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Potential for Developing Purinergic Drugs for Gastrointestinal Diseases

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Abstract

Treatments for IBD, IBS, FD or motility disorders are not adequate, and purinergic drugs offer exciting new possibilities. GI symptoms that could be targeted for therapy include *visceral pain, inflammatory pain, dysmotility, constipation and diarrhea*. The focus of this review is on potential for developing purinergic drugs for clinical trials to treat GI symptoms. Purinergic receptors are divided into adenosine P1 (A_1,A_{2A},A_{2B},A_3), ionotropic ATP-gated P2X ion channel ($P2X_{1–7}$) or metabotropic $P2Y_{1,2,4,6,11–14}$ receptors. There is good experimental evidence for targeting A_{2A}, A_{2B}, A_3, $P2X_7$, $P2X_3$ receptors or increasing endogenous adenosine levels to treat IBD, inflammatory pain, IBS/visceral pain, inflammatory-diarrhea and motility disorders. Purine genes are also potential biomarkers of disease. Advances in medicinal-chemistry have an accelerated pace toward clinical trials: Methotrexate and sulfasalazine, used to treat IBD, act by stimulating CD73-dependent adenosine production. ATP protects against NSAID-induced enteropathy and has pain-relieving properties in humans. A $P2X_7$R antagonist AZD9056 is in clinical trials for CD. A_3 AR drugs target inflammatory diseases (e.g. CF101; CF102). Dipyridamole, a nucleoside uptake-inhibitor, is in trials for endotoxemia. Drugs for pain in clinical-trials include $P2X_3$/$P2X_{2/3}$(AF-219) and $P2X_7$(GSK1482160) antagonists and A_1(GW493838) or A_{2A}(BVT.115959) agonists. Iberogast^R is a phytopharmacon targeting purine-mechanisms with efficacy in IBS and FD. Purinergic drugs have excellent safety/efficacy profile for prospective clinical trials in IBD, IBS, FD and inflammatory-diarrhea. Genetic polymorphisms and caffeine consumption may affect susceptibility to treatment. Further studies in animals can clarify mechanisms and test new-generation drugs. Finally, there is still a huge gap in our knowledge of human pathophysiology of purinergic signaling.

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1.0 Introduction

The purinergic field has come a long way since the original experiments done to identify the non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in the gut. [1] Recent studies challenge the view that adenosine triphosphate (ATP) is the main purinergic transmitter involved in gut neuromuscular transmission in mice, primates and humans. [2–5] Cloning experiments roughly 2 decades ago identified four G protein-coupled adenosine (P1) receptors (A_1, A_{2A}, A_{2B}, A_3), seven subtypes of P2X ion channel receptors for nucleotides ($P2X_{1–7}$) and 8 G protein-coupled receptors for nucleotides ($P2Y_{1,2,4,6,\ 11–14}$ receptors). [6] The P2X receptor channels consist of subunit trimers, which often are heterogeneous combinations of the various P2X proteins. Therefore, the medicinal chemistry of the P2X system is more challenging than the adenosine and P2Y receptors. Nevertheless, advances in medicinal chemistry are providing highly selective compounds for all 3 families of purinoceptors (P1, P2X and P2Y) to study the pharmacology, pathophysiology and therapeutic potential of targeting such receptors. A recent comprehensive review by Burnstock [7] covers purinergic signaling in the GI tract and related organs in health and diseases, in all cell types that express purinergic receptors. The focus of this review is on potential for developing purinergic drugs for clinical trials for GI diseases or disorders, and in particular inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), functional dyspepsia (FD), motility disorders and diarrheal disorders. Current treatments strategies are inadequate, and purinergic drugs offer exciting new possibilities. GI symptoms that can be targeted for therapy include *visceral pain, dysmotility, constipation, gastroparesis and diarrhea*.

Purinergic signaling plays an important role in gut neural reflexes, and most if not all purinergic receptors have been identified in the gut, differentially distributed on different cellular components of the gut, including several types of neurons in the enteric nervous system (ENS, sensory, interneurons and motor neurons), epithelial cells, immune/inflammatory cells, enterochromaffin cells (EC), glial cells, interstitial cells of Cajal and smooth muscle. [8,9] Purines in the gut are involved in secretion, immunomodulation, synaptic transmission, neuromuscular transmission, gliotransmission, and visceral sensation. Key studies utilizing gene knockout models to investigate the role of purines in IBD or functional GI disorders (e.g. IBS and FD) are referenced in Table 1. As discussed later, purines act as ‘danger signals’ and are very sensitive to inflammation or the health-disease state of tissues and organs. Therefore, abnormalities in purinergic signaling are a hallmark of IBS, IBD, chronic inflammation, diabetic neuropathy or other disease-states. Purines are important regulators of the neurophysiology of the gut, and therefore such abnormalities are linked to significant pathophysiology. Abnormalities in purinergic signaling are summarized in Table 2.

There is good experimental evidence for targeting A_{2A}, A_{2B}, A_3, $P2X_7$, $P2X_3$ receptors or increasing adenosine levels to treat IBD, inflammatory pain, visceral pain in IBS or FD and inflammatory-diarrhea, and key studies will be discussed. More comprehensive reviews have been written on the basic physiology, pathophysiology and signaling mechanisms of purinergic signaling in the GI tract. [7,10–14] There is also an expanding list of 'purinergic drugs' in clinical trials for relevant diseases that will be reviewed, and several drugs have clinical efficacy in IBD, IBS or FD. Some of the challenges of developing purinergic drugs are given special consideration.

2.0 Medicinal chemistry

Adenosine (**1,** or derivatives) is used as a drug since the 1990's. **Adenocard and adenoscan** are the generic forms of adenosine used for the treatment of supraventricular tachycardia and for cardiac stress testing, respectively. The medicinal chemistry of adenosine A_1, A_{2A} and A_3 receptors (P1 family) is well developed and many selective ligands are available for ligand binding, pharmacological analysis, in vivo studies in animal models of disease, and a significant number are being pursued in human clinical trials. Advances in medicinal chemistry are providing a pipeline of new potential drugs for testing in animals and humans. **Regadenoson** (**68**, **Lexiscan**, Astellas Pharma) is the first selective A_{2A} agonist approved by the FDA, which acts as a potent vasodilator. Drugs targeting adenosine receptors or which elevate endogenous adenosine levels are currently in advanced clinical trials as treatment for chronic heart failure, inflammatory and autoimmune disorders, dry eye syndrome, neurological disorders (e.g. Parkinson's disease), hepatocellular carcinoma, uveitis, cardioplegia, neuropathic pain, FD, IBS, perioperative pain as well as stress/diagnostic agents. The medicinal chemistry of P2X and P2Y receptors is not as well-developed [15–17], although various P2X and P2Y drugs have progressed into clinical trials, and $P2Y_{12}$ antagonists including clopidogrel (Plavix; Sanofi-Aventis/Bristol-Myers Squibb) is FDA approved and used widely to block platelet aggregation in the management of clot related cardiovascular events. [18]

The affinities of commonly used adenosine receptor ligands for studying adenosine receptors are summarized in Table 3. Structures of commonly used ligands of adenosine (A_1, A_{2A}, A_{2B}, A_3) and $P2Y_{1,2,4,6,11–14}$ receptors are shown in Figures 1A and 1B, respectively. Some of those compounds are currently in clinical trials, but the structures of most of the compounds mentioned in this review in a clinical context are shown in Figure 2A (adenosine system) and Figure 2B (P2X and P2Y systems). Table 4 summarizes selected purinergic drugs in clinical trials found on ClinicalTrials.gov that are described throughout the review. Table 5 lists selected ligands that represent new generation drugs that have been shown to have efficacy in animal models of IBD or IBS but have not yet made it to clinical trials.

Polymorphisms of genes involved in purinergic signaling are important considerations in designing clinical trials to test safety and efficacy of new potential drugs. Important genetic variants, such as for $ADOA_{2A}R$, $P2X_7R$, CD39 and PON1, can alter susceptibility to disease or efficacy to treatment, which will be further discussed latter. [18–26]

3.0 Adenosinergic drugs

3.1 A_3 AR medicinal candidates in Inflammatory Bowel Diseases

3.1a) Experimental therapeutics of A_3 AR—Adenosine receptors (A_1, A_{2a}, A_{2B} and A_3) are being investigated as therapeutic targets for chronic inflammatory disorders including IBD, autoimmune disorders and cancer. Adenosine is a potent anti-inflammatory agent and its actions are mediated in part through A_3AR activation. A_3AR agonists have been shown to be beneficial in experimental models of colitis. [27,28] In a model of colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), the prototypical A_3AR agonist IB-MECA (**9**, CF101) was very effective in ameliorating colitis in rats treated with 3mg/kg IB-MECA i.b.d. for 7 days, and the drug protected animals against weight loss, developing GI symptoms (*diarrhea, occult blood, mucosal inflammation*) and prevented changes in gene-expression profiles associated with chronic mucosal inflammation. [28] The beneficial effect of IB-MECA in murine models of colitis (including IL-10 KO mice and dextran sodium sulfate [DSS]-induced colitis) was less impressive, and species or model differences may explain the outcomes – this deserves further consideration. Differences in different experimental models of IBD may be a result of differential sensitivity to IB-MECA in models of Crohn's disease (CD) (e.g. TNBS colitis with transmural inflammation) and ulcerative colitis (UC) (e.g. DSS colitis with mucosal inflammation). This may be important because there are seversal clincial phenotypes of UC and CD, and it is possible that IB-MECA would be more effective in CD than UC, and in a particular phenotype.

Recent studies in $A_3^{-/-}$ deficient mice suggested instead a pro-inflammatory role for A_3 AR activation during development of colitis. [29] Mice lacking a functional A_3AR ($A_3^{-/-}$ AR phenotype) was less susceptible to DSS-induced colitis [29], and mice were protected against development of severe colitis. The implication is that A_3AR activation contributes to the development of colitis under these experimental conditions. In another study by Butler et al [30], A_3-deficient mice exhibited reduced colon pathology and decreased levels of myeloperoxidase, but the degree of protection was not as pronounced as that seen by Ren et al [29]. Another difference in the latter study is that by day 21 wild-type animals recovered, whereas A_3 deficient mice displayed significantly greater inflammation and a significantly higher burden of tissue-associated bacteria. Together with other findings, their data suggested that disruption of the A_3 AR interferes with neutrophil migration, and impairment of innate immunity prevents the clearance of invading microorganisms in the intestinal mucosa. This suggests that clinical use of A_3 drugs (agonists or antagonists) in IBD or gut infection induced inflammation, could potentially raise the risk of opportunistic infections, [30] making them more risky to use in the clinical setting. Differences in outcomes in the two separate $A_3^{-/-}$ knockout studies may be due to environmental variations, and housing of animals in different facilities could result in variations in microbiota that are known to affect the severity of various colitis models. [31,32] Differences in protocols or mouse strains could also contribute to outcomes. It should also be pointed out that adaptations in other adenosine receptors can occur with global KO mice of adenosine receptors – For instance, $A_{2a}^{-/-}$ AR KO mice exhibit A_{2B} up-regulation. [33] If such adaptation in other receptors occurs in A_3- deficient mice, it could serve to explain outcomes

in colitis. More studies with agonists and antagonists of A_3 receptors and conditional KO mice are needed to clarify the mechanisms of protection.

