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Medicinal Chemistry of P2 and Adenosine Receptors: Common Scaffolds Adapted for Multiple Targets

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Abstract

Prof. Geoffrey Burnstock originated the concept of purinergic signaling. He demonstrated the interactions and biological roles of ionotropic P2X and metabotropic P2Y receptors. This review paper traces the historical origins of many currently used antagonists and agonists for P2 receptors, as well as adenosine receptors, in early attempts to identify ligands for these receptors – prior to the use of chemical libraries for screening. Rather than presenting a general review of current purinergic ligands, we focus on common chemical scaffolds (privileged scaffolds) that can be adapted for multiple receptor targets. By carefully analyzing the structure activity relationships, one can direct the selectivity of these scaffolds toward different receptor subtypes. For example, the weak and non-selective P2 antagonist reactive blue 2 (RB-2) was derivatized using combinatorial synthetic approaches, leading to the identification of selective $P2Y_2$, $P2Y_4$, $P2Y_{12}$ or P2X2 receptor antagonists. A P2X4 antagonist NC-2600 is in a clinical trial, and A_3 adenosine agonists, show promise for chronic pain. P2X7 antagonists have been in clinical trials for depression (JNJ-54175446), inflammatory bowel disease (IBD), Crohn's disease, rheumatoid arthritis, inflammatory pain and chronic obstructive pulmonary disease (COPD). P2X3 antagonists are in clinical trials for chronic cough, and an antagonist named after Burnstock, gefapixant, is expected to be the first P2X3 antagonist filed for approval. We are seeing that the vision of Prof. Burnstock to use purinergic signaling modulators, most recently at P2XRs, for treating disease is coming to fruition.

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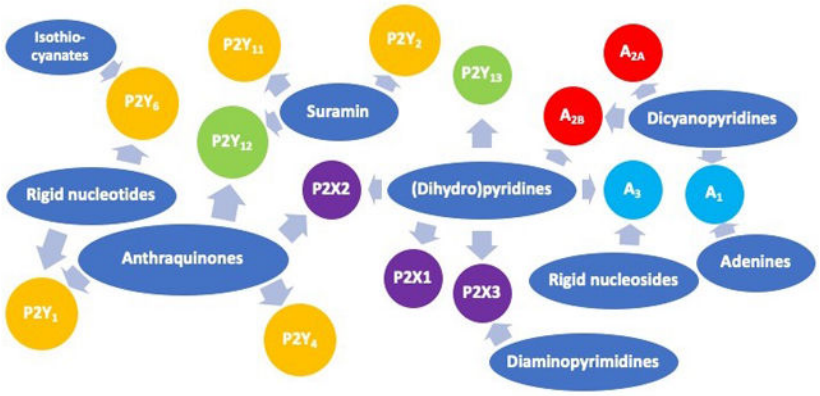
Graphical Abstract



Decades-long exploration of structure-activity relationships of known P2/adenosine ligand scaffolds and eventually novel scaffolds has directed the selectivity toward various receptor subtypes, leading to receptor probes and clinical drugs.

Keywords

P2X receptor; P2Y receptor; adenosine receptor; purinergic agonist; purinergic antagonist; scaffold

1. Introduction

This paper is dedicated to the memory of our inspirational friend and colleague Prof. Geoffrey Burnstock (1929–2020), who began the field of purinergic signaling and guided it to ever greater achievements throughout his long, active research career of roughly six decades. He discovered non-adrenergic, non-cholinergic nerves and found their neurotransmitter to be ATP [1]. Although not a medicinal chemist himself, he recognized the need for tool compounds in order to adequately characterize a receptor, and especially because his own proposal of purinergic receptors [1,2] was initially met with skepticism and even hostility. In 1976, Burnstock addressed the reluctance of biologists to accept his concepts of co-transmission (ATP coreleased with other transmitters), and purinergic signaling in general, by stating: “Gifted and meticulous workers will perform remarkable contortions to fit their data into accepted dogma, especially if established by powerful and brilliant personalities at the forefront of the field. They will often dismiss or ignore data that fall outside interpretation by current theory, searching hard for technical or artefactual explanations. Once a new attitude becomes acceptable, then the same data can be miraculously redeployed to support it.” [3]. Burnstock’s concepts proposed in the early 1970s were eventually fully accepted, but only after two decades.

Thus, selective agonists and antagonists that could distinguish effects of extracellular ATP (Figure 1A) in different tissues, e.g. inhibitory vs. stimulatory effects on smooth muscle, were desperately needed to validate the concept of purinergic receptors prior to their cloning in the 1990s. Eventually it became clear that there were two families of purinergic P2

receptors, eight P2Y (GPCRs, numbered 1, 2, 4, 6, 11, 12, 13 and 14) and seven P2X (ligand-gated ion channels, numbered 1–7) receptors [4–6]. Burnstock's early studies with ATP as a neurotransmitter included a search for P2 receptor antagonists, by analogy to competitive antagonists, theophylline and other alkylxanthines, at the adenosine (P1) receptors. He was limited to available chemicals, and the first compounds he and others discovered to block ATP effects were polyanionic compounds, including organic dyes, or a peptide (apamin, later found to inhibit P2Y-downstream small conductance calcium-activated potassium channels instead of P2 receptors) that only weakly antagonized. Although initially considered difficult scaffolds for determining the structure-activity relationship (SAR) at P2 receptors, much later some of the chemical scaffolds, so-called "privileged structures" explored by Geoff Burnstock as P2 antagonists were re-engineered chemically for higher affinity and selectivity. Thus, we have chosen to focus in this review paper on common chemical scaffolds that can often be adapted for multiple purinergic receptor targets. We wished to provide a different perspective in this review on the topic of purine medicinal chemistry, which has been written about in elaborate detail many times in the past few years [6–14]. We provide a sampling of the more important structures in each class, focusing on both history and the cutting edge, especially for the P2X receptors.

Molecular scaffolds are used for multiple applications in medicinal chemistry [15], including establishing molecular hierarchies, their association with diverse biological activities, transferring biological activity at a given target to a similar scaffold (scaffold hopping) and their use as fragments in higher order ligand structures. Such scaffold databases now can serve the purposes of artificial intelligence (AI) approaches to drug discovery and prediction of absorption, distribution, metabolism, excretion, toxicity (ADMET) properties [16]. Even by empirical trial-and-error probing, a relatively nonselective lead compound that binds to multiple subtypes within a receptor family can be modified to be more selective for a subset within that family.

In addition to optimizing the affinity of common scaffolds and other receptor ligands by studying their SAR, medicinal chemists often have as a goal to discover the most selective compounds achievable. However, for some applications drugs of dual or multiple activities would be desirable, especially when there is additivity or synergism of the two or more pharmacological effects (so-called polypharmacology). Thus, by comparing the SAR of a given scaffold at multiple receptors, one can in some cases combine pharmacological activities in a therapeutically useful manner by fusing or tethering pharmacophores [17,18].

2. Early attempts to identify P2R agonists and antagonists

The P2 receptor selectivity of nucleotide agonists was explored on an ATP/ADP scaffold (Figure 1A), and the difference in order of potency at each of the tissues (and later defined subtypes) provided an initial pharmacological toolset for defining the P2 receptors prior to their complete cloning [19]. For example, there were different agonist potency orders at the inhibitory taenia coli P2 receptor, later shown to correspond to the $P2Y_1R$, compared to the excitatory guinea pig ileum receptor, which corresponds to the P2X1R [20]. However, the *in situ* instability of ATP analogues to rapid phospho-ester hydrolysis and the potentially opposing effect of the breakdown product adenosine (Figure 1B) that is produced, along

with its selective uptake, are all factors leading to Burnstock's statement that the "true order of potency may be obscured in isolated organ experiments." [20].

Nevertheless, P2 agonist SAR analysis begun in the 1970s was largely confirmed in later studies. It was clear that substitution of the adenine 2-position of ATP/ADP with a methylthio or chloro group increased potency selectively at the taenia coli (P2Y) receptor [19,20]. On the other hand, α,β-methylene-ATP and β,γ-methylene-ATP, containing more stable phosphonate bridges, were selective activators of the vas deferens and urinary bladder (P2X) receptor of guinea-pig and rat [4,19]. Also, modest stereoselectivity in agonist SAR, a hallmark of receptor action, was observed, especially at P2Y receptors [21]. Stereoisomers of several ATP analogues were used to further classify the P2 purinoceptors [4]. The chirality was compared either in the form of a non-natural (mirror image) L-ribose moiety or introduced as a non-bridging sulfur atom on the α-phosphate group of ATP or ADP. Of the latter, the Rp diastereoisomers were more potent than the corresponding Sp isomer at the taenia coli P2Y but not bladder P2X receptor [21,22]. The L-nucleotides were weaker as P2 agonists than the D-ribose-containing native nucleotides at the taenia coli and vas deferens, but not bladder P2 receptor (all guinea pig) [4]. Early, validated P2 receptor antagonists were an important addition to the P2 receptor ligand toolset beginning in the late 1980s. Burnstock not only studied compounds that activate or inhibit purinergic signaling, but also compounds that potentiate the actions of purinergic agents by inhibiting their enzymatic breakdown or uptake [1], which now relates to the emerging area of ecto-nucleotidase inhibitors for cancer and other diseases [23,24]. Inhibitors of adenosine kinase, adenosine deaminase and nucleoside transporters raise endogenous adenosine levels [25].

