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Purinergic regulation: From a risky hypothesis to a triumphant theory

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With the discovery of the ATP structure in 1929, significant progress was made in understanding the role of nucleosides and nucleotides in the body. One of the most important breakthroughs is associated with the determination of the function of an autacoid in ATP, a participant in purinergic signal transmission. For the first time, this function of ATP was pointed out by Professor Geoffrey Burnstock in 1972. Purinergic signaling activators are extracellular nucleotides including ATP, ADP, UTP, UDP, and adenosine nucleoside. The purinergic signaling pathway begins with the synthesis and intracellular accumulation of nucleotides, and then their release from the cell under various physiological and pathological conditions. In the extracellular spaces, nucleotides are hydrolyzed by various enzymes with the removal of phosphate groups, which leads to the appearance of various regulatory molecules that interact with P1 and P2 purinergic receptors. This ligand-receptor interaction changes the functional state of the target cell. In turn, the expression of purinergic receptors changes depending on the functional state of the cell. The participation of purinergic regulation in the development of many diseases indicates that by changing the concentration of signaling molecules, it is possible to change the course of pathological processes, in particular the activity of inflammation and the direction of immune responses. This article provides a brief review of the literature on the structure of nucleotide and nucleoside autacoids, enzymes involved in their metabolism, specific purinergic receptors.

Keywords: purinergic signaling pathway, Adenosine, ATP, Purinergic regulation, P1 and P2 receptors.

Introduction

The discovery of ATP in 1929 was first reported by 2 groups of researchers, Karl Lohmann [1] from Germany and Cyrus Hartwell Fiske, and Yellapragada Subbarow [2] from the USA. In the same year, Alan Drury and Albert Szent-Györgyi discovered that adenosine nucleoside and adenylic acid (adenosine 5'-monophosphate, 5'-AMP) act as signaling molecules in the cardiovascular system [3]. However, a more complete understanding of the adenosine-5′-triphosphoric acid (ATP) regulatory role and the creation of the concept of purinergic signaling pathway is associated with the Jeffrey Bernstock's research. In the 1960s, as a young researcher, he studied autonomic neurotransmission in which two main transmitters (acetylcholine and noradrenaline) were not involved [4]. The term purinergic signaling pathway was first time used by Burnstock in an article of Pharmacological Reviews in 1972 [5]. He suggested that stimulation of any nerves releases a whole "cocktail" of biologically active substances, transmitters, and modulators can significantly affect the effectiveness of the main transmitter. The idea of co-transmission was not accepted by the scientific community for a long time, since the scientific authority of the Nobel Prize laureate Henry Dale, who argued (Dale's principle) that each neuron emits only one type of neurotransmitter [6], was incomparably higher than that of Professor Burnstock who was not widely known in the scientific community. His other hypothesis was even more revolutionary. According to this hypothesis, there are also some "noncholinergic, nonadrenergic" nerves in the autonomic nervous system aside from cholinergic and adrenergic nerves, and the effects of which are not mediated by classical mediators, acetylcholine, and noradrenaline. After doing a lot of experiments and analysis, Burnstock suggested that purine compounds, such as adenosine and adenosine-5′-triphosphoric acid (ATP), serve as mediators in these nerves, and therefore, he called them purinergic nerves [7]. However, since both of these purines are widespread in the body and are present in absolutely all cells, it was difficult to imagine their role as specific signaling molecules in the nervous system. It was objected to Bernstock that ATP cannot be an intermediary due to its widespread occurrence, molecule instability, and high molecular electric charge. Although the extracellular hydrolysis of ATP in tissue was demonstrated as early as the 1930s, the analysis of these processes required the development of more advanced biochemical methods, which became available decades later. Important discoveries of the early 1990s confirmed the existence of the purinergic signaling pathway. The first G protein-coupled receptor (GPCR) for ATP (P2Y1 receptor) was cloned by Burnstock and Julius in 1993. The purinergic hypothesis shortly became one of the most hotly debated topics in neurophysiology and neuropharmacology [8].