Our preliminary findings (AGA abstract form) indicate that Cl-IB-MECA (**10,** CF-102) is more effective than IB-MECA (**9**) in attenuating mouse DSS colitis, but both are less effective than A_3 $^{-/-}$AR KO mice. Clearly, more studies in experimental models of IBD and conditional KO mice are warranted to clarify the use of A_3AR agonists or antagonists to treat IBD; as are more studies on therapeutic effect of A_3 drugs in experimental colitis models. What is most encouraging is that use of an A_3AR agonist is beneficial in both animal and human studies of patients with inflammatory diseases (namely RA). [34]

3.1b) Clinical trials with A_3 AR agonists—Studies in animals and humans (clinical trials) indicate that A_3AR is a therapeutic target in inflammatory diseases including rheumatoid arthritis, psoriasis and possibly dry eye syndrome, and A_3 drugs have an excellent safety profile. [34] There is good evidence that the A_3AR expression level is a useful indicator or predictor of a patient's eligibility for treatment with the A_3AR agonist in these diseases. [34]

Can-Fite BioPharma has several pipeline drugs targeting A_3 AR with a good safety profile and oral availability, currently at various stages of development for inflammatory diseases. The following compounds are A_3 agonists and allosteric modulators: CF101 (**9**, IB-MECA) is a prototypical directly-acting A_3 agonist. Results from a phase IIa study in RA patients indicate that the drug has anti-inflammatory activity and is efficacious in RA patients failing methotrexate therapy. Thus far, CF101 has shown a 20% improvement in disease symptoms. An exploratory randomized phase II clinical trial was conducted in 75 patients to evaluate the safety and efficacy of the drug in treating patients with plaque-type psoriasis. CF101 was safe and well tolerated. A 2mg dose given orally twice daily for 12 weeks resulted in progressive improvement in the severity of plaque psoriasis. [35] Another Phase II randomized, double-blind and placebo controlled trial showed that CF101 is beneficial in dry eye syndrome. Notably, doses that are shown to improve dry eye syndrome, do not cause cardiovascular or other side effects. [36,37] CF602 is a new generation drug, a positive allosteric modulator of A_3AR that is being developed as a second-generation anti-inflammatory drug, its efficacy to enhance the protective action of agonist Cl-IB-MECA was shown in an animal model of cardiac ischemia [38]. Other possible indications of A_3 drugs are in the treatment of hepatocellular carcinoma and hepatitis (Phase II trial), as well as in analgesia to control murine and rat chronic neuropathic pain. [34,39]

Recent updates [40] by OphthaliX (subsidiary to Can-Fite) indicate that the CF101 drug failed to meet primary efficacy endpoint in a phase III study for dry eye syndrome; it was however well tolerated. This was a 24-week, placebo-controlled phase III study of 237 patients with moderate to severe dry eye syndrome. Patients received two oral doses of CF101 (0.1mg or 1.0mg) or placebo. As a follow up, OphthaliX is planning a phase III retrospective analysis on the basis of A_3 AR adenosine biomarker status. Can-Fite also announced positive data for a phase II trial in RA. A positive interim analysis was disclosed for a separate phase II/III clinical trial in patients with Psoriasis. Ophthalix is developing the CF101 for uveitis as well but no results are yet available.

There are currently no reported clinical trials with A_3AR drugs in CD, UC, or bacterial induced colitis. Such studies are worth pursuit given the safety and tolerability of these drugs in clinical trials in health subjects or in treating disease. [36,41] Studies are also needed to clarify the cellular mechanisms of A_3AR in healthy and inflamed gut tissues, including human surgical specimens or mucosal biopsies.

Other adenosine receptor agonists are of interest for pain treatment, including selective A_1 (GW493838, **66**) or A_{2A} (BVT.115959, structure not disclosed) agonists.

3.2 Biomarkers of disease

3.2a) A_3AR as a biomarker of disease—A_3AR overexpression occurs in inflammatory cells of both experimental animal models of inflammation and humans. A_3 AR expression was up-regulated in the colons of rats with TNBS-colitis [28] and in the lungs of LPS-induced pulmonary inflammation. [42] Over-expression of A_3 AR was also detected in synovial cells from patients with RA, and in animals with adjuvant-induced arthritis (in synovial cells, paw tissue and drainage lymph nodes). [43] In comparison to healthy control patients, there was over-expression of the receptor in tissues derived from eyes of patients with pseudoexfoliation syndrome. [44] Higher expression of the A_3AR was also found in patients with autoimmune inflammatory diseases including CD, RA and psoriasis [45,46] and animal models of RA. [43] In a phase II clinical trial with CF101 in RA, A_3AR expression levels at baseline was a good predictor of patients responses to the drug in predicting clinical response/efficacy. [31] Finally, a retrospective analysis of gene expression data in mucosal biopsy from CD patients also indicated that the chronicity of the disease (ranging from 2 to 20 years after diagnosis) was inversely related to the A_3AR expression. [47] However, a much bigger cohort study is needed to confirm this finding, if so it needs to be given consideration in any future potential clinical trial with A_3AR drugs.

3.2b) ADA activity as a biomarker of disease—Adenosine deaminase **(ADA)** is the enzyme involved in the metabolism of adenosine and its conversion to the inactive (or less active) metabolite inosine. It is a marker of inflammation and activated leukocytes, [48] and inhibition of ADA in animal models has been suggested to be a potential therapeutic strategy in IBD. [49] A recent study showed that ADA activity in patients with CD could distinguish between active and non-active disease. In this study the activity of total ADA (tADA) and its isoenzymes, ADA1 and ADA2, were measured in serum and neutrophils (mucosal infiltration in response to inflammation) obtained from 20 patients with active CD, 20 patients in remission, and 15 healthy controls. It is claimed that tADA and ADA2 are serum biomarkers of inflammation, and may provide a useful indicator of CD activity, since their levels decrease approaching normal values in patients who go into remission. [50] These findings are potentially very important, and deserve further consideration and confirmation.

3.2c) Purine gene dysregulation profile as a biomarker of disease—A retrospective analysis of existing gene-array data sets in IBD versus controls, showed that UC and CD could be distinguished based on their unique purine gene dysregulation profiles in mucosal biopsy or polymorphonuclear leukocytes. [47] Therefore, unique changes in the expression profiles occur in UC or CD compared to healthy controls for purine genes,

including receptors for P1, P2X and P2Y families, and enzymes involved in purinergic signaling. For example, in UC, there was up-regulation in mRNA levels of ADORA3, AMPD3, P2RY13, P2RY14, DPP4, and NT5E and no change in ADORA2A or ADAR expression. [47] In contrast in CD, there were down-regulation of ADORA3, AMPD3, P2RY14 and P2RY13, and upregulation of ADORA2A and ADAR. Gene expression and dysregulation was strongly associated with mucosal inflammation. [47] Overall, factors that influenced the expression of purine genes were inflammation, severity of inflammation/disease, chronicity of disease, and in some cases sex-dependent differences. Studies in part, supported by the National Institutes of Health and our Neuroscience Signature Program at The Ohio State University Wexner Medical Center, are underway to carry out a prospective analysis in cohorts of IBD and IBS patients to test the suitability of '*the purine gene dysregulation profile*' as a biomarker of disease that could potentially distinguish UC, CD and IBS.

Expression of other genes for $P2Y_2$, $P2Y_6$, CD39 enzyme, $P2X_7$ or A_{2B} receptor proteins were also shown in different studies to be sensitive to inflammation. [51–54] (Refer to Table 2). In addition to their potential value as biomarkers of disease or inflammation, alterations in the expression of various purine genes are likely to contribute significantly in the pathophysiology of GI diseases, especially IBD. [7,14] The functional significance of these changes deserves further study. Definitive information on purine gene dysregulation and their functional consequences in human gut of UC or CD patients in comparison to control, is necessary to fully appreciate the potential for targeting these receptors to treat IBD patients. Given the variability in disease models and responses observed, such translational studies become increasingly more important.

3.3 Clinical Relevance of other 'adenosinergic drugs' to block inflammation and diarrhea

The potential of adenosine receptors as therapeutic targets has been the subject of numerous reviews. [10,12,55–57] These articles cover the biology of adenosine signaling in health and disease, biomedical implications in a broad range of diseases including inflammatory diseases. A recent Nature review describes the challenges in developing drugs for adenosine receptors. [58] Ongoing efforts in medicinal chemistry are helping tremendously in drug discovery for adenosinergic or purinergic drugs by generating selective ligands for the human variants of the receptors, ligands with positron-emitting radioisotopes can be used to monitor in vivo occupancy of adenosine receptors in vivo, improved bio-distribution and tissue selectivity.

3.3a) Clinical trials with methotrexate and sulfasalazine, modulation of extracellular levels of adenosine by ectoenzymes—Purinergic signaling is involved in intestinal inflammation associated with IBD and with severe hypoxia of the inflamed mucosa. [59] The role of hypoxia in the regulation of extracellular adenosine production and signaling in intestinal inflammation was the subject of a recent review by Eltzschig et al. [55] In patients with intestinal inflammation such as occurs in IBD, profound hypoxia of the mucosa induces Sp1-dependent activation of CD39 [60] and a hypoxia-inducible factor (HIF) dependent induction of CD73 signaling [61] that favors extracellular adenosine production and signaling. In experimental colitis, enhancement of extracellular adenosine levels attenuates

intestinal inflammation. Adenosine deaminase (ADA) catalyzes the breakdown of adenosine to inosine, generally considered to be an inactive metabolite. However, inosine also has an anti-inflammatory and protective effect against TNBS-induced colitis, mediated by adenosine A_{2A} AR and uric acid, a metabolite of inosine. [62] The A_{2A} receptor is unique perhaps in that several distinct ligands can activate it in situ, e.g. adenosine, inosine, or 5′AMP (neural activation). [62,63]

The ectoenzymes *ectonucleoside triphosphate diphosphohydrolase* (CD39) and *ecto-5′-nucleotidase* (CD73) regulate nucleotide phosphohydrolysis of ATP and ADP to AMP, and conversion of AMP to its active metabolite adenosine, respectively. Studies in knockout mice of $CD39^{-/-}$ and $CD73^{-/-}$ highlight the importance of extracellular adenosine signaling in protecting against development of inflammation in pathologic situations. CD39 or CD73 deletion exacerbates experimental murine colitis (see Table 1). [26,64] In $CD39^{-/-}$ mice, reduction in extracellular adenosine level together with increase in ATP and ADP is suggested to result in increase in susceptibility to developing pathologic inflammation in disease states. Regulatory T-cells ($CD4^+$ T lymphocytes) inhibit antigen-specific T-cell responses and prevents colitis. Therefore they are critical players in suppressing intestinal inflammation. Adenosine generation produced by activation of CD39 and CD73 expressed on regulatory T-cells leads to immune suppression. [65] CD73 is a critical enzyme in PMN-mediated human intestinal epithelial Cl^- secretion. [66] A_{2A} and A_{2B} receptors are implicated in the protective effects of endogenous adenosine – the A_{2A}AR has a critical role in T-cell mediated regulation of colitis. [67–69]

Methotrexate (**64**) or **sulfasalazine** (**65**) are 2 commonly used drugs to treat IBD, autoimmune disease and rheumatoid arthritis. Their anti-inflammatory actions involve in part enhanced release of extracellular adenosine via a CD73-dependent signaling pathway. [70,71] Sulfasalazine acts through multiple mechanisms, by inhibiting 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) transformylase, via A_2 receptors on inflammatory cells to attenuate inflammation, and enhances release of adenosine at inflamed sites. An adenosine A_{2A} agonist **regadenoson** (**68**) is the first approved drug for clinical use in stress echocardiography.