The first P2 receptor antagonists were mainly of high molecular weight and not typical drug-like molecules. Suramin (Figure 2A) is a trypanocidal drug developed during World War I from colorless intermediates used in dye synthesis [26]. Based on a hint from its inhibition of intracellular Na-K-ATPase, suramin was tested against another activity involving ATP and was found to antagonize P2X (later shown to be P2X1) receptor-induced contraction of the vas deferens in 1988 [27]. Later, suramin was shown to be an ATP antagonist in neuronal-derived cells (PC12 cells, from which the P2X2 receptor was later cloned [28]) as well as other smooth muscle cells, including the guinea pig taenia coli [29, 30]. Suramin was a more potent antagonist at the bladder smooth muscle P2X receptor (i.e. P2X1) compared to the PC12 cell P2X receptor (i.e. P2X2) [31].

Following a proceedings abstract in 1979, Reactive Blue 2 (RB2) (Figure 2B) was shown to be a moderately potent antagonist of P2YRs [32,33] and P2XRs [34]. There was ambiguity about the structure of the RB2 family of dyes, which was finally analyzed by Glänzel et al. [31] to show that the *para*-substituted isomer held the desired P2Y receptor activity. High concentrations of the early P2 receptor antagonists were often used, for example 10–100 μM of RB2 and suramin [27,35,36]. In addition to some of the dyes coloring tissue preparations, most of these early P2 receptor antagonists were promiscuous in their interaction with diverse proteins [37] and, thus, were of limited use in studying P2 receptors pharmacologically. For example, other actions of suramin include inhibition of protein-tyrosine phosphatases, sirtuins and reverse transcriptase [27,38]. RB2 is also an inhibitor of glutathione S-transferase and protein kinases [39,40]. Therefore, more definitive P2 receptor

ligands were evidently needed - but it took decades after the early phases of Burnstock's work to achieve this goal, and many of the P2X and P2Y receptors still lack potent and selective agonists and antagonists.

PPADS (pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid, Figure 3A) was first reported in 1992 by Lambrecht and coworkers as a P2X receptor antagonist [41] and soon thereafter used by Burnstock's group to characterize P2X receptors in the rabbit urinary bladder [42]. PPADS was also shown to block $P2Y_1$ but not $P2Y_{12}$ receptors [28]. However, PPADS and some derivatives were later found to inhibit NTPDase1 (ecto-apyrase, CD39) and NTPDase2 (ecto-ATPase, CD39L1) [43] that convert ATP/UTP to AMP/UMP, which complicates their use as tool compounds at P2 receptors.

3. Subsequently analyzed SAR of early P2 ligand scaffolds

3.1. P2 Receptor Agonists.

ATP was first noted by Burnstock and colleagues to contract vas deferens and bladder smooth muscles by activating what were later categorized as P2X receptors [4]. However, P2Y receptors were initially described by Burnstock as "inhibitory" due to ATP's inducing relaxation of the taenia coli (gastrointestinal inhibitory junction potential [44]) and the vasculature through endothelial cells, later designated as $P2Y_1$ receptor effects [4]. Now it is evident that five of the P2Y receptor subtypes (1, 2, 4, 6 and 11) couple to G_q protein, the $P2Y_{11}$ receptor in addition to G_s, while the remaining three subtypes couple to G_i. Although G_q-coupled receptors may be stimulatory in neurons and astrocytes in the brain, e.g. by inducing Ca^{2+} waves, they can also be inhibitory [45]. For example, various presynaptic G_q-coupled P2Y receptors inhibit the release of neurotransmitters such as glutamate [46].

3.1.1. Mononucleotides.—The high P2 receptor potency when a 2-alkylthio group was installed on the ATP scaffold (Figure 1A) was first discovered in 1972 by Gough et al. [22,47] and later elaborated chemically by Cusack and Hourani for activation of $P2Y_1$ and $P2Y_{12}$ receptors [21, 22] and by Jacobson and coworkers [48]. The series of extended 2-alkylthio derivatives of ATP, ADP and AMP, based originally on 2-MeS-ATP, showed high affinity at P2 receptors, particularly $P2Y_1R$ and $P2Y_{12}R$ [49]. The substitution of a ring-constrained bicyclic system for ribose, known as (*N*)-methanocarba (bicyclo[3.1.0]hexane), introduced $P2Y_1R$ selectivity in potent agonist MRS2365 (Figure 1C) [50]. The SAR progression of 2-alkylthio-ATP analogues at the $P2Y_{12}R$ led eventually to a 5′-triphosphate analogue having a β,γ-dichloromethylene bridge for stability, i.e. cangrelor (AR-C69931MX) [51], as an approved antithrombotic drug, and even to an uncharged, potent $P2Y_{12}R$ antagonist, as the approved drug ticagrelor (AZD6140, a nucleoside analogue) [51].

Novel uracil mononucleotides as $P2Y_{2,4,6}$ receptor agonists have been introduced [52,53]. This followed the discovery that UTP (Figure 1A) and its analogues induced P2Y-like activities, which was later confirmed with the cloning of these receptors beginning with the mouse $P2Y_2$ receptor from neuroblastoma cells [54]. Nucleotides with alternative nucleobases have been shown to bind to P2Y and P2X receptors [55,56]. Efforts to chemically stabilize the di- or triphosphate moieties of P2R agonists include the introduction

of a chiral boranophosphate moiety, for example in an analogue of $P2Y_6R$ agonist UDP [57].

3.1.2. Dinucleotides.—The P2 receptors can accommodate dinucleotides as well as mononucleotides [58], and an extracellular dinucleoside tetraphosphate (Up_4A) is an endothelial-derived transmitter substance [59]. Each P2R subtype has a different preferred number of phosphate units in the chain [58], e.g. Np_3N (where N is a nucleoside) for $P2Y_6R$ [52] and Np_4N for P2XRs, $P2Y_2R$ and $P2Y_4R$ [10,58,60]. Other examples of dinucleotides that are useful P2R pharmacological probes are: Ip_5I (P2X1 [61]), Ap_4A ($P2Y_{12}$), Up_4U ($P2Y_{2,4}$) and MRS2957 ($P2Y_6$) [53]. Ap_4A analogues with defined stereochemistry have also been studied as dual $P2Y_1R/P2Y_{12}R$ antagonists [17]. Dinucleotide Up_4U (diquafosol, a long-acting $P2Y_2R$ agonist) is approved for treating dry eye syndrome in Japan and Korea, while its dinucleotide analogue denufosol failed to receive FDA approval after multiple clinical trials for cystic fibrosis [62]. Diquafosol, used as a 3% ophthalmic solution for treating dry eye, increases secretion from the meibomian glands in the eyelid [63].

3.1.3. Nucleotide sugars.—UDP-glucose potently activates the $P2Y_{14}R$ and acts as a damage associated molecular pattern (DAMP) in various inflammatory conditions [64]. Thus, a series of $P2Y_{14}R$ antagonists based on well-explored naphthalene and phenyl-triazole scaffolds have shown efficacy in models of inflammation, chronic neuropathic pain and asthma [64,65]. Other UDP-sugars are less potent than UDP-glucose, and UDP also acts as a $P2Y_{14}R$ agonist or partial agonist [11,58].

3.2. P2 Antagonists.

In recent years, the discovery of subtype-selective P2YR and P2XR antagonists has accelerated [6,9,10]. Novel scaffolds have been discovered that can be directed toward multiple P2R subtypes by appropriate derivatization [6,66]. Nevertheless, as described here, earlier scaffolds have also been useful in the discovery of P2R antagonists (Figure 2A). Multiple studies have shown that weak, non-drug-like, nonselective P2 receptor antagonists, such as RB-2, can still be utilized as a starting point leading to potent receptor subtype-selective compounds.

3.2.1. Suramin analogues.—A tetraphosphonate analogue related to antagonist suramin (Figure 2A), NF546, acts as a selective $P2Y_{11}R$ agonist, which is an example of using a common scaffold both to shift to a different receptor subtype and to change the pharmacological efficacy [67]. A suramin derivative, NF272, was identified as a competitive $P2Y_2$ receptor antagonist, although it was <10-fold selective compared to $P2Y_2$ and $P2Y_6$ and at least as potent at $P2Y_1$, $P2Y_{11}$ and $P2Y_{12}$ [68]. NF272 also blocks P2X1R. The suramin derivative NF770 is a relatively potent and selective P2X2R antagonist, which was proposed to display a competitive mechanism of action [69].