The model developed by Bernstock for the synthesis, release, storage, and inactivation of ATP at the purinergic neuromuscular junction is still relevant. Per this model, ATP is released by various cells, and this process is significantly enhanced under conditions of cellular stress or damage. Further, ATP is rapidly converted to adenosine by the enzymes ectonucleotidases. The original term ecto-ATPase (1955), ectoenzyme and ecto-apyrase were used at the International Symposium on Enzyme Chemistry in 1957 by Wladimir A. Engelhardt and Tatjana Wenkstern [9]. The main stages of the study of the purinergic system are shown in figure 1. In this review, we present the structures of nucleotide and nucleoside mediators, the types of enzymes involved in their metabolism, and specific purinergic receptors.

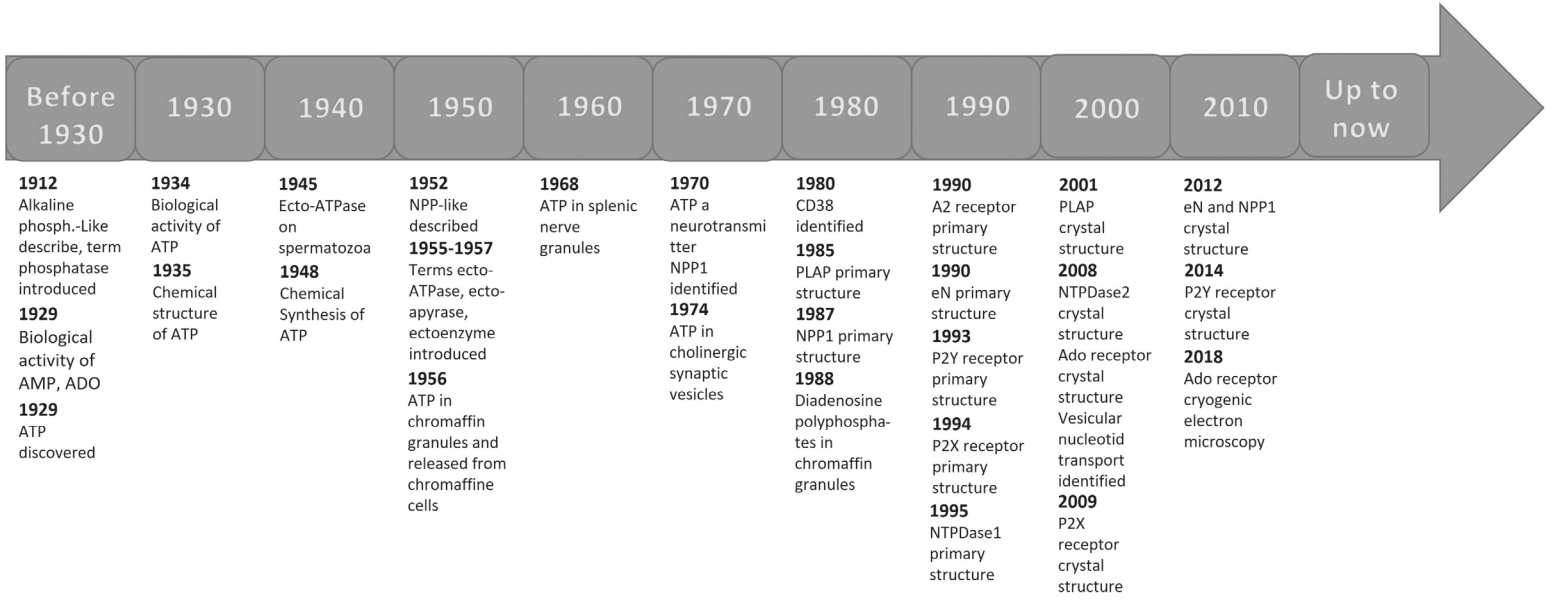

Fig. 1. A brief history of the purinergic signaling pathway discovery (adapted from [10]). In the 1980s the first efforts were made to purify ectoenzymes of this pathway. For the first time, ATP diphosphohydrolase was purified to homogeneity from the human placenta in 1995. In addition, in 1996 the apyrase enzyme that catalyzes the hydrolysis of ATP to yield AMP and inorganic phosphate (Pi) was cloned from potato tubers and was demonstrated to be related to CD39. Then a single mammalian ecto-apyrase was sequenced and expressed in 1997. Later it was demonstrated that this enzyme preferentially hydrolyzes ATP and appeared to function as an ecto-ATPase rather than an ecto-apyrase. In 1998, the identification of four paralog enzymes in the human displayed that an entire gene and protein family must exist. Now eight paralogs are encoded in the mammalian genome

Extracellular nucleotides and nucleosides

Nucleotides and nucleosides are ubiquitous molecules that are involved in many cellular processes such as the formation of nucleic acids, energy intermediates, allosteric modulators, coenzymes, and signaling. Almost all cells secrete nucleosides and nucleotides in various physiological and pathological conditions, such as cellular stress, infections, inflammation, pain, and cancer. Under normal conditions, ATP and other nucleotides are found in the extracellular space in the nanomolar concentration. But in the intracellular space, their concentration is much higher, from 5 to 10 mmol/L [11]. The peculiarities of nucleotides are hydrophilicity and rapid hydrolysis in the extracellular space.

