 3188, 3150, 2199

7-Amino-5-(phenethylamino)-2-phenylpyrazolo[1,5-c]pyrimidine-4-carbonitrile (73)

Yield: 60%; mp: 232-234°C (MeOH); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): 8.43 (br s, 1H, NH\(_2\), exc D\(_2\)O), 8.02 (d, \(J = 7.6\) Hz, 2H, Ar), 7.97 (br s, 1H, NH\(_2\), exc D\(_2\)O), 7.45 (dt, \(J = 21.6, 7.1\) Hz, 3H, Ar), 7.37 – 7.15 (m, 6H, Ar + NH, partially exc D\(_2\)O), 6.53 (s, 1H, CH), 3.61 (dd, \(J = 14.6, 6.2\) Hz, 2H, N-CH\(_2\)), 2.95 – 2.82 (m, 2H, CH\(_2\)-Ph); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): 157.88, 155.65, 148.68, 144.21, 139.97, 132.50, 129.57, 129.24, 129.13, 128.75, 126.90, 126.52, 118.09, 90.27, 43.20, 36.05; IR (cm\(^{-1}\)): 3333, 3157, 2193
10. Visiting period at Universität Bonn

During my Ph.D. course I spent six months at Universität Bonn, Pharmazeutische Chemie I department in the group of Professor Christa E. Müller under the supervision of Dr. Thanigaimalai Pillayiar. For many years, the group of Professor Müller has been specialized on the study of GPCRs and in particular on purinergic signaling. Recently, one of the most important aim of the group has been the deorphanization of the so called orphan receptors. In particular, the completion of the human genome sequencing project has identified approximately 720 genes that belong to the GPCR superfamily. Approximately half of these genes are thought to encode sensory receptors. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving 150 so-called orphan GPCRs with no known ligand or function (377). The identification of ligands active at orphan GPCRs has been achieved through the development of a number of experimental approaches, including the screening of putative small molecule and peptide ligands, reverse pharmacology, and the use of bioinformatics to predict candidate ligands (378). Following expression, candidate ligands are screened against the receptor to identify molecules capable of specific regulation of that receptor. Such ligands can include tissue extracts, expressed or purified proteins or small peptides, natural and synthetic small molecules, and lipids. Once a candidate ligand is identified, further studies are performed to confirm that the activity of the ligand is specific to the receptor. In recent years, small-molecule, peptide, protein, and lipid ligands have been identi- fied for many orphan GPCRs. However, approximately 160 orphan GPCRs remain for which there is no known ligand. Efforts continue to identify ligands for these receptors, however, it is now necessary to investigate alternative strategies. Such studies may involve the identification of new ligands using bioinformatics to predict likely peptide or protein ligands. It may be that the remaining orphan GPCRs are atypical in that they do not signal through heterotrimeric G-proteins. If this is the case, then the assays used to date, which all rely upon G-protein activation of a signal transduction cascade, cannot be used for these receptors. In support of this hypothesis, there is an emerging literature describing G-protein-independent signaling by GPCRs. The identification of ligands at such receptors depends on the development of new assays. It is also possible that not all of these receptors will have ligands; receptors have been identified that act as trafficking factors, are believed to act as ligand sinks, and exhibit constitutive signaling in the absence of ligand. The challenge in the coming years is to identify the biological function of the remaining orphan GPCRs.

MrgX-4 is an orphan G-protein coupled receptor (GPCR) that belongs to a family called MAS-related genes (Mgrs) (379). This GPCRs family takes its name from the first receptor discovered, MAS1 oncogene that is activated by binding angiotensin (1-7): it mediates a range of effect mostly opposed to the activation of angiotensin-II activated angiotensin receptor. MrgX-4 belongs to a class of receptors (MrgX1-7) which seems
to be selectively expressed in the small-diameter sensory neurons of dorsal root ganglia: this is one of the nowadays known evidences that lead to expect a promising involvement in neuropathic pain for this class of GPCRs. Besides, this family has not been found in rodents, making a deepening of the physiopathological role more complicated. MrgX receptors were cloned from rhesus monkey and functionally characterized alongside their human orthologues. Most of the human and rhesus MrgX receptors displayed high constitutive activity in a cellular proliferation assay. Proliferative responses mediated by human or rhesus MrgX-1, or rhesus MrgX-2 were partially blocked by pertussis toxin (PTX). Proliferative responses mediated by rhesus MrgX-3 and both human and rhesus MrgX-4 were PTX insensitive. These results indicate that human and rhesus MrgX-1 and MrgX-2 receptors activate both Gq- and Gi-regulated pathways, while MrgX-3 and MrgX-4 receptors primarily stimulate Gq-regulated pathways (379). The aim of my work was synthesizing agonist at MrgX-4 through a synthetic pathway partially shown in figure I. Neither complete synthesis (scheme 23), nor the twelve synthesized compounds and related pharmacological data can be showed in this thesis, in order to safeguard a future patent application or future publications.

(a) HMDS, (NH₄)₂SO₄, reflux, 60-70 °C, I₂, alkylbromide, 120 °C, 2 h, NaHCO₃, 70-75%; (b) Aq. AcOH, HNO₃, 60-65 °C, 68-75%; (c) Ac-NH₃ (15% or 25%), Na₂S₂O₃, 70 °C, 50-70%; (d) R-COOH, ethylcarbodiimide, HCl, Methanol, 24 h, rt, 70-85%; (e) 3-iodopropyl acetate, K₂CO₃, DMF, rt, 24 h, 60-70%; (f) P₂O₅, DMF, 100 °C, 10-30 min, 70-80%; (g) CH₂I, K₂CO₃, DMF, rt, 24 h, 80-90%; (h) KOH/methanol/H₂O, 90-100%; (i) PO(OMe)₃/POCl₃, rt, 30 min, H₂O, 60-80%.

Scheme 23
References


Bibliography


Bibliography


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