Recently, suramin-derived benzenesulfonates, benzenesulfonamides and related derivatives were found to act as $P2Y_2R$ antagonists with ancillary inhibitory activity at the related orphan GPCR GPR17. Dual inhibitors of these proinflammatory receptors may be advantageous for the therapy of inflammatory diseases [70].

3.2.2. RB2 analogues.—The group of August W. Frahm was the first to synthesize RB-2 analogs (anthraquinone derivatives) with the aim of optimizing their interactions with P2Y receptor subtypes. MG 50–3-1 (structure not shown) was reported to be a nanomolar antagonist at the $P2Y_1$-like receptor of guinea-pig taenia coli, showing only weak activity at the P2X1 receptor in rat vas deferens [71]. Subsequently, an improved synthetic procedure was developed for MG 50–3-1 to provide sufficient amounts of the compound for additional experiments [72]. At the human $P2Y_1R$ it was weaker (IC_{50} 350 nM) than at the guinea pig preparation and also blocked the human $P2Y_4R$ (153 nM).

Broad, systematic SAR analysis of truncated RB-2 derivatives became possible by the development of combinatorial synthetic approaches [73] and, later on, by an improved Ullmann coupling reaction of bromo-substituted anthraquinone derivatives and amines/anilines [74,75]. This led to the identification of antagonists with selectivity for $P2Y_2$, $P2Y_4$, $P2Y_{12}$ or P2X2 receptors. The highly potent and selective $P2Y_{12}R$ antagonist PSB-0739 (Figure 2B) showing low nano- to subnanomolar potency has become a useful pharmacological tool [76,77]. For example, this antagonist was used to demonstrate that blocking the $P2Y_{12}$ receptor in the spinal cord reduces chronic pain and cytokine production [78]. Mutagenesis and docking studies confirmed that it acts as a competitive antagonist [77,79]. Recently, the first $P2Y_4$-selective antagonists, namely the anthraquinone derivatives PSB-1699 and PSB-16133 (Figure 2B), were reported [80]. Mutagenesis and molecular modeling studies, based on X-ray structures of related GPCRs, defined their $P2Y_4R$ binding sites and interactions [81–83]. Optimization of anthraquinone derivatives related to RB-2 also led to potent, selective P2X2R antagonists (PSB-10211 and PSB-1011) and positive allosteric modulators (e.g. PSB-10129) [84].

3.2.3. PPADS and other pyridine analogues.—Aryl-diazo-bridged pyridoxal phosphate derivative PPADS serves as a versatile P2 receptor antagonist scaffold, and its binding region on a P2X1 receptor homology model was recently characterized [85]. PPADS analogues (Figure 3) have been developed as useful research tools with selectivity for various P2 receptor subtypes, although they contain several non-druglike structural features (aryl-azo, aldehyde and phosphate ester groups) [41,42,86–89]. Analogues of PPADS have been applied to antagonize both P2YRs and P2XRs. MRS2211 is a selective $P2Y_{13}R$ antagonist. PPADS analogues were found by mutagenesis to occupy the same binding region of the $P2Y_1R$ as nucleotide antagonists, and the extracellular loops participate in the ligand selectivity in comparison to the $P2Y_6R$ [82]. A P2X1 receptor positive allosteric modulator (PAM) MRS2219 was derived from a PPADS scaffold [90]. High affinity at P2X3R was also found for the PPADS scaffold [89]. Recently, a PPADS analogue lacking azo, aldehyde and phosphate groups was found to relieve pain in the mouse by blocking the P2X3 receptor (Figure 3A) [84]. Dihydropyridines (including nicardipine) and related anionic 5-phosphonate analogues were found to antagonize the rat P2X2R and P2X4R [91].

3.2.4. DIDS analogues.—The P2Y receptor antagonism by the irreversible chloride channel blocker DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) was used as a lead compound for several reactive and sparingly water soluble diisothiocyanate derivatives as P2Y antagonists, including $P2Y_6$-selective MRS2578 (structure not shown) [92]. Other

diisothiocyanate analogues favored various P2Y receptor combinations. DIDS was earlier found to antagonize the rat $P2Y_6$ receptor [92], as well as the bladder smooth muscle P2X1 receptor (IC_{50} 3 μM), but not the PC12 cell P2X2 receptor [31].

3.2.5. P2 agonists that became P2X antagonists by structural modification.— Some of the first P2 nucleotide ligands derived from ATP could not readily be examined by analogue synthesis, due to difficulty of synthesis and uncertainty about the precise structure. For example, the potent P2X1/P2X7 receptor agonist Bz-ATP [13,97] appears to be a mixture of 2′- and 3′-monoesters. Some of the 2′- and 3′-derivatives of ATP have been found to antagonize P2 receptors [98]. Notably, an early antagonist photoaffinity probe arylazidoaminopropionyl-ATP ($ANAPP_3$, structure not shown) also had an ambiguous structure and its use was curtailed after the 1980s. Nevertheless, $ANAPP_3$ was the only P2R antagonist described when the distinction between P2X and P2Y receptors was proposed by Burnstock and Kennedy in 1985 [4], which illustrated the pressing need at that time to identify new ligands. In the absence of potent antagonists, pre-exposure to α,β-methylene-ATP was used to block the subsequently termed P2X1 (but not P2X2) receptor effects by desensitization. Oxidized ATP (o-ATP, structure not shown) was originally used to block and affinity-label nucleotide binding sites in mast cells [99], later cloned as the P2X7 (originally termed P2Z) receptor [100]. It was shown to be an irreversible antagonist at recombinant P2X1, P2X2 and P2X7 receptors [31,100]. However, o-ATP is also not suitable for analogue synthesis, because it is both unstable and chemically reactive towards amines. Furthermore, the possibility of off-target interactions, for example with other ATP-binding proteins is enormous.

The first ATP analogue generally useful as a P2X antagonist was 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP, structure not shown), which has some selectivity for P2X2 and P2X2/3 and P2X4 receptors [101–103] but also binds to diverse ATP-recognizing proteins such as protein kinases and ATPases [104]. Dal Ben et al. [98] recently explored the SAR of ATP analogues with large acetal groups at the 2′- and 3′-hydroxyls as P2X receptor antagonists.

3.2.6. P2 agonists that became P2Y antagonists by structural modification.— In 1996, Boyer et al. discovered that naturally occurring bis-phosphate derivatives of adenosine block the $P2Y_1$ receptor [110]. Based on this finding, Jacobson, Harden and coworkers explored the SAR in great detail [50,94–96]. Bioisosteric substitutes for the ribose moiety, i.e. cyclic alkyl and acyclic groups have been discovered (Figure 1C), including acyclic phosphonate MRS2298 [96]. Introduction of a rigid (N)-methanocarba ring system as in place of ribose resulted in MRS2500 as a sub-nM antagonist that is highly selective for the $P2Y_1R$ [97]. The SAR for nucleotide antagonists at the $P2Y_1$ receptor largely resembles agonist SAR, except that a 3′,5′-bisphosphate is required, instead of a 5′-diphosphate, and a 2′-deoxy modification is favored [111]. Nevertheless, the bicyclic (N)-methanocarba modification was highly affinity-enhancing for both $P2Y_1R$ nucleotide agonists and antagonists. Attempts to apply the same strategy of separating the diphosphate moiety into bisphosphates to another 5′-diphosphate activated receptor, $P2Y_6$, failed to produce antagonists, indicating a major structural difference between these two G_q-coupled receptors. In fact, $P2Y_6$ agonists require the opposite ring twist of ribose, and a South (S)-

methanocarba modification, but not (N) is tolerated in UDP analogues [53,58]. Among non-nucleotide $P2Y_2R$ antagonists, such as AR-C118925XX (structure not shown), a uracil core is shared with $P2Y_2R$ agonists [9].

4. Recently identified scaffolds for P2X, P2Y and adenosine receptors

4.1. Novel P2X receptor ligand scaffolds and antagonist clinical trials.

As reviewed recently by Burnstock [112], there are numerous potential clinical applications of P2 receptor-based drugs. Geoff Burnstock and colleagues reported that the P2X3 receptor is localized in sensory neurons of the dorsal root ganglia [113], and perhaps in the future P2X3R antagonists may be applied to inflammatory or neuropathic pain control [66,114,115]. P2X3 antagonists have also been considered for further indications including control of neuropathic pain, chronic pain, e.g. associated with endometriosis, sleep apnea, and overactive bladder [116].