Adenosine formed after enzymatic dephosphorylation of ATP can transport across cell membranes by the special carrier proteins [12]. Adenosine has a multidirectional effect on cells [13; 14]. With an increase in extracellular concentration, adenosine can dump the immune response, as it amplifies the signal from certain immune mediators that suppress the activity of immune cells. This situation is observed in chronic inflammation or malignant tumors [15]. Taking into account the possibilities of purinergic regulation, therapeutic agents that can affect the components of this system in various diseases are being developed.

The interaction of nucleotides and nucleosides with two main groups of purinergic receptors (P1 and P2) on cell membranes [16] determines the activity of neurons, glia, platelets, as well as various types of cells of the cardiovascular, immune, endocrine, gastrointestinal and other systems [11; 17].

Enzymes involved in the purinergic pathway

The degradation of nucleotides and nucleosides is mediated by a wide range of enzymes, some of which are shown in figure 2.

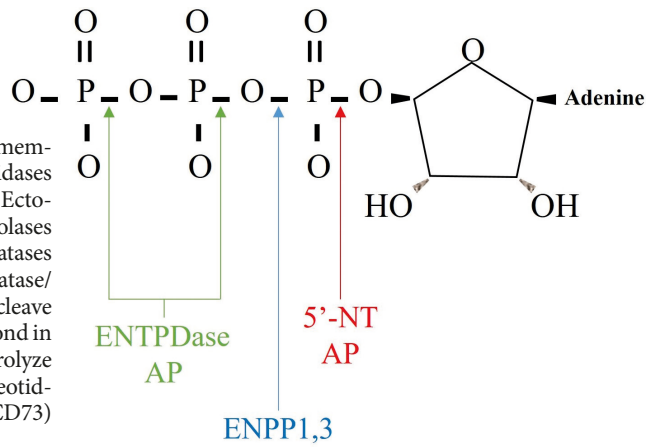

Fig. 2. Different cleavage sites of members of the four types of ectonucleotidases on extracellular ATP, ADP, and AMP. Ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases, CD39), alkaline phosphatases (APs), and ecto-nucleotide pyrophosphatase/phosphodiesterases (ENPPs) 1 and 3 cleave ATP and ADP. ENPPs cleave the same bond in ATP and ADP whereas ENTPDases hydrolyze different bonds. Eventually, ecto-5′ nucleotidase (5′-NT) and APs hydrolyze AMP (CD73) (adapted from [11])

Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases). E-NTPDases (CD39 family) are the most important nucleotide-hydrolyzing enzymes involved in purinergic signaling. They catalyze the hydrolysis of the γ and β-phosphate residues of triphosphonucleosides (ATP, UTP), and diphosphonucleosides (ADP, UDP). NTPDases

are involved in various aspects of adenosine receptor signaling, including termination of P2 receptor activation, protection of sensitive P2 receptors from desensitization, and enhancement of some receptor activation [18].