In contrast to adenosine, ATP (the precursor of adenosine), is involved in purinergic chemotaxis, the process of cell migration during host defense responses by neutrophils to engulf and destroy foreign microorganisms. [72] Furthermore, during colitis inflammatory cells (platelets or epithelial cells) release nucleotides to activate P2 receptors, and in particular a neuronal $P2X_7$-pannexin-1 signaling pathway leading to death of enteric neurons and exacerbation of tissue injury and inflammation. [73]

Studies in animals or humans provide compelling evidence that enhancing the production of extracellular levels of adenosine (rather than ATP) in inflamed states provides protection against intestinal inflammation and injury. Furthermore, it is worth further investigation into drug strategies favoring a shift in the balance from pro-inflammatory nucleotide signaling (P_2/$P2X_7$ activation) to anti-inflammatory adenosinergic signaling (via A_{2A}, A_{2B} or perhaps A_3 receptors).

Human polymorphisms in the noncoding region of **CD39** cause a decrease in ectonucleotidase expression that leads to an increased susceptibility to IBD and multiple sclerosis. [25,26] In Chagas disease (caused by infection with the protozoan parasite *Trypanosoma cruzi*) E-NTPDase 1 (CD39) and ectoadenosine deaminase activity are reduced in lymphocytes of patients with the disease. [74] Upregulation in CD39 is also implicated in the loss of purinergic vascular regulation in the colon during colitis. The resulting impaired regulation of GI blood flow may have contributed to a more compromised permeability barrier provided by epithelial cells lining the gut mucosa. [75]

3.3b) Clinical trials for dipyridamole—Adenosine plays an important role in gut immune and inflammatory responses. [12] As discussed, adenosine is involved in the therapeutic actions of anti-inflammatory drugs such as methotrexate or sulfasalazine. [76] Adenosine inhibits cytokine production, reduces neutrophil activity and increases the production of IL-10, an anti-inflammatory cytokine. [49,67,77] Adenosinergic drugs are potential anti-diarrheal drugs by virtue of their anti-inflammatory properties. Drugs shown to have efficacy in experimental models of colitis include ATL-146e (**69**, agonist of A_{2A} AR), ATL-801 (**78**, A_{2B} antagonist), IB-MECA (**9**, A_3AR agonist), pentostatin (**62**, adenosine deaminase inhibitor) or dipyridamole **61** (a nucleoside transport inhibitor). Some inconsistencies in the literature on the efficacy of these drugs in pre-clinical models suggest further work is needed to clarify mechanisms and sites of action of A_{2A}, A_{2B} and A_3 receptors. However, overall, the potential of adenosinergic agents as anti-diarrheal drugs should be further explored in clinical trials, not done to date.

Dipyridamole acts as a nucleoside uptake inhibitor by blocking the equilibrative nucleoside transporters (ENT1 and ENT2). Dipyridamole is particularly effective when there is an increased extracellular level of endogenous adenosine, such as in inflammation or hypoxia. Blockers of ENT are shown to reduce the severity of tissue injury in models of inflammation. Effects are mediated via A_{2A} AR since antagonists could abolish the therapeutic effects of dipyridamole. [78–81]

Seven-day oral treatment with dipyridamole increased circulating adenosine concentration, and augmented the anti-inflammatory response in experimental human endotoxemia. [82] Dipyridamole treatment enhanced the anti-inflammatory IL-10 response during endotoxemia that is produced by cells of the innate immune system, and it was able to inhibit production of proinflammatory cytokines like TNFα. These effects were also seen in human cultured mononuclear cells, and in patients undergoing coronary bypass surgery, dipyridamole inhibited post-op PBMC cell adhesion to endothelial cells. [83–85] Further studies in systemic inflammatory diseases, including IBD and inflammatory diarrhea are suggested, given that it has limited side effects (e.g. oral drug is associated with bleeding tendency; intravenous drug can cause chest pain and angina in patients with coronary artery disease).

Dipyridamole is a coronary vasodilator used in patients for pharmacological stress echocardiography or to prevent platelet aggregation (to protect after cardiac bypass surgery, NCT01295567). Dipyridamole is also in clinical trial as a supplement with prednisolone in RA (NCT01369745). The drug dipyridamole could easily be used to enhance the anti-inflammatory actions of endogenous adenosine in IBD, in both pre-clinical and clinical

trials. [83] Considering the widespread distribution of adenosine receptors, targeting receptors with agonist or antagonist drugs could have a wide range of effects in cells and tissues including potential untoward side effects. Therefore, it is possible that using drugs that elevate endogenous adenosine levels locally at sites of release (e.g. with dipyridamole) may offer potential advantages over drugs targeting a specific receptor. As such, despite encouraging pre-clinical pharmacology, several phase III clinical trials of A_{2A} receptor antagonists in Parkinson's Disease were with little or insufficient clinical efficacy. [86,87] The largest clinical Phase III trial (PROTECT) of the A_1 receptor antagonist **rolofylline** (**18**) in acute heart failure has failed because of its toxicity, [88] but we still don't know if its toxicity this is specific to the drug in the disease or a result of general targeting of the A_1 receptor. [57]

3.3c) Caffeine (methylxanthines) use in humans, and clinical trials—Caffeine (**13, a methylxanthine**) has biological effects as an antagonist at adenosine receptors, e.g. used to treat premature apnoea, chronic obstructive pulmonary disease (POCD), cardiac ischemia/reperfusion injury. Caffeine's actions can be explained in part by its effects to reduce adenosine receptors to ~50% the normal expression levels. [89] It has been estimated that the doses consumed in coffee are sufficient to exert biological effects at adenosine receptors. Long-term use of **caffeine** in coffee in sufficient doses to influence behavior [90] is not associated with any severe side effect(s) or increased morbidity. This knowledge is encouraging, and may suggest that long-term use of drugs like caffeine that act as antagonists at adenosine receptors, especially the A_{2A} AR can be fairly safe. [58] In a recent nature review [58] it was stressed that clinical trials of adenosine drugs (and in particular A_{2A} drugs) need to take caffeine consumption into considerations in clinical trials. As a cofounding variable, caffeine is indicated for headache and fatigue, but it can cause CNS excitation.

Single nucleotide polymorphisms (SNPs) in **ADORA2A** are associated with age of onset of Huntington's disease, and reduced risk of Parkinson's disease. Caffeine and others A_{2A} antagonists, having a common target, have similar pharmacological effects in brain. The identification of SNPs in association with caffeine consumption (in relationship to Parkinson's disease), suggests that clinical trials should consider subdividing patients according to their genotypes for A_{2A}, CYP1A1, CYP1A2 genes, etc. [58] Genetic studies with caffeine may offer unique prospects for individualized medicine by identifying useful pharmacogenetic markers to predict individual responses to caffeine and adenosine drugs in clinical trials. An earlier example, arguing for individualized medicine, is the use of A_3AR upregulation as a predictor of susceptibility to treatment with an A_3AR agonist (CF101).

3.3d) Experimental therapeutics with purinergic drugs in diarrheal diseases targeting ENaC—UC and CD and infection-induced inflammation causes diarrhea. There is potential to exploit purinergic signaling-mechanisms in the treatment of inflammatory diarrhea. In normal colon and rectum, the electrogenic Na^+ absorption via the epithelial Na^+ channel (ENaC) accounts for the lumen-negative transmucosal electrical potential difference (PD). In active UC, TNFα/IFNγ cause down-regulation of the ENaC leading to impairment of electrogenic Na^+ absorption and consequent reduction/loss of PD. [91,92] In UC reduction in Na absorption could also be involved from impairment in electroneutral NaCl transport in

colon. ENaC absorption of Na^+ is also impaired in CD in non-inflamed sigmoid colon of patients with active CD of the terminal ileum. Therefore, impaired absorption of Na^+ is likely to contribute to the pathogenesis of diarrhea in CD and UC. [93] The potential application of purinergic compounds as anti-diarrhea drugs was the subject of a recent review by Sandle and co-workers. [94] Drugs shown in Table 6 have efficacy in experimental models of gut inflammation–induced diarrhea, which may act in part to restore normal Na^+ absorption. Studies with purinergic drugs targeting the ENaC are worth pursuit.

3.3e) Epithelial A_{2B} in diarrhea and inflammation—The role of A_{2B}AR in immunity and inflammation has been comprehensively reviewed. [95] We will restrict our focus to studies indicating a role of A_{2B}AR as a therapeutic target in IBD and inflammatory diarrhea. Briefly, during active intestinal inflammation, polymorphonuclear leukocytes transmigrating into the lumen, release 5′AMP that is converted to adenosine, which then activates electrogenic Clsecretion via apical A_{2B}AR that likely contributes to secretory diarrhea. [67,96] A_{2B} AR blockade by pharmacological antagonism [97] or $A_{2B}^{-/-}$ gene deletion [98] suppresses gut inflammation and ameliorates murine colitis. A_{2B}AR regulates Cl^- secretion from intestinal epithelial cells, a process that is critical in the development of diarrhea. Stimulation of A_{2B} AR increases cAMP and triggers the release of IL-6. Neutrophil-epithelial crosstalk at the intestinal luminal surface involves reciprocal secretion of adenosine and IL-6 [99] thus providing an amplification mechanism for intestinal inflammation. Furthermore, TNF-α upregulates the A_{2B} receptor gene in gut tissue in human IBD and murine colitis, propagating a vicious cycle of inflammation in the intestinal tract. [100] Protection afforded by A_{2B}AR inactivation is associated with a decrease in the production of IL-6, a reduction in neutrophil infiltration in mucosal tissues, and keratinocyte – derived chemokine.

It is notable that not all studies have yielded consistent results. In contrast to the above mentioned studies, a separate study by Frick et al [68] found that $A_{2B}^{-/-}$ deletion or an A_{2B} antagonist PSB-1115 (**27**) increased the severity of DSS colitis. It remains puzzling as to why one study reveal an anti-inflammatory and tissue-protective role of A_{2B} AR, whereas others indicate a pro-inflammatory effect of A_{2B} AR in colitis. Differences in murine strains of genetic deleted mice or bacterial flora of the mice were offered as potential explanations. More studies are warranted to identify the mechanism, and to test whether the A_{2B} AR is a viable therapeutic target in a mucosal inflammatory disease like IBD.

3.3f) Potential for purinergic drugs targeting neurogenic diarrhea—The ENS is important for secretion, mixing, and propulsion of intestinal contents. [101,102] Fluid secretion involves a predominant neurogenic component. Estimates suggest that neurogenic secretion [101–104] is responsible for >60% of that to luminal secretagogues [104] and excessive secretion is often associated with clinical symptoms of diarrhea, whereas low rates of secretion may be a contributing factor in constipation. [104] Diarrhea is a prominent feature of IBD, ranging in frequency from >50% to 99% of acute flare-ups of CD or UC, respectively and is often a leading symptom of distress in these patients. [104] Diarrhea-predominant IBS (D-IBS) occurs in a subset of IBS patients. [105] Colonic inflammation or agents like immune/inflammatory mediators that cause ENS excitation increase fluid volume and liquidity of

luminal contents, ion secretion and the potential for neurogenic diarrhea. [106–108] A better understanding of purinergic mechanisms regulating human gut reflexes is necessary to fully-understand the basis of disturbances in secretomotor function in UC, CD or D-IBS. Both EC [109–114] and ENS [101,103] are implicated in the pathophysiology of intestinal secretory states suggesting potential new sites of action for drugs to treat diarrhea or constipation. [106] Use of P2X antagonists as drugs [13,115] to target motility and slow intestinal transit is another approach, since activation of P2X receptors in the ENS is expected to have pro-kinetic effects. [12,116] Of the many purine receptors known to exist in the human gut, $P2Y_1$ (stimulation) and A_3 AR (inhibition) are primary regulators of neurogenic secretion and early human data supports it. [12,101,108,117–120] $P2X_2$ and $P2X_3$ ion channel receptors are expressed on human submucousal neurons [121], and they are involved in stimulatory purinergic transmission in human ENS (Linan-Rico, Wunderlich and Christofi, unpublished observations). Release of ATP or a related nucleotide evokes a slow EPSP response in secretomotor neurons via $P2Y_1$ receptors resulting in increase in fluid and electrolyte secretion. [103] Mechanically evoked reflex electrogenic chloride secretion in rat distal colon is triggered by endogenous nucleotides acting at $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors. [117] Mechanical stimulation also releases nucleotides that activate $P2Y_1$ receptors to trigger neural reflex chloride secretion in guinea pig distal colon. [119] Antagonist drugs at $P2Y_1$ or P2X (or agonists at A_3 AR) could suppress intestinal secretion by acting at both EC and ENS, and lead to harder, drier stools and could be beneficial for neurogenic diarrhea – studies in animals on mucosal diarrhea and fluid secretion are needed to prove their efficacy in vivo.