A large pharmaceutical effort identified the class of diaminopyrimidines (DAPs) as a scaffold for P2X2/3 or P2X3R antagonists (Figure 3C) [63,103]. One the P2X3 receptor antagonists in this series, gefapixant [100,104], is a hopeful drug for chronic cough that has recently completed two Phase 3 clinical trials (COUGH-1 and COUGH-2) [117]. The higher dose of 45 mg twice per day significantly improved symptoms of chronic cough and thus met the primary clinical endpoint. As an adverse event, disturbance of taste perception was observed. Geoff participated in the discovery of Gefapixant, and the compound is named after him (**Gef** = **Ge**o**f**f; **p**i**xant** = **P**2**X** receptor **ant**agonist). Gefapixant is expected to be the first P2X3 antagonist that will be filed for approval. The compound acts as a negative allosteric modulator (NAM) at a novel P2XR site, bridging the left flipper, lower body, and dorsal fin regions of the P2X3R [118].

Further P2X3 receptor antagonists containing diverse scaffolds have been developed that are clinically evaluated, including BLU-5937 [66,119] (Figure 3C), BAY 1817080 [120] and S-600918 (structures undisclosed). All of these antagonists are allosteric modulators, which may bind to different sites on the ion channel receptor. BLU-5937 displays selectivity for P2X3R homomers over P2X2/3 heteromers, which is expected to reduce or abolish taste sensitivity (dysgeusia) [119]. In a Phase 2 clinical study in chronic cough (RELIEF), BLU-5937 had low impact on taste perception, but also failed to reach its primary endpoint [121]. BAY 1817080 was evaluated in a Phase 1/2A clinical trial in chronic cough and, like gefapixant, the antagonist did show efficacy. S-600918, another P2X3 antagonist, probably belonging to a previously disclosed series of pyrrolinones [122] that was evaluated in a phase 2a study, also showed a potential to reduce cough with moderate effects on taste perception [123].

One P2X4R antagonist, NC-2600 (structure not disclosed), is in clinical trials for chronic neuropathic pain [124], and further P2X4R antagonists for the treatment of peripheral inflammatory pain and for chronic neuropathic pain are in the pipeline. Microglial P2X4R is upregulated in pain and injury, leading to release of brain-derived neurotrophic factor (BDNF), a short-term promoter of pain. P2X4R antagonists are also a potential treatment during the early stages after a stroke [125]. Putative mechanisms of P2X4 anti-ischemic

neuroprotection include fewer pro-inflammatory myeloid cells entering the brain, reduced plasma membrane P2X4R expression, and increased blood brain barrier integrity [125].

Chemically diverse heterocyclic P2X7 receptor antagonists (Figure 3D) have been developed, and several have been in multiple clinical trials for inflammatory bowel disease (IBD), Crohn's disease, rheumatoid arthritis, inflammatory pain, chronic obstructive pulmonary disease (COPD) and more recently for CNS disorders, in particular depression [66,115,126–129]. P2X7 activation induces the production of pro-inflammatory IL-1β and reactive oxygen species (ROS) by macrophages, and mast cells may also be involved in the protective effects of P2X7 antagonists in IBD [129,130]. P2X7 antagonist JNJ-54175446 is currently in a Phase II clinical trial for major depressive disorder [127]. Brain-penetrant P2X7 receptor antagonist PET imaging agents have been designed [66,127].

4.2. Novel P2Y receptor ligand scaffolds, clinical trials and approved drugs.

In addition to orthosteric antagonists cangrelor and ticagrelor (mentioned above), there are two allosteric $P2Y_{12}R$ antagonists, approved as antithrombotic agents, clopidogrel and prasugrel, that act as prodrugs that require conversion in vivo; further compounds are in development [131]. The thienopyridine class of $P2Y_{12}R$ prodrugs was first discovered empirically as antithrombotic agents, and only later associated with this receptor subtype [115]. Various other non-nucleotide heterocyclic classes of high affinity $P2Y_{12}R$ antagonists have been reported (Figure 3C) [11,132], but none have been approved as antithrombotic drugs. The X-ray structure of the $P2Y_{12}R$ complex of one such orthosteric antagonist AZD1283 (a cyanopyridine derivative containing a polar sulfonylcarbamoyl group as a diphosphate bioisostere), was reported [133]. Similarly, elinogrel contains a polar sulfonylurea group, but its Phase 2 clinical trial was discontinued in 2012.

Activation of both $P2Y_1R$ and $P2Y_{12}R$ is needed for stable thrombus formation; thus, blocking each individually has an antithrombotic effect [11]. A class of *N*,*N*′-diarylureas was discovered (Figure 3B), initially from screening of chemical libraries, to be orally active $P2Y_1R$ antagonists, and their antithrombotic activity was established [134]. The allosteric antagonist BPTU binds to the lipid-exposed outer surface of the $P2Y_1R$ and its SAR has been analyzed in light of the determination of the X-ray structure of its $P2Y_1R$ complex [50]. An in silico screen using the X-ray structure identified 1-indolinoalkyl-2-phenolic derivatives as $P2Y_1R$ agonists with anticancer potential [135]. However, no $P2Y_1R$ ligands are yet approved for clinical use.

4.3. Adenosine receptor ligand scaffolds.

The physiological effects of adenosine were discovered decades before Burnstock's pioneering studies on ATP [4], and the search for new ligands for adenosine receptors developed earlier than for the P2 receptors. Since adenosine derivatives (Figure 1B) and the prototypical receptor antagonists, xanthines (Figure 4), were already commonly found in the compound collections of academic and pharmaceutical laboratories by the 1970s [136,137], it was relatively straightforward to begin the SAR process using compounds off the shelf as each of the four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) was discovered [6–8,138]. Purine nucleobases, such as adenine derivatives were also plentiful, and their SAR as

antagonists was explored early (Figure 5). One of the first entries into adenosine receptor SAR was by Bruns [139], who wrote to numerous laboratories around the world requesting samples of adenine nucleosides and other purines to test for activation or inhibition in fibroblasts of what was later termed the A_{2B} receptor. The subsequent process of structural optimization of adenosine receptor agonists and antagonists through *de novo* chemical synthesis was not as challenging as for the P2 receptors, as there was already a large literature surrounding preparation of xanthines and nucleoside analogues [137]. Unlike P2 receptors, each of the adenosine receptor subtypes already has definitive agonists and antagonists. Numerous clinical trials of adenosine receptor agonists and antagonists have occurred. The only synthetic ligands thus far approved for human use are an A_{2A} agonist and A_{2A} antagonist [140,141]. Nevertheless, interest in developing adenosine receptor ligands therapeutically has accelerated recently, for example, with A_{2A} antagonists for boosting the innate immune response in the tumor microenvironment [142,143].

4.3.1. Adenosine agonist scaffolds.—Most adenosine receptor agonists are derivatives of adenosine (Figure 1B), but several classes of nonnucleoside agonists have been found (Figure 6C) [144]. Although the generalized SAR patterns of adenosine derivatives as agonists are shown in Figure 1B, specific agonist structures are not depicted here but rather are found in references [6–8]. Adenine is the address portion of the adenosine scaffold, while the ribose is the message portion. Thus, modification of the ribose ring has been shown to decrease efficacy at the A_1, A_{2A} and A_3 receptor, leading to partial agonists and antagonists [145]. Adenines lacking the ribose moiety entirely are often adenosine receptor antagonists (Figure 5), and this is one of the widely explored scaffolds for such antagonists besides xanthines [105,146].

Adenine nucleosides are well explored, either as single receptor subtype-selective agonists or agonists of mixed selectivity. For example, modification at both C2 and 5′ positions resulted in the first A_{2A}-selective agonist, CGS21680 [147]. 2-Arylalkylether MRS3997 (structure not shown) is a potent, full agonist at both A_{2A} and A_{2B} receptors, but not at the other two subtypes [148]. 2-Alkynyladenosine derivatives have been developed to act at A_{2A} and A_3 receptors [7,8,149]. The effects on selectivity of substitution at the C2, N^6 and 5′ positions of adenosine (Figure 1B) are often interdependent [126]. Certain novel nucleobases in place of adenine in nucleoside derivatives are also known to act as adenosine receptor agonists [150]. Rigid pseudoribose moieties, such as the (*N*)-methanocarba ring system, have been used to gain selectivity of adenosine agonists for the A_3 receptor [7].

Most of the adenosine agonists in clinical trials so far have failed in development [140], either for problems of efficacy or side effects, or for commercial reasons. One example of the latter is *O*-methyl-isoguanosine (BVT.115959), which was terminated although it showed efficacy in a randomized, placebo-controlled Phase II trial for diabetic neuropathic pain. Although the ascribed mechanism was activation of the A_{2A} receptor, it proved to be 6-fold more potent in hA_1 and hA_3 receptor binding than at the hA_{2A} receptor [151]. This is a hopeful indicator of the potential future use of A_1/A_3 receptor agonists for chronic pain treatment. Both A_1 and A_3 agonists demonstrate efficacy for treating neuropathic pain [124]. For example, highly selective (*N*)-methanocarba A_3 agonist MRS5980, potently prevents or reverses mechano-hypersensitivity in chronic pain models and, when combined with

morphine, makes the opioid effective upon chronic use with no tolerance or withdrawal behaviors [152]. Of note, the A_3-induced mast cell degranulation that occurs in rodent species is absent in higher species including human [140]. A_3 agonists have progressed to advanced clinical trials for autoimmune inflammatory diseases (psoriasis and rheumatoid arthritis) and for liver diseases (cancer and non-alcoholic steatoheaptitis) [8].