Four members of NTPDases including NTPDase1/CD39, NTPDase2/CD39L, NTPDase3/CD39L3, and NTPDase8 are located at the cell surface and the NTPDase5 and NTPDase6 are in secretory form. Membrane-bound NTPDase1 (NTPDase1/CD39) hydrolyzes ATP almost directly to AMP with the temporary formation of small amounts of free ADP [19]. In contrast, NTPDase2, upon hydrolysis of ATP, releases mainly ADP, an agonist for nucleoside diphosphate-sensitive receptors such as platelet receptors P2Y1 and P2Y12 [20]. Further, ADP is slowly dephosphorylated to AMP by ecto-5'-nucleotidase (Ecto5'NTase/CD73). NTPD1 is expressed on natural killer cells (NK), dendritic cells (DC), monocytes, and subpopulations of activated T cells [21; 22]. This enzyme plays an important role in the creation of regulatory nucleotides that regulate neutrophil chemotaxis and inflammatory activity [21].

Ecto-nucleotide phosphodiesterase/pyrophosphatases (E-NPPs). In this enzyme family, there are seven members include ENPP1 to 7. They can hydrolyze ATP to AMP but not to adenosine because their cleavage sites are different from ENTPDase (Figure 2). ENPP1, 2, and 3 hydrolyze pyrophosphate or phosphodiester bonds in ATP and ADP, nicotinamide adenine dinucleotide (NAD^+), flavin adenine dinucleotide (FAD), and possibly also cyclic AMP (cAMP). These enzymes are expressed on epithelial surfaces of respiratory epithelium, liver epithelium, kidney epithelium, and intestinal epithelium [23].

ENPP1 (CD203a) can hydrolyze both NAD^+ and adenosine diphosphate ribose (ADPR) to AMP. ADPR is produced by cyclic ADP ribose hydrolase (CD38), and further, it is processed by CD203a into AMP. The CD38-CD203a enzyme axis, operating independently or in synergy with the CD39/CD73 pathway, and contributes to the generation of the adenosine [24]. ENPP1 hydrolyzes extracellular ATP to form inorganic pyrophosphates (PPi), which are involved in bone mineralization, the calcification of vascular smooth muscle cells, and other tissues during inflammation [25]. ENPP1 is expressed in several immune cells and also is involved in changing the phenotype of macrophages from M1 to M2. As a result, inhibition of ENPP1 may provide immune regulation in the treatment of cancers and pathogenic infections like tuberculosis [26].

ENPP1, 3, 6, and 7 can also hydrolyze phospholipids which are singled out by lysophospholipase D (lysoPLD), this process is associated with the production of the bioactive lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) [27].

Ecto-5′-nucleotidase (5′-NT). 5′-nucleotidase (CD73) is an enzyme that produces adenosine from AMP. CD73 has enzymatic function as a nucleotidase and non-enzymatic function as an adhesive molecule that can regulate cell interaction with extracellular matrix components [28]. 5′-NT is expressed by several types of cells such as stromal cells, follicular dendritic cells, endothelial cells, regulatory T cells (Treg), B lymphocytes, and many tumor cells. Under conditions of hypoxia and the action of inflammatory mediators, the activity of this enzyme is significantly increased [29; 30]. Lung cells, including the epithelium, vascular endothelium, or immune cells, express high levels of CD73 and can provide high production of extracellular adenosine during acute lung injury [31]. The activity of 5′-NT also rises as the concentration of adenosine increases. 5′-NT is upregulated in neutrophils, monocytes, and macrophages which is paralleled by enhanced expression of A2A receptors in them. Modulation of 5'-NT activity allows the conversion of inactive

precursors of the A2A receptor agonist into an active anti-inflammatory form at the sites of inflammation [32].