3.3g EC cell signaling in IBD—Enterochromaffin cells (EC) lining the intestinal mucosa release serotonin (5-HT) to regulate gut secretion, motility, pain signaling to the brain, nausea and immune modulation in IBD. [122] Alterations in 5-HT signaling are associated with IBD and IBS in both animals and humans. [109] 5-HT signaling is tightly regulated by adenosine and ATP. [10,12,123]

Hypoxia is a key feature of IBD that can activate HIF-1α signaling and 5-HT release from EC cells isolated from the human GI tract. [124] Responses are augmented by inflammation. Hypoxia stimulates release of adenosine [125,126] and it also acts to stabilize HIF-1α. [127] Hypoxia induces 5-HT synthesis and secretion from EC cells. Adenosine acts to decrease serotonin transporter (SERT) activity [128] that would serve to increase 5-HT signaling in the gut. Activation of A_{2B} AR via MAPK/CREB and TPH-1 signaling amplifies the effect of hypoxia in human EC cells. [129] Overall, effects of adenosine in IBD are very complex, and much more work needs to be done, but targeting this pathway in EC cells is of potential interest as a therapeutic target in IBD.

Adenosine A_1, A_{2A}, A_{2B} and A_3 ARs provide fine tune modulation and autocrine regulation of 5-HT release from EC cells in response to mechanical stimulation. [123] Comprehensive reviews have been written on the role of purinergic signaling in health and disease of the GI tract, mechanosensory reflexes and secretomotor function. [10,12] Recent findings indicate that ATP-gated $P2X_3$ channels and metabotropic $P2Y_1$ receptors provide fast and slow – regulation of mechanically evoked 5-HT release, respectively. A putative $P2Y_{12}$ receptor provides inhibitory modulation of 5-HT secretion. Therefore, these receptors are likely to

play a critical role in the physiological regulation of peristaltic and secretory reflexes. [130] Any change in the expression of these receptors or signaling pathways in disease states such as IBD (or IBS) would be expected to have significant consequences. So for instance, $P2X_3$ – immunoreactivity is normally expressed in 15% of human EC cells lining the colonic mucosa. However, in patients with ulcerative colitis (UC), $P2X_3$ could no longer be detected by a selective $P2X_3$ antiserum indicating that the fast-purinergic autocrine regulation of 5-HT release is impaired. [130] This needs confirmation in functional studies, but if so, it may be an important mechanism in the pathophysiology of UC. Alternatively, impairment in the $P2X_3$ mechanism may actually be a compensatory mechanism in a futile attempt to try and restore normal 5-HT signaling that is known to be altered in IBD (and IBS).

3.3h) Immunomodulation via A_{2A} AR and experimental therapeutics— Adenosine accumulation in inflamed (or hypoxic) tissues occurs via a two-enzyme dephosphorylation process involving CD39 (nucleoside triphosphate dephosphorylase) that converts ATP to ADP then to 5′AMP. Next, CD73 (a 5′ectonucleotidase) converts 5′AMP to adenosine. [26,131] It is well known that activation of A_{2A} AR attenuates gut inflammation in animal models of IBD. [132] A_{2A} AR is expressed on several types of immune cells involved in the mucosal inflammatory response in IBD, including myeloid cells, endothelial cells, T-lymphocytes. Adenosine analogs can ameliorate colitis and *Clostridium difficile* toxin-induced diarrhea, as well as gastric mucosal inflammation. [133] $A_{2A}AR^{-/-}$ mice exhibit a more inflamed phenotype, for example after infection with *Helicobacter pylori* that causes gastritis. Activation of A_{2A} AR on $CD4^+$T (Th) cells causes an anti-inflammatory response. A_{2A} receptors play a critical role in mucosal immune regulation by suppressing T-cell cytokine production including TNFα, IFNγ and IL-2 and it regulates *Helicobacter*-induced gastritis and bacterial persistence. In IL-10 KO mice, the inflammatory response is sufficient to clear (resolve) *H. pylori* infection. Infection of mice lacking the $A_{2A}{}^{-/-}$AR exacerbates the inflammation/gastritis in comparison to wild type mice. Administration of an A_{2A} agonist ATL313 during infection suppresses inflammatory responses of Th cells, and reduces gastritis, but it also impairs immunity to *H. pylori* infection that could favor persistence observed as an increase in bacterial load. [133] Notwithstanding this potential ‘risk’ in its activity as an immunomodulator, adenosine’s A_{2A} AR anti-inflammatory properties are worth pursuit in IBD. It remains unknown whether A_{2A} agonists are effective in clinical trials of IBD.

It is not yet clear whether adenosine receptor heterodimerization with other purine or different types of receptors represents a significant challenge to the use of adenosine drugs and their clinical pharmacology (e.g. A_{2A}-A_{2B}; A_{2A}-D_2; A_{2A}-A_1). [134–139]

3.3i) Immune modulation in epithelial cells—Epithelial cells respond to invading pathogens by producing inflammatory mediators. Perception of microbial molecular recognition receptors with various pattern recognition receptors (PRRs) stimulates the production of inflammatory mediators that can recruit and activate innate and adaptive immune responses. The immune response to non-pathogenic bacteria (e.g. commensal flora) is normally regulated to avoid a state of chronic inflammation. ATP has been proposed to serve as an endogenous ‘danger signal’ of adaptive immunity. [140] ATP was shown to alter

human epithelial responses to commensal bacterial products in vivo, provoking an inappropriate immune response that could potentially favor development of IBD. [141] Activation of $P2X_7$ receptors by ATP induces apoptosis and autophagy (possibly via production of free radicals) in human epithelial cells, an effect that could have implications for gut inflammation. [142] The epithelial $P2X_7$ is suggested to play a critical role in initiating a positive amplification loop of polymorphonuclear leukocyte recruitment into the intestinal mucosa during the acute phase of inflammation. It was inferred from that study that dysregulation of the $P2X_7$ apoptotic mechanism could result in the development of chronic IBD. [51] Other purinoceptors are implicated in IBD as well. Therefore, inflammatory stress associated with IBD elevates extracellular nucleotide concentrations at tissue sites of inflammation, in association with increased $P2Y_2$ mRNA expression in colonic epithelia from mice with experimental colitis or from patients with Crohn's Disease (CD) and UC. [143] $P2Y_2$ expression is regulated by an NF-kB dependent mechanism and it is suggested it may contribute to IBD or other inflammatory diseases by stimulating prostaglandin release. C/EBPβ is a regulator of $P2Y_2$ expression. [144] Further studies are needed to explore the pathophysiology and therapeutic potential of targeting epithelial $P2X_7$, $P2Y_2$ or other nucleotide receptors. [10,12]

4.0 A_3, A_1, A_{2A}, P2X and $P2Y_1$ receptors and implications for motility disorders, constipation and diarrhea

Neural A_3AR are involved in the regulation of both neuromuscular functions [145] and coordination of motility and secretion in the colon. [146] A_3AR is distributed throughout myenteric ganglia in the colon, with highest expression in distal colon of the rat. RT-PCR indicated that A_3 and ADA mRNA increased in inflamed tissues from experimental colitis. [145] The A_3AR – mediated tonic inhibitory control of colonic cholinergic contractions was shown to be impaired in the inflamed bowel despite an increase in functional A_3AR.

In a model of neurogenic diarrhea, a mast-cell mediator, histamine (or dimaprit, H_2 agonist) was used to activate a stereotype cyclical pattern of chloride secretion that could be sustained for hours in the presence of drug. [146] Endogenous adenosine provided ongoing inhibitory modulation, and A_3 antagonists could cause profound augmentation of the secretory response. Neural activation of histamine receptors in submucosal neurons activates a neural program leading to a coordinated motor and secretory response. A_3AR tightly regulates the coordinated response to the mast cell mediator histamine. Histamine excites neurons in human submucosal plexus through activation of H_1, H_2, H_3 and H_4 receptors. [147] Furthermore, ENS excitation by supernatants collected from biopsy in IBS patients was sensitive to blockade with an H_1–H_4 antagonist cocktail, indicating that release of histamine from mucosal mast cells can cause activation of the ENS.

Functional disruption of A_3 receptors in $A_3^{-/-}$ mice alters intestinal motility. A_3AR-immunoreactivity in the distal colon ≫ proximal colon, by a ratio of 2:1. [146] The receptors in the mouse ENS are restricted to varicose fibers (sites of transmitter release) and glia. Notable species differences exist in the distribution of A_3AR. Therefore, in rat unlike the mouse, cell bodies of enteric neurons (postsynaptic sites in transmitter release) highly express the A_3AR. [146] Interestingly, as in the mouse the highest expression of A_3 is in the

distal colon. Intestinal transit and colonic evacuation reflex were accelerated in $A_3^{-/-}$ mice, and stool retention was lower in these mice. [29] It was suggested that activation of A_3AR by eADO attenuates the evacuation reflex and slows down intestinal transit, colonic emptying and mass movement in the colon. Therefore, the A_3AR is a potential target for motility disorders. The $A_3^{-/-}$ phenotype also protected against colitis, diarrhea, occult fecal blood, weight loss, neutrophil or $CD4^+$ infiltration, and tissue injury. [29]

Overall, taken together, these studies support the concept that A_3AR selective agonists are potential therapeutic agents for the management of diarrhea and abnormal bowel motor activity or secretion associated with IBD, IBS or neurogenic diarrhea. A_3AR selective agonists are already in clinical trials for inflammatory diseases, and their safety profile is excellent. It remains to be shown whether adenosine can suppress ENS activation evoked by supernatants from mucosal biopsies collected from patients with IBS (or IBD).

In contrast to A_3AR, A_{2B}AR plays a key role in regulating distal colon relaxation, and the mechanisms is linked to NO signaling [148] It has been suggested that targeting colonic A_{2B} AR could represent a therapeutic strategy to treat constipation. $A_{2B}^{-/-}$ AR mice have a constipated phenotype whereas $A_3^{-/-}$ AR mice have accelerated motility and colorectal evacuation reflex. [29] Therefore, an A_{2B}AR agonist or A_3AR antagonist are potential drugs for constipation by promoting motility. Further animal studies with A_3AR antagonists are needed to confirm the physiological relevance of A_3 in motility and its efficacy in ameliorating colitis. For both A_{2B}AR and A_3AR, more studies are needed to further clarify the mechanism of action on motility and secretion.