There are two classes of non-nucleoside adenosine agonists, dicyanopyridines and (cyano)pyrimidines (Figure 6C) [106,144]. With appropriate functionalization, this scaffold can be directed to bind with A_1, A_{2A} and A_{2B} receptors, either individually or as mixed agonists. For example, the commonly used A_{2B} receptor agonist BAY 60–6583, which is actually a partial agonist at the A_{2B}AR [107] and an antagonist at A_1 and A_3 receptors at higher concentrations [108], several A_1 receptor agonists (Capadenoson and Neladenoson) that were in clinical trials for cardioprotection/heart failure [109], and a mixed A_1 /A_{2A} tool compound (agonist LUF5834) all contain a common 2-amino-4-phenyl-pyridine-3,5-dicarbonitrile scaffold. Not only is this scaffold variable in its target adenosine receptor subtype(s), small changes in functional groups dramatically alter the relative efficacy in this series, even inducing receptor antagonism/inverse agonism in selected cases [153]. Molecular modeling combined with site-directed mutagenesis provided evidence for the accommodation of this scaffold in the adenosine receptor orthosteric binding site [154, 155].

4.3.1. Adenosine antagonist scaffolds—We have selected a few illustrative examples from the myriad of adenosine receptor antagonist scaffolds [6–8]. The alkylxanthines are the prototypical adenosine receptor antagonists, and countless analogues have been reported, leading to selectivity for each of the four adenosine receptor subtypes (Figure 4) [12]. The inclusion of an 8-phenyl or 8-cycloalkyl moiety on the xanthine scaffold provided antagonist selectivity for the A_1 receptor, in parallel to similar affinity-enhancing derivatization of adenosine at the N^6 position. The 8-cycloalkyl group could be bridged to further enhance the A_1 receptor affinity as with PSB-36 [108]. An 8-phenylxanthine with an amine-functionalized chain (XAC) has provided many fluorescent probes and other conjugates for detection and characterization of adenosine receptors [156], as this alkylamino group extends out of the orthosteric binding site and is not constrained. 8-Phenylxanthines later were found to be more versatile in their applicability to the A_{2B} receptor, for which the first selective antagonist was MRS1754 [157,158]. The xanthine scaffold later showed a preference for 3-H at the A_{2B} receptor (PSB-603), compared to 3-propyl substitution in A_1 receptor antagonists [159]. 8-Styryl groups on the xanthine scaffold provide A_{2A} receptor antagonists, including the approved drug istradefylline (KW-6002) for Parkinson’s disease treatment [160]. Cyclized xanthines have provided antagonists of the A_3 receptor [8]. Examples are PSB-11, KF-26777 and Cmpd 6 (Figure 4).

Many adenine derivatives, without the ribose moiety, have been reported as mentioned above as subtype-selective adenosine receptor antagonists (Figure 5) [161]. The original discovery that adenine derivatives, per se, antagonize the adenosine receptors was reported by John Daly and coworkers, e.g. with A_1 antagonist N-0840 [105]. A survey of non-xanthine heterocylic antagonists of the adenosine receptors was published in 1988 [162]. During the same time period, basing alternative heterocycles on the adenine/purine scaffold lacking ribose, i.e. fused 5- and 6-membered nitrogen heterocycles, led to early antagonists of the

A_{2A} receptor from pharmaceutical labs, i.e. CGS15943 [163, first disclosed in an abstract in 1983] and ZM241,385 [164]. Further elaboration of the heterocyclic core resulted in more selective A_{2A}AR antagonists, such as pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine derivative SCH442416 [165]. This tricyclic series has progressed to an A_{2A}AR antagonist, preladenant (SCH420814, MK-3814) [166], that was in clinical trials for Parkinson's disease and is currently being repurposed to cancer immunotherapy.

Flavones, of the flavonoid family were discovered in early library screening to bind as antagonists to various adenosine receptors, although flavonoids notoriously have many off-target actions [167]. Another class of phytochemicals, coumarins, which have a rearranged backbone relative to flavonoids, have provided selective A_3AR antagonists [168].

1,4-Dihydropyridines (DHP) have long been used clinically as calcium channel blockers. Jacobson and colleagues detected A_3 adenosine receptor interactions of some common DHPs (Figure 6A) by screening several thousand members of a small, manually collected chemical library, prior to the widespread use of commercial libraries [8,169]. Subsequently, the calcium channel interactions were deselected by chemical modification of this scaffold, and at the same time A_3 receptor affinity was greatly increased, leading to the identification of often-used A_3-selective 1,4-dihydropyridine antagonists MRS1191 and its 4-nitrobenzyl derivative MRS1334. The same scaffold was oxidized systematically to direct the A_3 SAR to pyridine derivative MRS1523, an effective antagonist at rodent homologues as well as the human A_3 receptor. Recently, the DHP nifedipine was found to block the A_{2B} receptor at clinically relevant concentrations, suggesting its possible use in treating diarrhoea [170]. To illustrate the versatility of DHPs as a privileged scaffold, they were also directed toward modulation of the P2X2 receptor [91]. Sotelo and coworkers discovered novel, selective AR antagonist scaffolds containing a pyrimidine core that can be assembled from condensation of multiple prefunctionalized components [171]. These antagonists can bind to different AR subtypes depending on the functionalization, but recently high affinity and selectivity at the A_{2B} receptor was reported for (*S*)-ICAM-C032 [171]. Quinoline and isoquinoline scaffolds (Figure 6B) have also been explored as A_3 antagonist scaffolds [172].

5. Structure-based approaches for purinergic agonists and antagonists

Since the first X-ray structure of an A_{2A} adenosine receptor was reported in 2008 [173], ligand design at purinergic receptors has become increasingly structure-based. A recent count yielded fifty-seven purinergic GPCR structures (52 AR structures and 5 P2YR structures) deposited in the Protein Data Bank (PDB), comprising an impressive ~14% of all GPCR structures known [174]. Thus, the structures of $P2Y_1R$, $P2Y_{12}R$, A_1AR and especially the A_{2A}AR have been leading examples to help guide the application of structure-based design to medicinal chemistry [50,173–177]. P2XR structures are beginning to be useful in medicinal chemistry, with their more complex structure of three interwound subunits, each approximating the form of a dolphin [178]. For example, the interaction of antagonists having a pyroglutamide scaffold with the P2X7R has been modeled [130].

Each of these receptor classes presented unanticipated features in their 3D structures. For example, an allosteric antagonist BPTU was the first GPCR ligand to be discovered to bind

entirely outside the heptahelical bundle and in close contact with the membrane phospholipids [175]. The same study found that the binding site for nucleotide antagonist MRS2500 did not share any contact points in common with BPTU. The orientation of typical adenosine receptor agonists and antagonists in the A_{2A} receptor showed a portion that is facing the extracellular medium, rather than being localized in the canonical binding site at ~1/3 the total depth through the transmembrane region [173,176].

An A_{2A} antagonist that was designed completely by structure-based methods, a 1,2,4-triazin-3-amine derivative AZD-4365 (HTL-1071), is in clinical trials for cancer immunotherapy [142]. The design of this antagonist series was guided by biophysical mapping of the binding site, based on multiple A_{2A} receptor X-ray structures. Its effects on multiple cell types contribute to its in vivo efficacy, including improved function of $CD103^+$ dendritic cells and T cells in the tumor microenvironment [143].

6. Conclusions

Ligand development at P2 and at P1 (adenosine) receptors followed different paths. The adenosine receptors are currently most advanced in ligand discovery, and the discovery of potent ligands of P2Y and then P2X receptors followed chronologically. The search for ligands of the purinergic receptors progressed through phases from the early need to prove the existence of P2 receptors until its present relevance for disease treatment was established. Initially, the P2 agonists and antagonists were poorly suited for pharmacological studies. However, intense efforts to modify known scaffolds and eventually to discover novel scaffolds have been sustained since the 1990s. Diverse scaffolds were chemically modified to direct the selectivity toward different subtypes of P2X, P2Y and adenosine receptors. Some of the interactions of the versatile scaffolds presented here are summarized in Figure 7. The medicinal chemistry of purinergic receptors has progressed from useful tool compounds to, in some cases, promising experimental drugs. With Geoffrey Burnstock's inspiration and encouragement, numerous clinical trials have ensued using various P2XR antagonists. Thus, the vision of Prof. Burnstock to use purinergic signaling modulators, most recently at P2Rs, for treating disease is coming to fruition.