Alkaline phosphatases (APs). Alkaline phosphatases (APs) are a family of endogenous metalloenzymes that are present in various organs and blood serum [33]. AP has hydrolytic phosphatase and transphosphorylase activity targeting a variety of molecules [34].

AP is involved in immune responses to tissue damage and inflammation in many diseases [35; 36]. This enzyme binds to immunoglobulins in the blood and with an increase in the concentration of immunoglobulins, its activity also increases. The increased activity of serum AP in many inflammatory conditions can be used as a protective agent to reduce systemic inflammation. There is a strong positive correlation between AP activity and adenosine production [37]. It was shown that AP may be the main soluble blood ectonucleotidase in the setting of cardiopulmonary bypass surgery [38].

Adenosine deaminase (ADA). Adenosine deaminase (ADA) is one of the most important enzymes involved in the conversion of adenosine to inosine. Inosine has immunomodulatory, cardioprotective, and cytoprotective effects [39; 40]. ADA isoforms in humans include ADA-1, -2, -3. ADA-1 may exist either as low molecular weight or as a high molecular complex with the ADA-binding enzyme dipeptidyl-peptidase IV (CD26) or the adenosine receptor subtypes A1 and A2B [41]. Besides taking part in adenosine catabolism, ADA-1 may interact with cell surface anchoring proteins, acting as an ectoenzyme. ADA-1 can be expressed in different tissues and cells like the thymus, spleen, intestine, dendritic cells, and lymphocytes [11; 42; 43]. In pathological conditions such as inflammation, myocardial ischemic injury, leukemia, and lymphomas, this enzyme is a suitable drug target for their management [44; 45].

ADA-2 displays high similarity with the enzymatic mechanism of ADA-1, but it has been mainly localized in the extracellular space. Scarce information is currently available about the recently discovered ADA-3 or ADA-like protein (ADAL) [46].

ADA deficiency disrupts the metabolism of deoxynucleotides and S-adenosyl-L-methionine (AdoHcy) — dependent reactions of cellular transmethylation, which leads to the accumulation of toxic deoxyadenosine, which can kill cells, especially thymocytes [47]. In patients with partial or complete ADA deficiency, disorders of purine metabolism can be found, such as an increase in adenosine and deoxyadenosine in blood plasma, deoxyadenosine in urine, and a decrease in ADA activity in erythrocytes [47; 48]. In patients with milder disorders, T-cell lymphopenia often precedes overt immunodeficiency [48].

Purinergic receptors

Most cells express two main groups of purinergic receptors — P1 for adenosine and P2 for ATP, ADP, UTP, and UDP. In turn, receptors of the P2 family are defined as ionotropic (P2X) and metabotropic (P2I). The latter, like the P1 receptors, belong to class A (rhodopsin-like family) of the superfamily of G-protein coupled receptors (GPCRs) [49].

P1 (ADORAs) adenosine receptors. There are four different types of these receptors in this family include A1, A2A, A2B, and A3 receptors, which result in different biological functions [50]. In general, the A1 receptor (A1R) and A3R decrease the cAMP levels, but A2AR and A2BR increase it [24].

A1R mediates the inhibition of adenylate cyclase, induces activation of phospholipase C, and inhibits G-protein-coupled activation of voltage-dependent Ca^{2+} chan-

nels [51]. A1 receptors are found extremely in the CNS, adipose tissue, heart muscle, inflammatory cells especially in neutrophils, and immature DC. These receptors can be activated at 0.3–3 nM concentration of adenosine [52].

It has been shown that their agonist ligands A1R have the therapeutic potential as atrioventricular node block and supraventricular tachyarrhythmia and are candidates for the treatment of bradyarrhythmia associated with interior myocardial infarction, cardiac arrest, cardiac transplant rejection [53].

A2 receptors are classified into the A2A (high affinity, activated by adenosine concentration 1–20 nM) and A2B (low affinity, activated by adenosine concentration more than 1 μM) receptors [54].