In chemically induced colitis in the rat, there is also molecular and functional rearrangement of neural A_1 and A_{2A} receptors, favoring A_{2A} receptor regulation of inhibitory control of colonic neuromuscular activity. Both A_1 and A_{2A} receptors contribute to inhibitory neuromuscular control in normal bowel. In inflamed bowel, neuronal A_1 receptor function is lost, and A_{2A} function becomes more prominent, in part due to A_{2A} and CD73-dependent upregulation. [149] A_1 and A_2 receptors mediate inhibitory effects of adenosine on motor activity of human colon. [150]

To date, a single study has been published on the direct effects of activating adenosine receptors in the human ENS. A study by Wunderlichet al [108] provided proof for inhibitory A_3AR in human submucous plexus involved in suppressing synaptic neurotransmission. A_3AR inhibited nucleotide or cholinergic synaptic transmission in the human ENS. Neural A_1 receptors could not be revealed in contrast to animal studies. [151] In the study of Antonioli et al [149], the inhibitory effect of A_1 activation on motor activity in human colon also differs from that in rodents – they found that A_1 inhibition was restricted to the muscle, while A_{2A} receptors operated through inhibitory nitrergic nerve pathways. [150] Species differences in other purinergic receptors are also known to occur between the mouse, rat and guinea-pig (e.g. for $P2X_2$ and $P2X_3$ receptors). For the development of purinergic drugs for motility disorders or neurogenic diarrhea for instance (associated with specific phases of IBD), it is imperative that translational studies in human surgical specimens are done to fully characterize receptors, enzymatic pathways and inactivation mechanisms for adenosine receptors, as well as $P2X_{1–7}$ and $P2Y_{1,2,4,6,11–14}$ receptors in both normal and inflamed gut.

It is also necessary to identify receptors that are conserved in mouse and human ENS-muscular tissues for pre-clinical testing of drug candidates for IBD or IBS. Our unpublished observations indicate that endogenous adenosine and nucleotides play a critical role in purinergic regulation of neurotransmission in the human ENS (Linan-Rico, Wunderlich and Christofi, unpublished observations).

An earlier study provided encouraging results on the use of an A_1 AR antagonist to treat post-operative ileus (POI) in a rat model. [152] The selective A_1AR antagonist DPCPX (**17,** or CPX) reversed the slowed colonic propulsion in the rat. The sites or mechanisms of action are not understood, and more studies are warranted. Alterations in the purinergic pathway also occur in POI [153] (e.g. increase in ATP production in myenteric neurons and P2Y expression on smooth muscle) and drugs targeting these pathways may be relevant in alleviating POI. Little is known about purinergic signaling in postoperative ileus. In ulcerated regions of inflamed guinea-pig distal colon, neuromuscular transmission and propulsive motility are attenuated, an effect that is associated with a decrease in the purinergic component of the descending inhibitory limb of the peristaltic reflex. [154]

Derivatives of benzimidazol-2-ylquinoline and benzimidazol-2-ylisoquinoline are selective A_1AR antagonists with stimulant activity on human colon. Nanomolar concentrations (~10 nM) of these compounds enhanced EFS – contractions of the human colon, and this property makes them highly attractive agents for stimulating motility in humans. [155] As pointed out, this is a first step to developing new drugs for the therapeutic management of digestive disorders that are characterized by alterations GI propulsion (e.g. idiopathic chronic constipation, post-operative paralytic ileus and IBS). Their suitability in these disorders could be rather limited if inflammation (as occurs in post-operative paralytic ileus) or the disease impairs A_1 regulation of neuromuscular transmission as occurs in experimental colitis. [149] Furthermore, A_1 receptors were identified on smooth muscle but not in human ENS. Perhaps targeting other neural adenosine receptors might be a better option, and they should be given further consideration.

Studies in P2ry1$^{-/-}$ knockout mice demonstrated the physiological relevance of $P2Y_1$ purinoceptors in inhibitory motor control of murine colonic excitability and transit. [4] Overall, inhibitory neuromuscular transmission is mediated via $P2Y_1$ purinergic receptors in mouse, guinea pig, primate and humans. [4,5,156,157] It is not clear what effects $P2Y_1$ receptor antagonists would have on in vivo transit or constipation, but P2ry1$^{-/-}$ KO mice should prove helpful in such studies in animal models of constipation. In Hirschsprung's Disease, $P2Y_1$ and $P2Y_2$ receptors were absent in the aganglionic segment in both myenteric and submucous plexuses. $P2Y_1$ receptors are involved in inhibitory transmission to smooth muscle of the human colon [5,156] and their absence could at least in part, explain the contracted state of the aganglionic gut. Functional studies are needed to test this hypothesis.

The purinergic hypothesis is based on ATP (or a related nucleotide, e.g. ADP or AMP) release as the neurotransmitter at synapses or in neuromuscular transmission. However, a significant body of evidence is emerging to suggest that β-NAD^+ and ADP ribose are involved in neurotransmission and inhibitory neuromuscular transmission in rodents, primates and humans. [2–4] For example, in P2ry1$^{-/-}$ KO mice, purinergic fast inhibitory

junction potentials (fIJPs) and responses to β-NAD^+ or ADP ribose were abolished, whereas those to ATP or ADP were retained. The findings of the group at Reno support the intriguing hypothesis that β-NAD^+ or ADPR meet the criteria for a neurotransmitter in neuromuscular transmission. [3] Gallego et al [5] concluded from their study that β-NAD^+ only partially fulfills the criteria for the transmitter involved in inhibitory neuromuscular transmission of the human colon. Nevertheless, the direct actions of these mediators (β-NAD^+ or ADP ribose), their involvement in 'purinergic transmission', P2Y-inhibitory transmission or $P2Y_1$ stimulatory transmission in the human ENS have yet to be determined.

P2X agonists on the ENS may enhance GI transit and secretion and they could be useful in treating constipation or constipation-predominant IBS. Alternatively, P2X antagonists could be useful in treating diarrhea-predominant IBS or the neurogenic component of inflammation–induced diarrhea, since majorities of the secretory diarrhea observed in IBD or infection-induced gut inflammatory states is estimated to be neurogenic in nature. [104] Blockade of excitatory $P2Y_1$ receptors on secretomotor neurons in animals [103] or humans [108] is another target to regulate neurogenic diarrhea, and studies in $P2ry^{-/-}$ mice can provide molecular proof.

5.0 ATP and P2X ion channel receptors antagonists as potential analgesic drugs for visceral pain in IBD and IBS

P2X ion channel receptors are distributed on subsets of myenteric and submucous neurons of the ENS, glia, ICC, smooth muscle, epithelia and EC cells, and immunochemical studies have revealed their discrete localization in subsets of neurons with distinct chemical coding and function. Electrophysiological and calcium imaging studies confirmed the role of P2X ion channel receptors in excitatory neurotransmission and information transfer between neurons and glia. [158] ATP is a potent stimulus for electrolyte secretion in the GI tract (colon, gall bladder, pancreatic duct, and from bile duct) and its release is likely mediated from both local epithelial cells and nerves to modulate peristalsis, secretion and nociception. ATP also exerts fine tune modulation of EC-cell function and 5-HT secretion that also triggers intrinsic gut and nociceptive reflexes. [130] Enteric glia express $P2X_7$ and ($P2Y_4$ receptors). [14] The ectoenzyme NTPDase2 is exclusively localized to glia in the ENS (where as NTPDase1 is localized to neurons) to regulate the availability of ATP [159] and gliotransmission. $P2X_7$ receptors (and $P2X_2$ receptors) are expressed on NOS-positive inhibitory neurons, cholinergic secretomotor neurons, and intrinsic sensory neurons. [115]

5.1 Pain and visceral hyperalgesia

Abnormalities in P2X signaling are implicated in diverse diseases such as IBS, IBD, Chaga's Disease, Hirschsprung's Disease and non-erosive esophagitis. Recent reviews have addressed the role of purines in gastrointestinal diseases and inflammation, and pain control. [7,14,158,160] IBS is more prevalent in females than males and severity of pain seems to fluctuate with the menstrual cycle. This implies that sex hormones could affect perception of painful stimuli, although a causal relationship between sex hormones and IBD is not yet clearly evident. However, $P2X_3$ mediated nociception in a colitis model is closely related to endogenous estrogen modulation. [161] $P2X_3$ channels on sensory EC cells are down-

regulated in UC [130] and up-regulated in enteric neurons in IBD [162] that would impact on both P2X signaling in intrinsic neural reflexes and nociceptive reflexes via the dorsal root ganglia to the brain.

Visceral pain is a debilitating symptom of IBD, IBS, FD and dysmotility disorders (e.g. gastroparesis), pseudo-obstruction, and GERD. The visceral purinergic system is a therapeutic target for pain control, and the experimental evidence support the hypothesis that mechanosensory signaling via P2X receptors can trigger visceral pain in visceral hollow organs (tubes or sacs) that include the bladder, ureter and the GI tract. [160,163] It is proposed that during distension, ATP released from sub-epithelial visceral afferents conveys pain information to the brain via the dorsal root ganglia. $P2X_3$ and $P2X_{2/3}$ receptors on low threshold fibers are involved in physiological reflexes whereas such receptors on high threshold sensory fibers transmit pain. $P2X_{2/3}$ receptors contribute to small intestinal afferent hypersensitivity in post-infectious bowel disease. [164] IBS is a functional GI disorder associated with pain and hypersensitivity in the absence of colonic inflammation or obvious structural changes. Several studies indicate that P2X receptors are involved in colonic hypersensitivity. [165,166] Visceral hypersensitivity in non-erosive reflux disease (in the absence of esophageal mucosal injury) may involve ATP sensitization of $P2X_3$ receptors. [160,167] Mice lacking $P2X_3^{-/-}$, $P2X_2^{-/-}$ or double knockout mice $P2X_2/P2X_3^{-/-}$ have provided proof for the involvement of $P2X_2$ and $P2X_{2/3}$ receptors in inflammatory pain, physiological voiding and bladder inflammation (in a model of interstitial cystitis using cyclophosphamide). [168–170]

5.2 Pain – relieving effects of intravenous ATP in chronic intractable orofacial pain

An earlier clinical study provided proof of concept in a small cohort of patients (n=16) that intravenous infusion of ATP at a rate of $100\mu g \cdot Kg^{-1} \cdot min^{-1}$ over 2 h has pain-relieving effects in chronic intractable orofacial pain. [171] They found that ATP caused a reduction of the VAS scores for spontaneous pain and allodynia by 82%±15% and 74%±9% respectively. These beneficial effects of ATP outlasted the infusion period (for medians of 7 and 12 h respectively). A later study by the same group conducted a double blind placebo controlled study to evaluate the effects of intraoperative intravenous ATP on postoperative pain in 30 patients scheduled for sagittal split ramus osteotomy. In this study the ATP infusion rate was $160\mu g \cdot Kg^{-1} \cdot min^{-1}$ throughout surgery. Data suggested that ATP infusion could blunt hemodynamic responses to surgical stimulation, and it produced prolonged analgesia in patients undergoing such orofacial surgery. ATP reduced the cumulative morphine consumption for 72 h postoperatively by 47% compared to placebo, and no adverse effect of ATP was reported. [172] These are compelling studies, albeit in small numbers of patients, but they provide proof of concept that the cognate ligand ATP can provide effective analgesia. Further studies are needed to determine the sites of action of ATP and whether $P2X_{2/3}$, P2X or multiple receptors are targeted by the non-selective ligand ATP, therefore, larger multi-center studies are warranted. Several medicinal candidate drugs exist for $P2X_3$, $P2X_{2/3}$ and $P2X_7$ for trials. A larger Phase II, double - blind, placebo-controlled, dose-response trial involved intravenous adenosine for perioperative analgesia in 166 subjects (125 subjects received adenosine and 41 received placebo). Women undergoing major gynecological surgery were randomized to receive 25, 50, 100 or $200\mu g \cdot Kg^{-1} \cdot min^{-1}$

or placebo. Adenosine was not different from placebo with respect to efficacy and safety for perioperative analgesia. [173] It would be important to ascertain whether adenosine's lack of effect is because ATP or a related nucleotide (ADP or AMP) is a requirement for efficacy.