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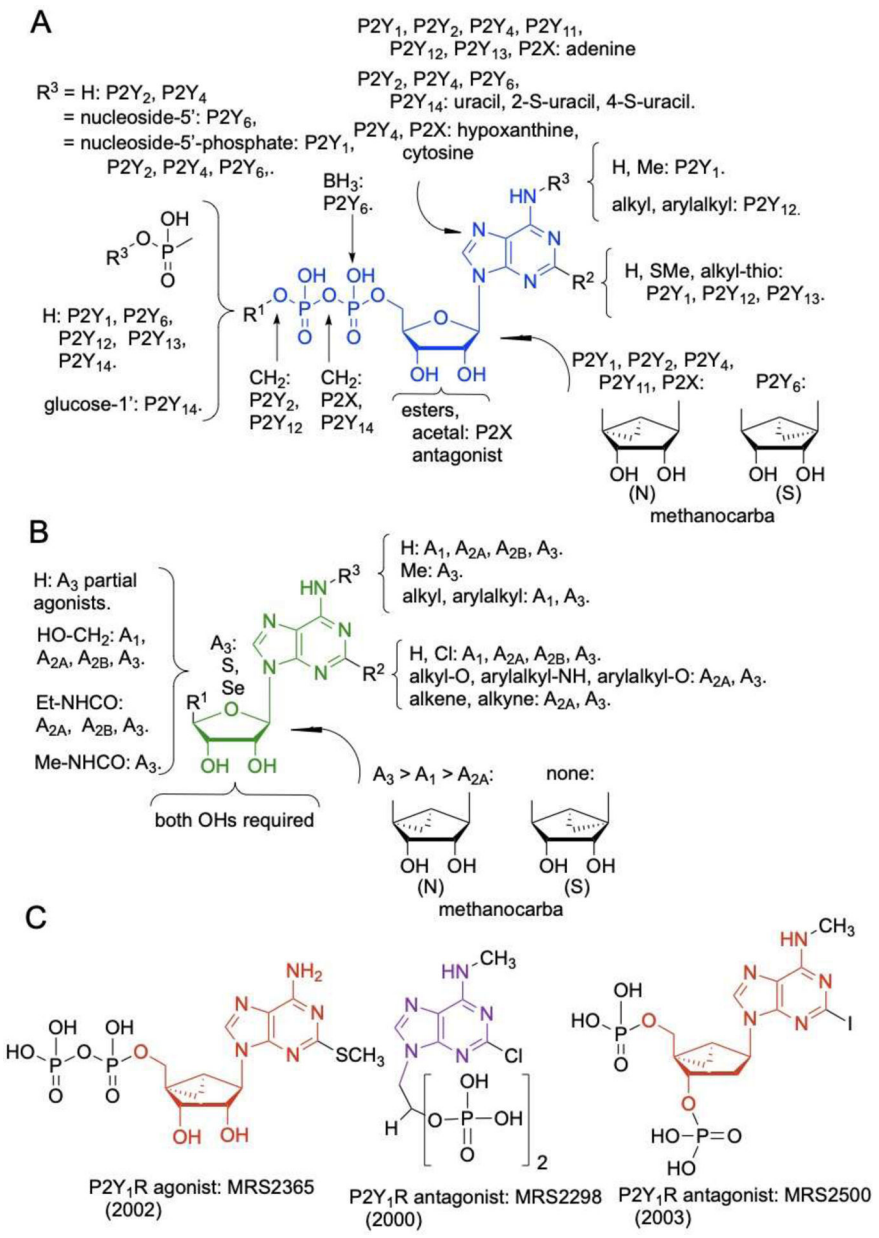

Figure 1.

Generalized SAR of the native scaffolds of the P2 (A, C) and adenosine (B) receptors. Note that there is often a complex relationship between multiple modifications. It is necessary to consult detailed original publications and reviews for individual structures [5–6–8,10–12,51,58,60,66,93–96]. The notations indicate general effects of the chemical substitution shown (although not applicable in all cases of the same substitution). For example, in part A: "R^3=H: $P2Y_2$, $P2Y_4$" means a H at this position is recognized by $P2Y_2$ and $P2Y_4$ receptors. Scaffold backbone structures are colored as follows: blue, ADP; green, 4′-truncated adenosine; red, (N)-methanocarba adenosine or deoxyadenosine; violet, 9-alkyladenine. The effects, on receptor affinity and selectivity, of substitution at different positions of the same scaffold are often interdependent. In some cases, agonists were converted into antagonists by

specific structural changes. Years shown for each compound indicate an early, major report in the literature.

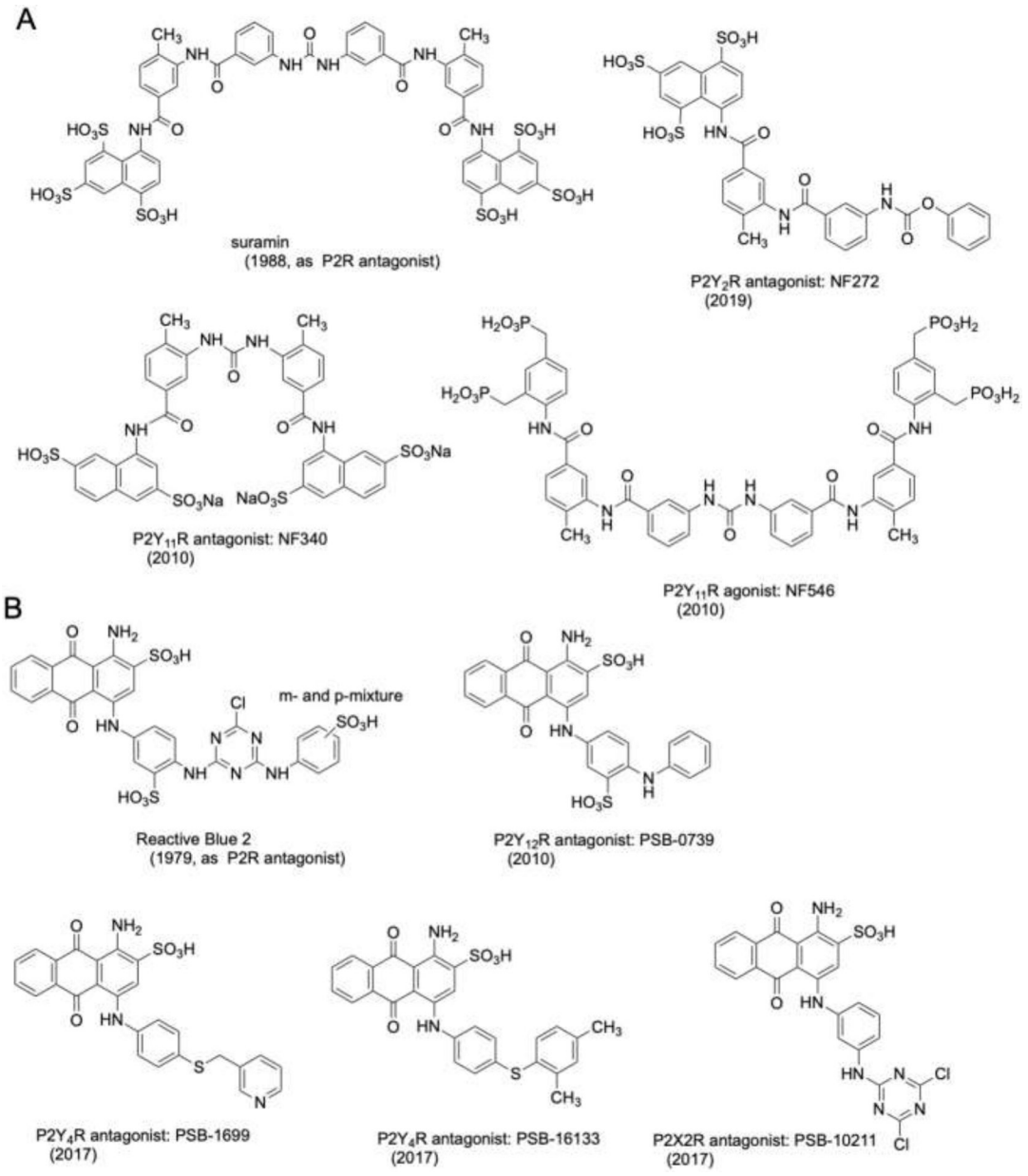

Figure 2.

Early-identified large scaffolds for P2R antagonists, suramin (A) and Reactive Blue 2, an anthraquinone (B). Affinities of many these compounds and their original references are collected [6,66–80]. Years shown for each compound indicate an early, major report in the literature.