A2A receptors are found in the neurons, blood platelets, olfactory bulb, spleen, thymus, leukocytes, heart, lung, and blood vessels [55]. A2A agonist receptor ligands are being studied to cure respiratory disorders, sepsis, reperfusion injury, thrombosis, hypertension, and inflammatory disorders by enforcing and blocking A2AR dependent immunomodulatory mechanisms [56]. Adenosine, by binding to A2A, exhibits anti-inflammatory properties and can suppress inflammation, protect against inflammation in trauma and neurodegeneration [57]. There is evidence that the expression of the A2A receptor on human monocytes is raised by pro-inflammatory cytokines like interleukin-1 (IL-1) and tumor necrosis factor (TNF) [58]. Ligation of A2A receptors on monocytes and macrophages increases the secretion of IL-10, which has immunosuppressive properties [59].

A2B receptors are expressed in the gastrointestinal tract, cecum, colon, bladder, lung, blood vessels, adipose tissue, adrenal gland, brain, kidney, mast cells, stem cells, lymphocytes, and macrophages [60]. IFN-γ prevents activation of the adenosine receptor (A2BR) on macrophages, thus maintaining their activation during inflammation. A2B receptors in mast cells cause an increase in the production of pro-inflammatory mediators in inflammatory airways disease. A2B agonist receptor ligands have therapeutic potential in allergic reactions, asthma, and pulmonary inflammation [61].

A3 receptors inhibit adenylate cyclase activity, while stimulate directly phospholipases C and D [57]. They are highly expressed in testis (rat), mast cells (rat), cerebellum, hippocampus, thyroid, most of the brain, adrenal gland, spleen, liver, kidney, heart, DC, lymphocytes, eosinophils, and macrophages, but not present on human lung mast cells [62]. A3 agonist receptor ligands are studied in experimental trials of cardiac ischemia, arrhythmias, glaucoma, asthma [63; 64].

P2 receptors. Unlike the P1 receptor, P2 receptors recognize nucleotides. They have an affinity range for extracellular nucleotides from 100 nM to 1 mM. The P2 receptors are classified into two main families, P2Y and P2X. P2Y (G protein-coupled) receptors trigger downstream effector signaling pathways ending with increased concentration of intracellular Ca^{2+} or cAMP. P2X, the ATP-gated ion channels, allow Na^{+}, Ca^{2+} influx, and K^{+} efflux [65]. P2Y receptors have eight subtypes include P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. P2X receptors have seven subtypes with six homomeric contain P2X1 to P2X5 and P2X7 and six heteromeric P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, and P2X4/6 receptors [66; 67].

The P2X7 receptor is a rather unusual receptor/channel since it generates a non-selective plasma membrane pore that allows the transit of aqueous molecules with a molecular weight up to 900 Da [68]. P2X7 receptor plays a key role in immunity and inflammation as a major activator of the NLRP3 inflammasome, and therefore a powerful trigger of IL-1β

and IL-18 maturation and secretion [11]. Also, P2X7 stimulates reactive oxygen species (ROS) production in macrophages via the mitogen-activated protein kinase (MAPK) and the nicotinamide adenine dinucleotide phosphate oxidase complex. This receptor is involved in several inflammatory conditions, such as sepsis, arthritis, granuloma formation, and others [68].

P2Y1, P2Y2, P2Y4, and P2Y6 receptors can activate Gq and phospholipase C-β (PLC-β), producing inositol triphosphate (IP3), which raise intracellular Ca^{2+} level through its release from intracellular stores, and diacylglycerol (DAG), which in turn activates protein kinase C (PKC) [16].

P2Y12, P2Y13, and P2Y14 receptors activate protein Gi and can inhibit adenylyl cyclase, and therefore the reduction of cAMP levels. Stimulation of P2YR11 via activation of Gq and Gs triggers the growth of intracellular Ca^{2+} and cAMP [69].