5.3 $P2X_{2/3}$, $P2X_3$ and $P2X_7$ antagonists are potential drugs for visceral pain

In the last decade advances in medicinal chemistry offer new selective $P2X_3$ and $P2X_{2/3}$ compounds with suitable potency, selectivity and bioavailability, to consider testing in clinical trials. These compounds are being developed by several pharmaceutical companies including Evotec, AstraZeneca, Merck and Shionogi. [174] Recent development of orally bioavailable $P2X_2$ and $P2X_{2/3}$ antagonists targeting these receptors makes these compounds potential therapeutic agents in treating visceral pain. A-317491 (**83**) is a selective $P2X_{2/3}$ and $P2X_3$ antagonist synthesized by Abbott Laboratories – it allowed studies to validate the role of these receptors in neuropathic and chronic inflammatory pain. AF-353 (**82**) is an antagonist at these receptors that is bioavailable and stable in vivo. [175] It is synthesized originally by Roche Palo Alto and is now being developed by Afferent Pharmaceuticals. Trinitrophenyl-ATP (**81**, TNP-ATP) is a high affinity antagonist at $P2X_3$ and $P2X_{2/3}$ that requires much higher concentrations to activate $P2X_2$ receptors. Several diaminopyridines were shown to be selective antagonists at $P2X_3$ and $P2X_{2/3}$ receptors, and had in vivo efficacy in a pain model. [176]

Clinical trials for some P2X compounds are in progress, but there is no information yet on the efficacy of these drugs to relieve pain. [175] The $P2X_3$ antagonist AF-219 (an aryloxy-diaminopyrimidine) from Afferent Pharmaceuticals is the first compound in clinical trials. [163] Several Phase I clinical studies in healthy volunteers indicate good safety and tolerability. In addition, several Phase II studies began in 2011 including a study in suppressing chronic cough (airway sensitization), joint pain (knee osteoarthritis) and visceral pain (in bladder pain syndrome). $P2X_3$ immunoreactivity is elevated in lingual mucosa in patients with burning mouth syndrome, suggesting that $P2X_3$ may be a therapeutic target for treating this type (trigeminal) of neuropathic pain. [177] Another P2X antagonist for treating pain is an orally available negative allosteric modulator of the $P2X_7$ (GSK1482160, **85**).

5.4 Potential for $P2X_7$ receptor antagonist drugs

$P2X_7$ receptor plays an important role in inflammation and immunity. The $P2X_7$ receptor antagonist is a potential therapeutic target for inflammatory diseases including *rheumatoid arthritis, IBD and glomerulonephritis, and for treating inflammatory pain, and amelioration of the pro-inflammatory phase of sepsis.* This is supported by animal studies with $P2X_7^{-/-}$ deletion or pharmacological studies with selective $P2X_7$ receptor antagonists. More than a dozen phase I and phase II clinical trials are ongoing or completed on the use of selective $P2X_7$ antagonists in the treatment of pain or inflammation in patients with RA. These trials are truly in the early stages of development, but the safety and tolerability of the drugs, and early results on efficacy are encouraging, to justify further study. For a comprehensive review of $P2X_7$ receptor antagonists in treating inflammatory diseases and clinical trials refer to Arulkumaran et al 2011. [178]

A number of patents have been filed for $P2X_7$ receptor antagonists for neuropathic pain and inflammatory disorders. [179] In addition, nociceptive signaling is dually modulated by Gi- and Gq-coupled P2Y receptors (ADP activated); Gq-coupled $P2Y_1$ activation is required for full expression of inflammatory hyperalgesia, while agonists for Gi-coupled P2Y receptors ($P2Y_{12–14}$) cause reduction in hyperalgesia. [180] Overall, a number of candidate drugs for P2X receptors (antagonists) are available for future clinical trials to treat painful conditions.

5.5 Inflammatory and neurological diseases

There is good evidence that the $P2X_7$ receptor has a pathogenic role in inflammatory glomerulonephritis, and pre-clinical studies in animal models suggest a possible therapeutic role of $P2X_7$ antagonists in the treatment of inflammatory renal diseases. [178] $P2X_7$ is important in the defense mechanism against *Mycobacterium tuberculosis*. [181] It was shown that the crucial bactericidal step of mycobacteria following phagocytosis by macrophages is $P2X_7$-mediated apoptosis of the macrophage. And, a polymorphism in the $P2X_7$ gene increases susceptibility to extrapulmonary tuberculosis by 3.5 fold. [181,182] Some data also suggest that targeting $P2X_7$ receptors might be a therapeutic option for treating COPD. Furthermore, extracellular ATP activation of $P2X_7$ receptors contributes to cigarette-smoke induced lung inflammation and emphysema. $P2X_7$ KO mice have reduced pulmonary inflammation after acute cigarette smoke exposure. The anti-nociceptive properties of $P2X_7$ antagonists have been researched extensively, and reports indicate reduction or amelioration of chronic inflammatory and neuropathic pain. [183,184] The levels of purines and pyrimidines in synovial fluid of patients with RA are high, and these nucleotides can produce joint inflammation through production of cytokines (IL1β, TNF-α, IL-2 and IL-6). [185,186]

$P2X_7$ receptors are implicated in the course/progression of Alzheimer's disease and of other neurodegenerative diseases via ATP-mediated cortical cell death and free radical release. In the gut, $P2X_7^{-/-}$ mice are protected against colitis. The mechanism involves $P2X_7$-pannexin 1 signaling, pore formation and caspase-3 leading to neuronal death in gut in animal models of IBD or CD [73] and may be relevant to human IBD. The $P2X_7$ receptor is expressed on enteric glia, NOS-positive inhibitory neurons, cholinergic secretomotor neurons and intrinsic sensory neurons. [7]

Therefore, $P2X_7$ activation in the inflamed state is likely to contribute to the symptoms of IBD, including motor abnormalities, diarrheal state, and visceral pain. The actions of $P2X_7$ receptor activation are not restricted to neurons and glia. Activation of the $P2X_7$ receptor leads to activation of inflammasome and release of interleukin-1β. [186] Recent findings also indicate that the extracellular ATP mediates mast cell-dependent intestinal inflammation through $P2X_7$ purinoceptors, and suggests that antagonists of $P2X_7$ receptors are potential therapeutic targets in both IBD and IBS (or other functional GI disorders) where mast cells are implicated in the immune/inflammatory response. In mast cells, $P2X_7$ receptors induce inflammatory cytokines, chemokines and leukotrienes. Activated MC's exacerbate inflammation by also recruiting neutrophils to produce TNFα. [54]

5.6 Clinical trials with $P2X_7$ antagonists

Phase I and II clinical trials on the safety and efficacy of $P2X_7$ antagonist drugs are ongoing. AZD9056, CE-224, 535 (**84**) and GSK1482160 (**85**) are compounds in phase II studies targeting IBD, RA and COPD (Table 4). These drugs are fairly well-tolerated and no serious concerns have been raised regarding their safety thus far. The main adverse events of these drugs are gastrointestinal (nausea, diarrhea and vomiting), dizziness and headaches. However, these side - effects were reported most frequently at the higher doses. Newer antagonists are entering clinical trials, and it is too early to draw conclusions. These are patients receiving background treatment with methotrexate or/and sulfasalazine without symptomatic relief (see Table 4).

CE-224, 535 (**84**) represents another antagonist drug of $P2X_7$ receptors, but its effect was not shown to be better than placebo for the treatment of RA in patients with an inadequate response to methotrexate. CE-224,535 exhibited an acceptable safety and tolerability profile. [187]

5.7 First clinical trial in CD patients with $P2X_7$ antagonist

AZD9056 is an adamantane amide and selective $P2X_7$ antagonist (structure not disclosed), being evaluated for safety and efficacy in causing clinical remission in CD patients – This is the first clinical trial in IBD patients with a P2X antagonist. It represents a phase II double blind, placebo-controlled, parallel group, and multicenter international study. The drug **AZD9056** is from Astra Zeneca (study code D8830C00002 2008 available from www.astrazenecaclinicaltrials.com). A 200 mg of AZD9056 once daily is given for 4 weeks to adult patients with active CD compared to placebo – 10 centers in 5 countries are participating in this first study in IBD patients. The aim of the study is to evaluate the safety and benefit of the drug in reducing the CD Activity Index (CDAI) score from a moderate to severe index (CDAI ≥ 220) to clinical remission (CDAI≤150) after 4 weeks of treatment in ileum and/or colon. Forty patients were enrolled, and 30 of the patients were randomized (20 to AZD9056 and 10 to placebo). Initial results were promising, and there was improvement in CDAI compared to placebo. Vital signs and laboratory values remained unchanged. The proportion of CD patients with a clinical response and those in remission was greater in the AZD9056; improvements in the IBD questionnaire score were seen in the ADZ group. Three of 4 patients who discontinued the study (of 30 patients) due to adverse effects were in the AZD9056 group. Abdominal pain was common and reported in both treatment groups. Also, GI disorders that included diarrhea were more frequent after treatment with the drug (54%) versus 30% with placebo. Overall, the drug was well – tolerated to continue development of the drug. Despite encouraging results in CD patients, **AZD9056** did not show significant efficacy in the treatment of RA, and targeting the $P2X_7$ with this antagonist does not appear to be a therapeutically useful target in RA.

5.8 $P2X_7$ polymorphisms

Functional $P2X_7$ receptor polymorphisms have been identified in patients with CD. [19] These include a gain-of-function single nucleotide polymorphism (SNP) His155Tyr and a loss-of-function SNP Arg307Gln and Glu496Ala. There is evidence for $P2X_7$ polymorphisms that renders an increased susceptibility to Alzheimer's disease, bipolar affective disorders or

major depressive illness, multiple sclerosis and diabetes or resistance to infection with *Chlamydia trachomatis.* [20–24] However, association analysis indicated that these SNP's of the $P2X_7$ receptor are not a susceptibility factor for CD.

6.0 Herbal medicines are natural purinergic drugs with efficacy in FD and IBS

6.1 STW 5 (Iberogast$_R$)

STW 5 is a liquid formulation of nine herbs (phytopharmacon) shown to be effective in randomized, double bind placebo controlled multi-center clinical trials in *functional dyspepsia* [188,189] and *IBS* [190]. Adenosine A_{2A} receptors contribute to the anti-inflammatory effect of Iberogast in rat TNBS colitis. [191] A double blind, randomized, placebo-controlled phase III study is ongoing on the efficacy of Iberogast (BAY98-7411) to reduce pain intensity in patients with IBS. No data is yet available.

6.2 Paeoniflorin

Natural products are also a potential source to obtain P2X antagonists for use in clinical applications as analgesics. [192] Several natural products are shown to cause analgesia on inflammatory pain or neuropathic pain by inhibiting $P2X_3$ or $P2X_7$ mechanisms, although the selectivity of these compounds for specific P2X receptors remains unclear. **Paeoniflorin** (**72**) is a chief ingredient in the root of *Paeonia lactiflora* Pall, and it has been shown to be effective in relieving colorectal distension induced visceral pain and hyperalgesia in a rat model of IBS (neonatal maternal separation). The effect is mediated through the adenosine A_1AR to inhibit glutamate/NMDA receptor – dependent ERK signaling. [193] Much more work is needed to validate these products as viable alternative 'medicinal candidates' for clinical trials.