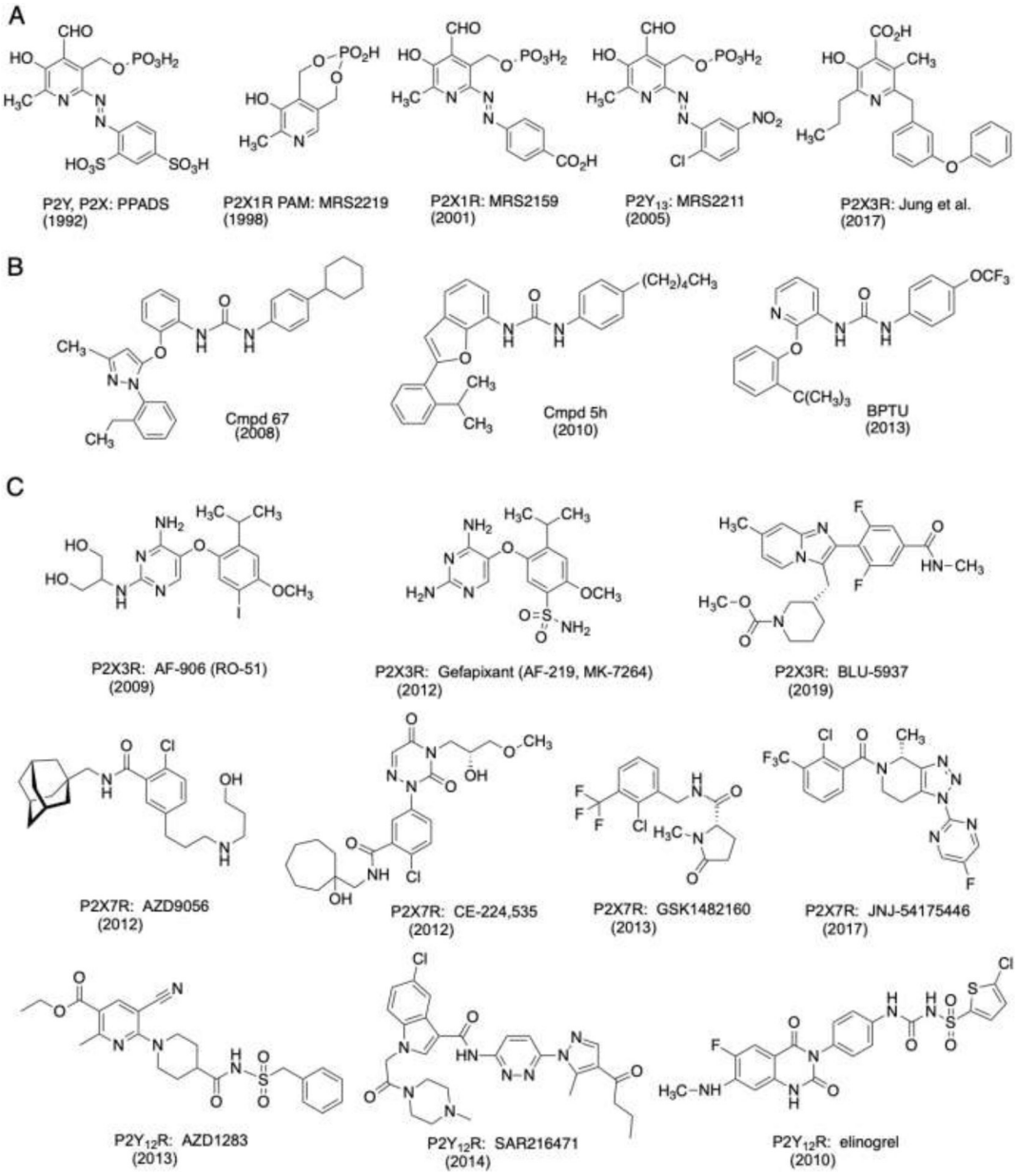

Figure 3.

Small scaffolds for P2R antagonists, including PAMs and NAMs related to pyridoxal phosphate (A), *N*,*N*′-diarylureas that allosterically antagonize $P2Y_1R$ (B) and various P2X3R, P2X7R and $P2Y_{12}R$ (C) antagonists. Gefapixant, BLU-5937, AZD9056, CE-224,535, GSK1482160 and JNJ-54175446 have been in clinical trials [66–68,116–120]. The pyridoxal phosphates are known to interact with various P2X and P2Y receptors as antagonists or PAMs, while the diarylureas are specific for $P2Y_1R$. This specificity is a function of their unusual binding site on the outer surface of the receptor where the urea forms a bidentate H-bond with a backbone carbonyl group. Affinities of many these compounds and their original references are collected [6, 66–68,116–120]. Years shown for each compound indicate an early, major report in the literature.

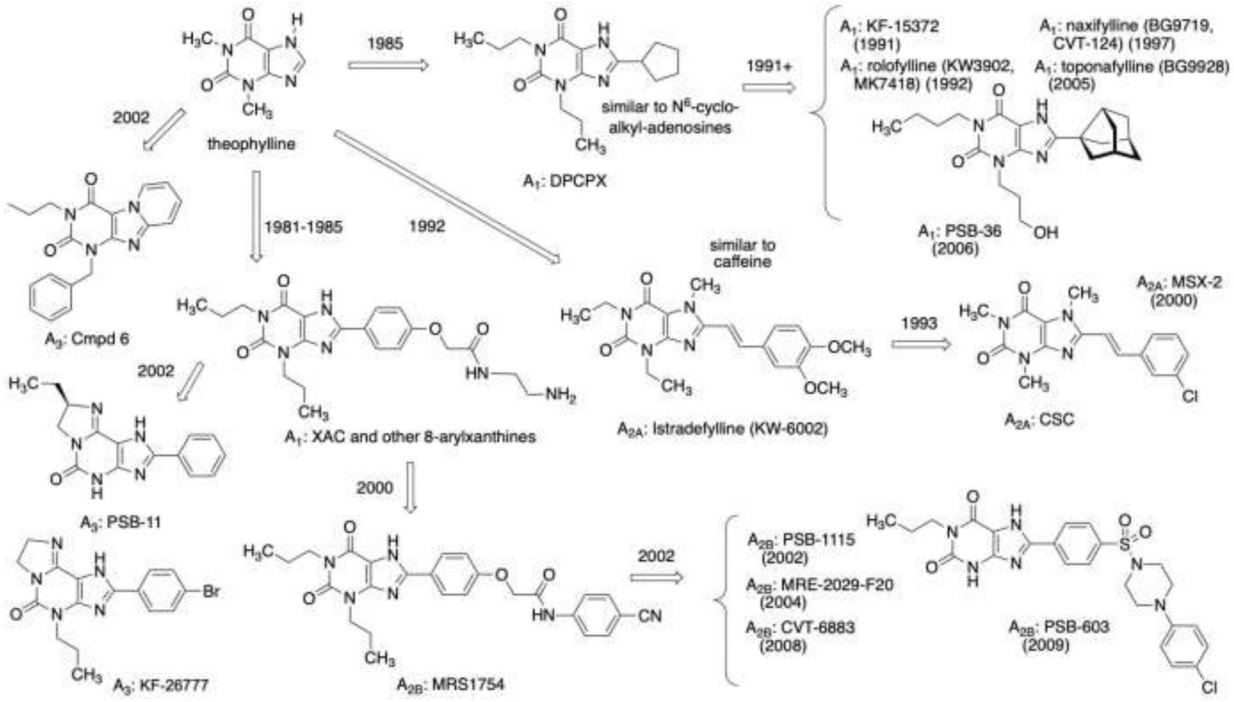

Figure 4.

Adenosine receptor antagonists derived from a xanthine scaffold. The prototypical xanthine is theophylline, which is slightly more potent than caffeine at the adenosine receptors. The receptor affinities of many of the compounds cited can be found in the original references [6–8,12,136–140,156–159]. Years shown for each compound indicate an early, major report in the literature.