Conclusion

The first evidence of purinergic cell-to-cell signaling was presented by Bernstock in the 1970s, and by 2009 all components of the purinergic signaling pathway had already been identified. Since 2015, the complex of proteins and cofactors that are involved in the fundamental aspects of purinergic signaling and cellular homeostasis is termed "purinom" [70]. There is no longer any doubt that purinergic signaling leads to a wide range of cellular responses. Purinergic regulation has been studied as an important factor of hormone secretion, neurotransmission, and neuromodulation, in the regulation of specialized functions of various organs, such as kidneys, liver, cardiovascular, immune, and respiratory systems [11]. In the study of cognitive functions, special attention was paid to the study of purinergic receptors on neurons and immune cells of the brain. However, the range of cells for which the purinergic regulation is extremely important continues to expand. Recent studies in mice revealed that signaling through the erythrocyte adenosine A2B receptor (ADORA2B) promotes O_2 release to counteract hypoxia, and the loss of erythrocyte–specific A2B receptors enhances brain hypoxia and accelerates the early onset of age-related impairments in spatial learning, memory, and hearing ability [71].

It should be noted that purinergic mediators/autacoids are involved in different ways in many tissues. For instance, if the level of adenosine in the blood raises, blood circulation in the coronary arteries may improve. However, in the lungs, adenosine causes a narrowing of the airways, and in the kidneys, it reduces the production of renin (angiotensinogenase), which reduces renal blood flow. In addition, adenosine is an inhibitory neurotransmitter in the brain. During nighttime sleep, the level of adenosine in the brain increases, improving sleep quality and suppressing arousal [72]. This versatility of activity creates conditions for many "side effects" and determines the difficulties in the clinical use of purine mediators. Currently, for the prevention of thrombotic complications in diseases of the cardiovascular system, clopidogrel, an antagonist of the P2Y12 receptor, is widely used. The efficacy and safety of this drug, which inhibits platelet activation, is because P2Y12 receptors are present only on platelets and microglia. Therefore, the structure of the drug, which does not allow penetration through the blood-brain barrier, ensures its accurate targeting on platelets. However, for other purines, it should be noted that purinergic mediators/autacoids are involved in different ways in many tissues. For instance, if the level of adenosine in the blood raises, blood circulation in the coronary

arteries may improve [72]. For other purine receptors, such selective localization is not typical, which complicates the use of their agonists/antagonists in clinical practice.

So far, purinergic regulators are being actively studied as resources for the diagnosis or treatment of various diseases (Table) [54].

Table. **Examples of purinergic receptor subtypes as therapeutic targets. Some of them are already in clinical use, while others are in clinical trials or proof of concept studies (adapted from [54])**

Receptor type	Family	Subfamily/Class		Diseases
Adenosine Receptors	P1	A1		Supraventricular tachycardia
		A2A		Scleroderma
				Parkinson's disease
				Coronary artery disease
				Neurodegenerative and psychiatric diseases
		A2B		Asthma
		A3		Psoriasis
	P2	P2X	P2X3	Chronic cough
				Hypertension
				Visceral pain
				Overactive bladder
				Migraine
				Atherosclerosis
			P2X4	Neuropathic pain
			P2X5	Inflammatory bone loss
			P2X7	Atherosclerosis
				Infection
				Abdominal pain
				Rheumatoid arthritis
				Neurodegenerative and psychiatric diseases
				Asthma
				Autoimmune diseases
				Ulcerative colitis/Crohn's disease
				Renal disease
				Cancer
		P2Y	P2Y2	Dry eye
				Cancer
				Muscular dystrophy
			P2Y12	Thrombosis and stroke
				Osteoporosis

Finally, it is important to note that purinergic signaling is a very early evolutionary mechanism, that was formed in bacteria [73]. It has been shown that bacteria have their enzymes of purine metabolism and in some cases use them to stimulate excessive inflammation. With uncontrolled inflammation, the conditions for stimulating a protective immune response are disrupted and bacteria escape from destruction in phagocytic cells, which makes it possible to establish chronic forms of infection [74]. Thus, the study of the characteristics of purinergic regulation in infections can indicate new ways to increase resistance to infectious and non-infection diseases.

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