7.0 GI side effects of the $P2Y_{12}$ antagonist clopidogrel (Plavix)

Clopidogrel (**79**) is a thienopyridine class antiplatelet drug used to inhibit vascular clot formation. After preactivation in the liver, its active metabolite binds irreversibly to $P2Y_{12}$ receptors on platelet membranes and prevents platelet aggregation. Typically patients with significant coronary artery disease who undergo percutaneous intervention and coronary artery stent placement begin dual anti-platelet therapy immediately following the procedure. [194] Adverse effects of clopidogrel include bleeding (3–10%), hypersensitivity reactions, thrombotic thrombocytopenic purpura, neutropenia. Among others, significant gastrointestinal symptoms are discomfort (27.1%), diarrhea (4.5%), dyspepsia (5.2%), nausea (3.4%), and abdominal pain (5.6%). CNS side effects also occur: headache 7.6%, dizziness 6.2% and occasional vertigo, numbness, neuralgia and paresthesias. It is likely that GI side-effects of Plavix are linked to receptors localized in the GI tract, and pharmacological studies suggest inhibitory $P2Y_{12}$ receptors on EC cells modulate mechanosensitive 5-HT release. [130] $P2Y_{12}$ immunoreactivity is distributed throughout the ENS on enteric neurons (Christofi, unpublished observations). Irreversible antagonist binding to $P2Y_{12}$ receptors would lead to dis-inhibition and over-activation of mucosal reflexes and perhaps activate nociceptive reflexes as well. Therefore, receptors on EC or

other sites in the ENS deserve further consideration, in better understanding the actions of Plavix and to test their hypothesis.

8.0 ATP protects against NSAID-induced enteropathy in humans

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed medications for their analgesic and anti-inflammatory properties. NSAIDs such as naproxen, ibuprofen and indomethacin have an elevated risk of mucosal damage in the GI tract. A significant increase in mucosal permeability (leakiness) could be the underlying cause of such enteropathy. [195,196] A clinical study in healthy humans showed that topical ATP administration into the duodenum during a short challenge of NSAID attenuates the NSAID-induced increase in intestinal permeability in vivo. This finding may have potential implications for the treatment of other intestinal diseases or disorders associated with an increase in mucosal permeability. [197] For instance, infection with *Helicobacter pylori* increases intestinal permeability of the stomach and intestine. [198] In CD, gut mucosal permeability changes are associated with increase in the severity of the disease and is an early predictor of relapse. [199–202] An increase in mucosal permeability is also implicated in Celiac Disease [203] and IBS [204]. Further studies are needed to prove the validity of this approach in animal models of disease, healthy humans and GI diseases, but certainly it would be a worthy pursuit.

The therapeutic potential of ATP as an immune modulator in the treatment of HIV/AIDS, in combination with highly active antiretroviral therapies has recently been described. [205] This intriguing possibility awaits a pilot proof of concept clinical study.

9.0 Neuroprotection of the Enteric Nervous System

There are 2 potential receptor targets for neuroprotection of the ENS in lipid induced enteric neuropathy [206] and IBD. [73] The neuroprotective effects observed in $P2X_7$ null mice have already been discussed elsewhere in this review, and provide experimental proof to suggest that $P2X_7$ antagonists may be beneficial in protecting the ENS against apoptosis of the neurons, [73] and in immunomodulation in mast cells. Another receptor with pro-apoptotic properties is the $P2Y_{13}$ receptor. [207] A new study indicates that a $P2Y_{13}$ receptor antagonist such as MRS2211 (55) could prevent neuronal loss caused by fat-diet and palmitic acid induced neuronal loss in mice. [206] The ADP sensitive $P2Y_{13}$ receptor is a potential therapeutic target in lipid-induced enteric neuropathy. Briefly, animals fed a high fat diet for 6 months developed enteric neuropathy and cell damage, whereas $P2Y_{13}{-/-}$ litermates were protected against neuropathy and the loss of myenteric neurons. Therefore, activation of the $P2Y_{13}$ receptor is important in the development of enteric neuropathy and apoptosis of the neurons.

10.0 Extracellular ATP as ‘alarmin’ or danger signal in IBD

In animals fed a diet supplemented with nucleosides and nucleotides, chemical induced colitis and colonic injury was exacerbated, in association with increased leukocyte, macrophage and lymphocyte infiltration of the colonic mucosa. [208,209] UDP activation of $P2Y_6$ receptors is involved in the innate mucosal response of the gut and it regulates T cell

activity in chronic colitis [210] Intestinal inflammation has been shown to increase the expression of $P2Y_2$ and $P2Y_6$ receptors on epithelial cells and the release of CXC chemokine ligand 8 by UDP, the cognate ligand of the $P2Y_6$ receptor. [143] Extracellular nucleotide signaling is therefore involved in the progression of intestinal inflammation. Extracellular nucleotides can act via $P2X_{1–7}$ ligand-gated cation channels and G protein-coupled $P2Y_{1,2,4,6,11–14}$ receptors. $P2Y_{2,4,6}$ receptors regulate Cl^-, Na^+ and K^+ secretion in the intestinal tract and absorption mechanisms. [10] In the inflamed state, P2Y receptors stimulate production and secretion of cytokines, proinflammatory molecules and cell adhesion molecules, and induce cell migration, immune cell recruitment and proliferation and differentiation processes. Overall, it has been suggested that extracellular nucleotides are ‘alarmins’ or danger signals that can be rapidly released to enhance the activity of the innate immune system of the gut. [143] It is too early to know whether nucleotide receptors are promising therapeutic targets in IBD.

Conclusions

Purinergic drugs methotrexate, sulfasalazine, Adenocard (adenosine), dipyridamole, caffeine, and many newer generation drugs developed as a result of progress in medicinal chemistry (targeting A_3, A_{2A}, $P2Y_{12}$, $P2X_{2/3}$, $P2X_3$, $P2X_7$ receptors) and several phytopharmaca have an excellent safety/efficacy profile for potential future clinical trials in IBD, IBS, FD and inflammatory diarrhea. The future for purinergic drugs on clinical trials seems hopeful, although it may be a bit risky, and somewhat of a ‘balancing act’ to obtain the benefit of treatment without compromise the physiology of the patient that is dually targeted by receptor drugs. Genetic polymorphisms, caffeine consumption, and other individual traits or differences in behavior between patients may potentially affect susceptibility to treatment. Therefore, a personalized medicine approach may ultimately be a suitable option to tailor treatment in every patient. Future studies in animals are needed to further clarify cellular and molecular mechanisms and to test new-generation drugs. There is still a huge gap in our knowledge of human pathophysiology of purinergic signaling, and such translational studies are of critical importance given that significant species differences likely exist in purinergic signaling between animals and humans and the receptors are differentially regulated in disease states.

Overall, given the safety, tolerability, efficacy of several purinergic drugs in clinical trials, a rather compelling case can be made for going ahead with FDA approved, designed clinical trials to treat GI symptoms in patients with IBD and IBS.

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Abbreviations

ADA	Adenosine deaminase
AR	Adenosine receptors
eADO	Endogenous adenosine
tADA	total adenosine deaminase
CNS	Central nervous system
CD	Crohn's Disease
COPD	Chronic obstructive pulmonary disease
CDAI	Crohn's Disease activity index
CD39	Ectoucleoside triphosphate diphosphohydrolase (ENTPDase, Nucleotidase CD39)
CD73	ecto-5′-nucleotidase (ecto-5′-NT)
CYP1A1	Cytochrome P450, 1A1
CYP1A2	Cytochrome P450, 1A2
DSS	Dextran sulfate sodium salt
EC	Enterochromaffin cell
EFS	Electrical field stimulation
ENaC	Epithelial Na^+ ion channel
ENS	Enteric nervous system
ENT1/2	Extracellular Nucleoside Transporters 1/2
FD	Functional disorder
GI	Gastrointestinal
GERD	Gastroesophageal reflux disease
HIF	Hypoxia-inducible factor
5-HT	5-Hydroxytryptamine (Serotonin)
IBD	Inflammatory Bowel Disease
IBS	Inflammatory Bowel Syndrome
ICC	Interstitial cells of Cajal
IL	Interleukine
INF-γ	Interferon-γ
KO	Knock-out
LPS	Lipopolysaccharides
NSAID	Non-steroidal anti-inflammatory drugs

NANC	non-adrenergic, non-cholinergic
PMN	Polymorphonuclear lukocytes
PBMC	Peripheral blood mononuclear cells
POI	Post-operative ileus
PON1	Paraoxonase-1
RA	Rheumatoid Arthritis
RT-PCR	Reverse transcription polymerase chain reaction
SNP	Single nucleotide polymorphism
TNFα	Tumor-necrosis Factor α
TNBS	2,4,6-Trinitrobenzenesulfoni acid
TPH-1	Tryptophan hydroxylase 1
UC	Ulcerative Colitis
UDP	Uridine-5′-diphosphate
VAS	Visual analog scale pain score

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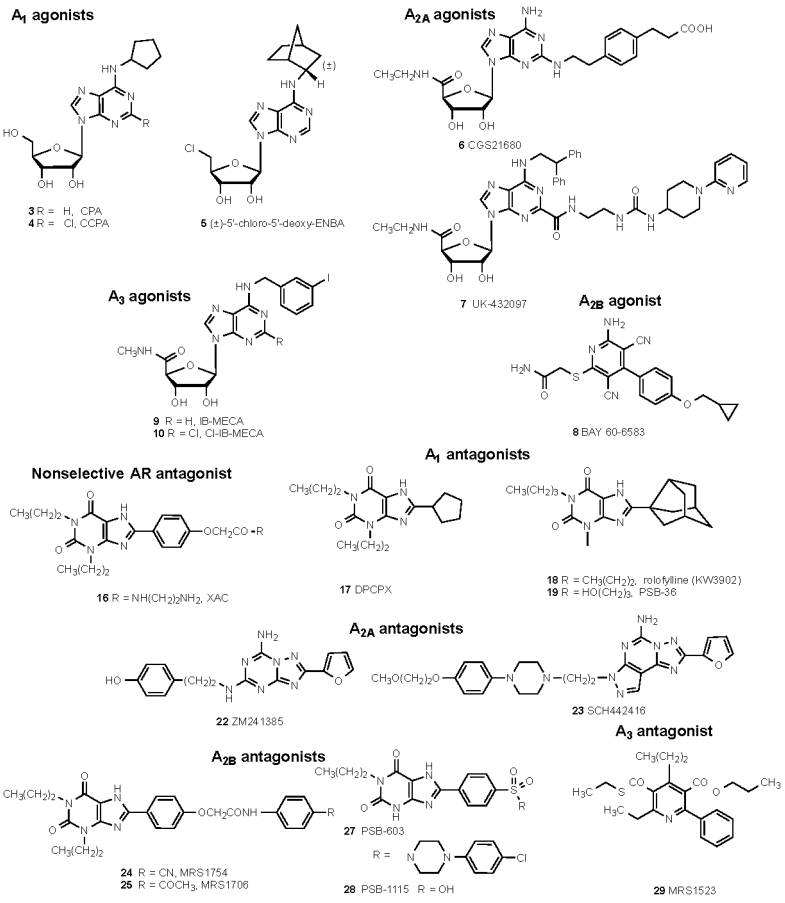