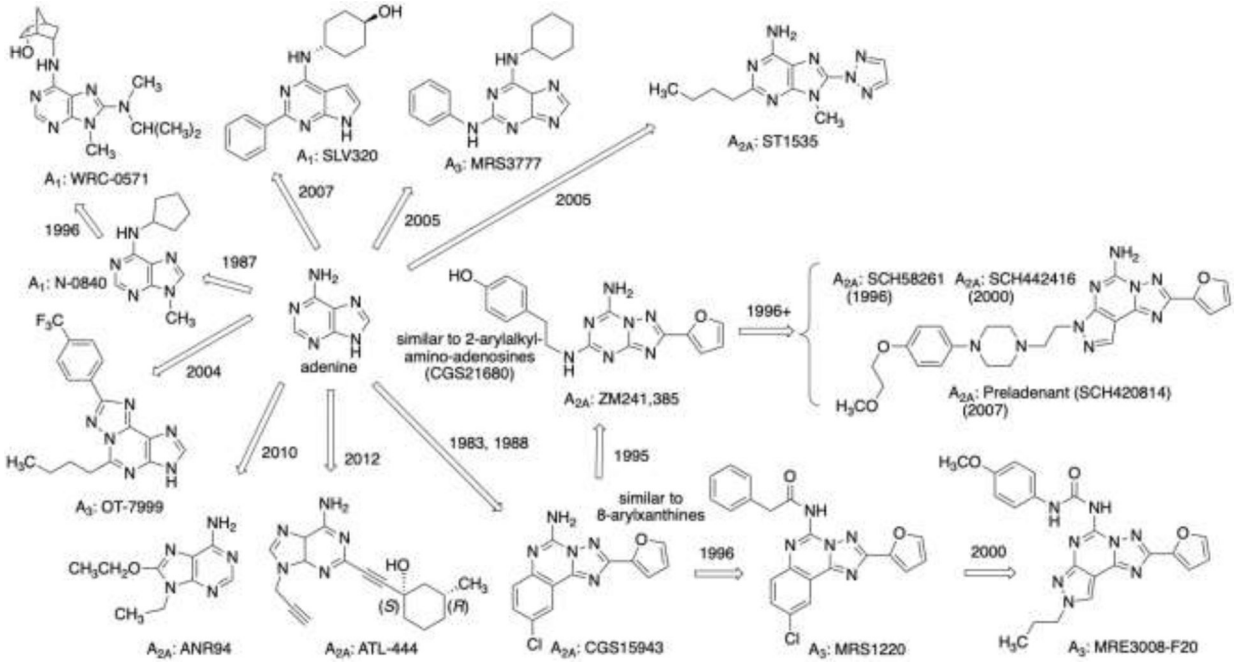

Figure 5.

Adenosine receptor antagonists derived from an adenine scaffold, stemming from early key reports [105,136,139]. Without the ribose moiety of adenosine, adenine derivatives are consistently antagonists or inverse agonists at the adenosine receptors. Diverse A_{2A} antagonists have been in clinical trials for Parkinson's disease and cancer immunotherapy. The receptor affinities of many of the compounds cited can be found in the original references [6–8,12,105,146,161]. Years shown for each compound indicate an early, major report in the literature.

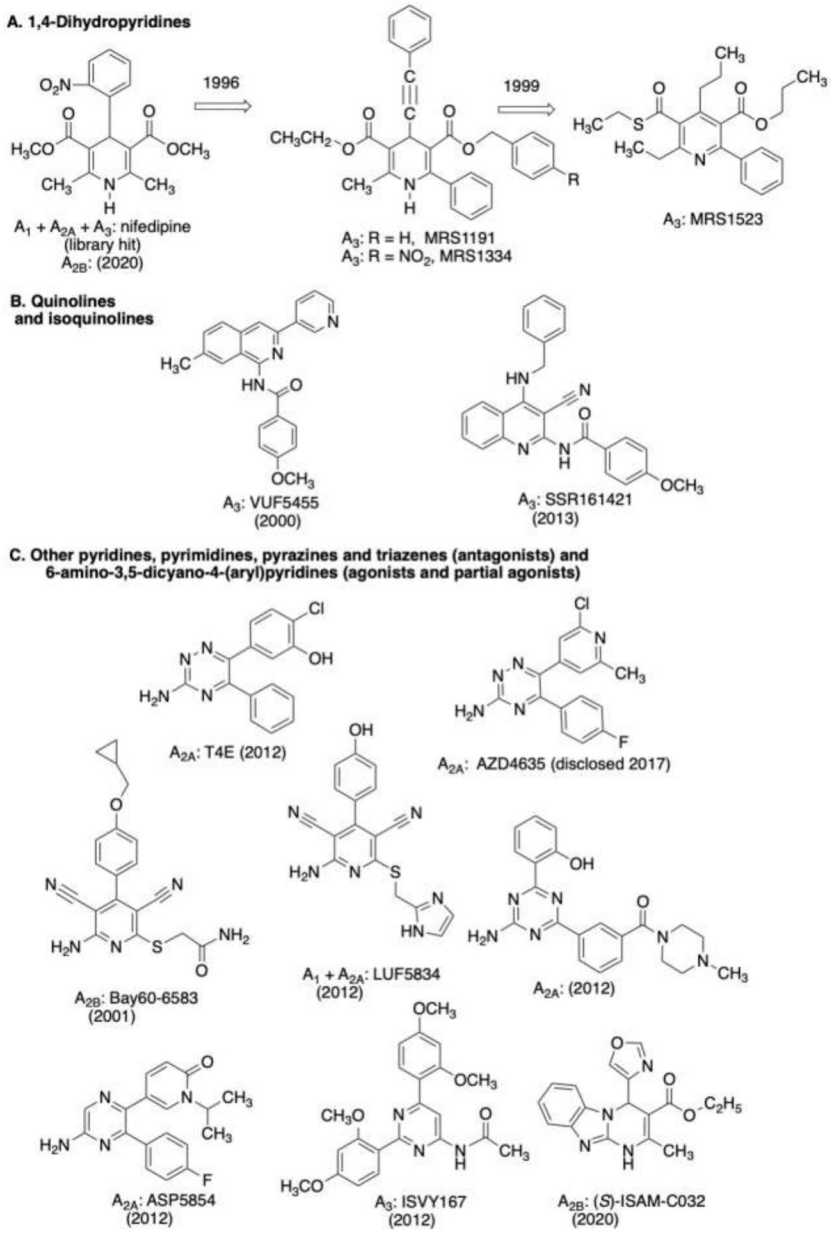

Figure 6.

Adenosine receptor ligands based on other selected heterocyclic scaffolds, particularly those that were modified from library screening hits and can be assembled by multicomponent condensation. Numerous non-purine heterocycles have been found to bind to adenosine receptors (particularly A_{2A} and A_3 receptors [7,8]), and these compounds represent a small fraction of those diverse structures. 3,5-Dicyanopyridines (C) are agonists or partial agonists [106,144], while the other heterocycles shown are antagonists. The receptor affinities of many of the compounds cited can be found in the original references [6–8,12, 106,107,144,154,169,170,172]. Years shown for each compound indicate an early, major report in the literature.

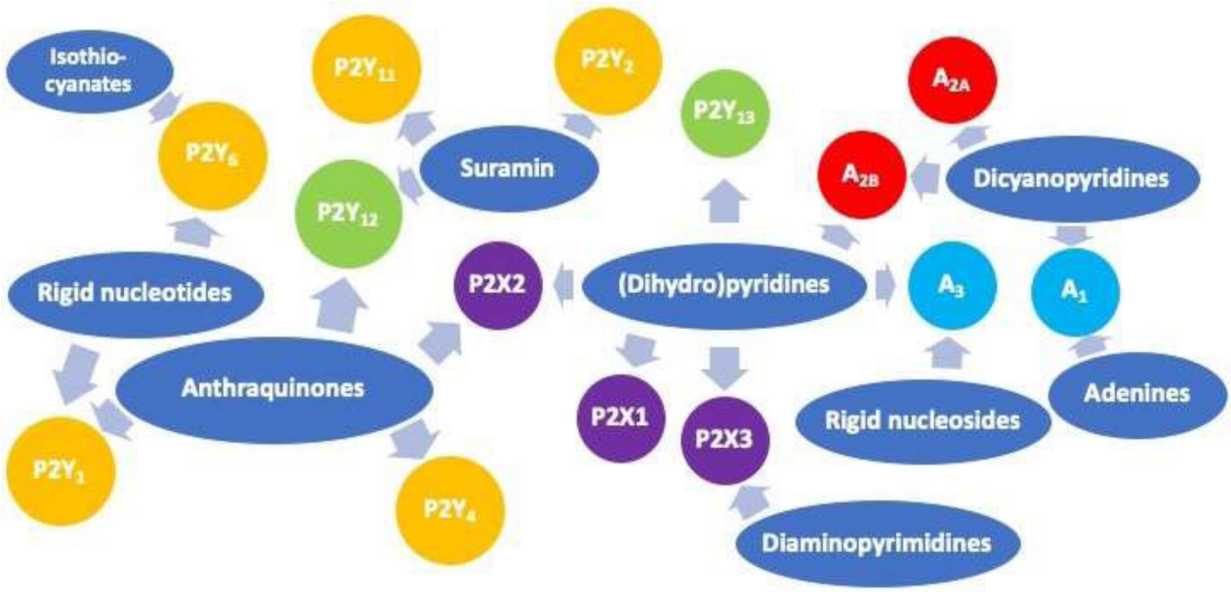

Figure 7.

Summary of some of the P2X, P2Y and adenosine receptor interactions of the versatile scaffold classes (blue) presented here. The receptor color coding is: P2X ion channels (violet), P2Y (G_q-coupled in orange, G_i-coupled in green) and adenosine (G_i-coupled in cyan, G_s-coupled in red). Agonist/antagonist ligands are not distinguished. The large class of xanthine antagonists (Figure 4) is not shown.