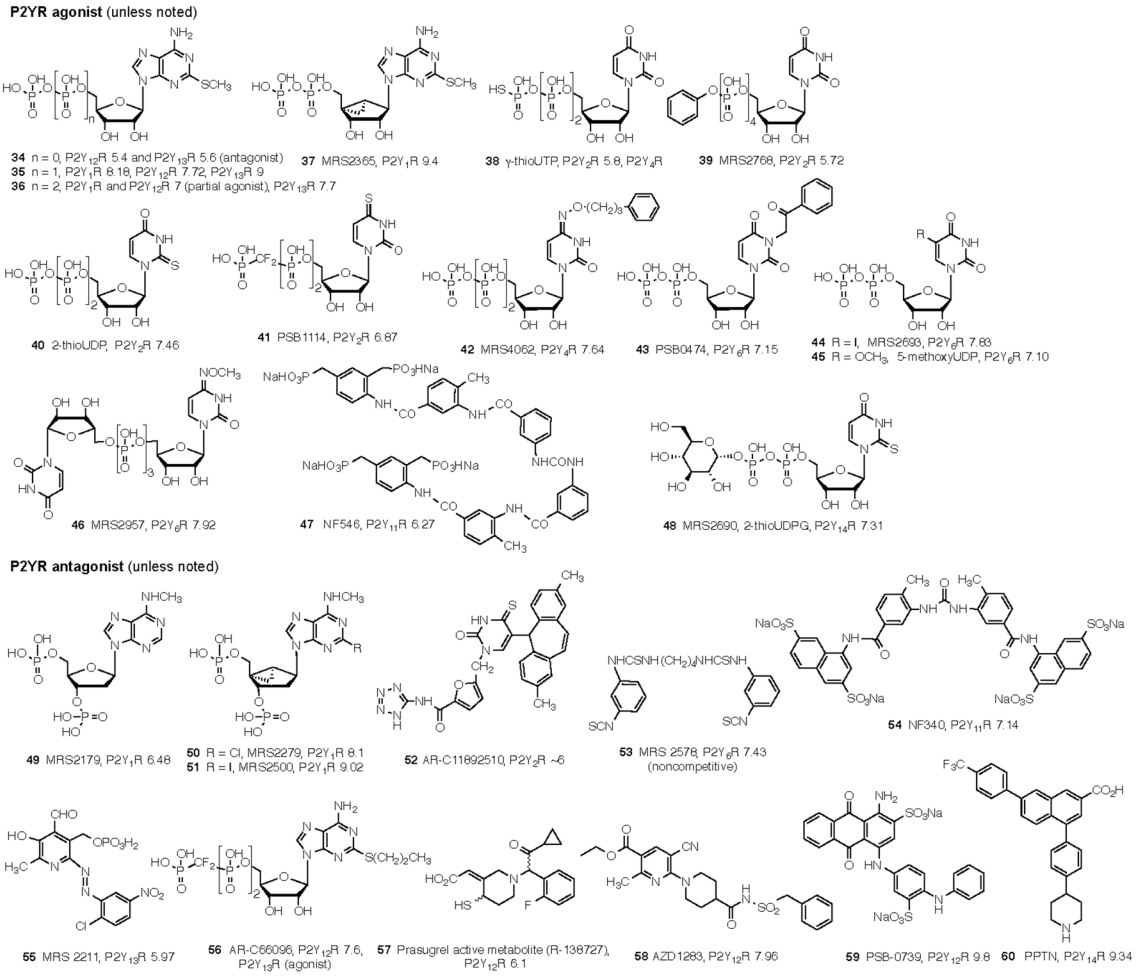
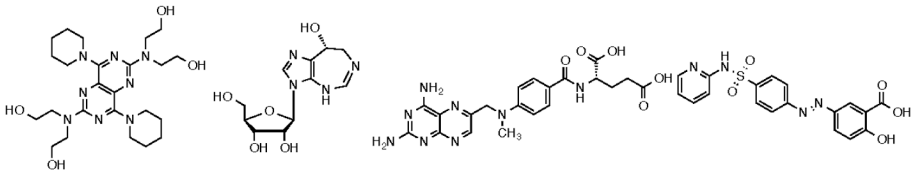


Figure 1.

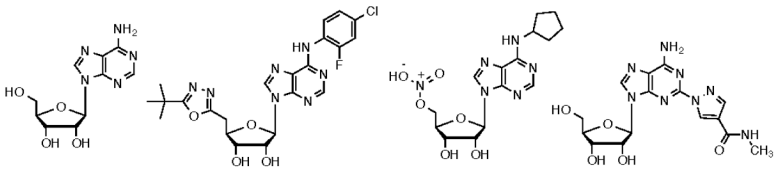
Structures of commonly used ligands for adenosine and P2Y receptor families are shown. **1A.** Selective agonist - and antagonist - probes of the adenosine receptors that are readily available as pharmacological probes. The compounds are numbered consecutively to correspond to the list in Table 3. For additional details refer to a review by Müller et al. [211] **1B.** Selective agonist - and antagonist - probes of the P2Y receptors that are readily available as pharmacological probes. The in vitro pEC^{50} or pIC^{50} is indicated at each relevant subtype. Compound **57** is the active metabolite of **80** (see Figure 2B). The compounds are numbered consecutively from the end of Figure 1A. More detail is available in reference [15] and on the website. [17]

indirectly acting on the adenosine system

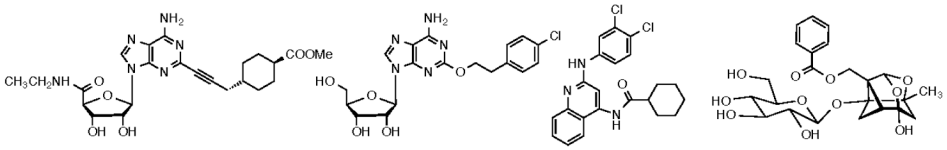


60 dipyridamole (Persantine) **61** pentostatin (deoxycoformycin) **62** methotrexate **63** sulfasalazine

directly acting as adenosine receptor agonists, or positive allosteric modulator (PAM) and modulator of unknown mechanism

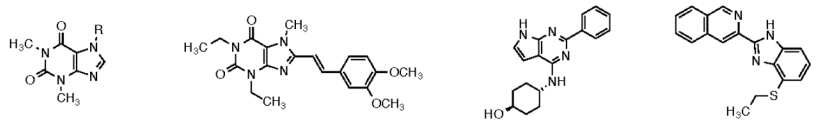


64 adenosine **65** GW493838 **66** INO-8875 **67** Lexiscan (CVT-3146)



68 Apadenoson (ATL-146e) **69** Sonedenoson (MRE-0094) **70** CF602, LUF6096 **71** paeoniflorin

adenosine receptor antagonists

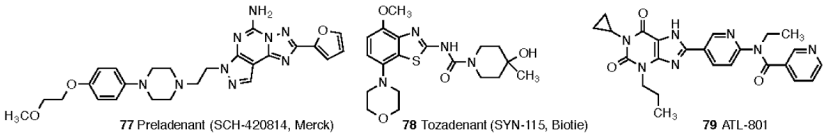


72 R = H, theophylline (aminophylline as ethylenediamine salt)
73 R = CH_3, caffeine

74 Istradefylline (KW-6002, Kyowa Hakko)

75 SLV-320

76 Compound 4b (3-[4-(ethylthio)-1H-benzimidazol-2-yl]isoquinoline)



77 Preladenant (SCH-420814, Merck) **78** Tozadenant (SYN-115, Biolie) **79** ATL-801

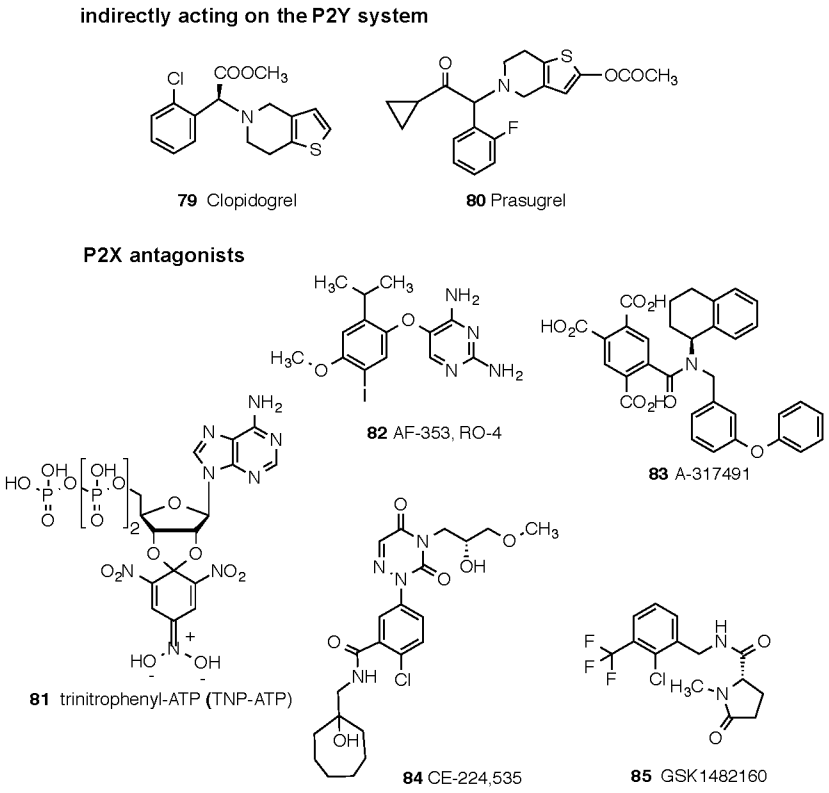


Figure 2.

Structures of ligands of clinical interest acting directly or indirectly through the adenosine receptor system (Fig. 2A), or through the P2X and P2Y receptor families (Fig. 2B). The compounds are numbered consecutively from the end of Figure 1B. Many of these compounds are proprietary

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Table 1

Key studies in IBD or functional GI disorders in mouse knockout models targeting purine genes

Model	Cellular Target	Functional Consequence	Disease/Functional disorder
$P2X_7$ $^{-/-}$ KO (DNBS, DSS, Oxazolone, IL-$10^{-/-}$)	nNOS + Myenteric Neurons	Prevents neuronal apoptosis and protects against IBD inflammation-induced colonic motor dysfunction	IBD [73]
Mouse Jejunum, Serum & Peritoneum (*T spirallis*) & $P2X_7^{-/-}$ KO	Macrophages	Increased IL-1β-mediated mechanosensitivity in mesenteric afferents, not present in KO	IBS [212]
$P2X_{2/3}^{-/-}$ KO (*T spirallis*)	Small Intestinal Afferent Nerves	Increased afferent hypersensitivity.	IBS [164]
Heterozygote and $P2X_3$ $^{-/-}$ KO (zymosan)	DRG Neurons	Reduced VMR to CRD Absent hypersensitivity in zymosan-treated $P2X_3^{-/-}$ KO	IBS [165]
$A_{2A}AR^{-/-}$ KO *(H. felis)*, IL-10 $^{-/-}$ KO (*H. pylori)*	Blood & Mucosal Th cells	Exacerbated gastritis with no difference in colonization (Diminished with ATL313 in IL-10 deficient)	GERD [133]
$A_{2A}AR$ $^{-/-}$KO and $A_{2B}AR$ $^{-/-}$KO (DSS)	Epithelial cells	Increased severity of DSS colitis and loss of mucosal IL-10 expression in $A_{2B}AR$ $^{-/-}$KO	IBD [68]
$A_{2B}AR$ $^{-/-}$ KO (DSS, TNBS, *S typhimurium*)	Epithelial Cells	Reduced inflammatory response Possible abnormal circular muscle	IBD [98]
CD39-null (TNBS, Oxazolone)	Lamina Propria	Decreased severity of disease (TNBS but no Oxazolone)	CD & UC [52]
CD39 null & heterozygote (DSS), Colon	Leukocytes	Increase severity of DSS colitis Increase leukocyte infiltration	IBD [26]
$A_{3A}R^{-/-}$KO(DSS)	Mucosa	Decreased severity of disease (prevent diarrhea, weight loss, inflammation) vs WT	IBD [29]
$A_{3A}R$ $^{-/-}$KO (DSS)	Epithelial Cells, ENS/Glial cells	Increased intestinal transit and colonic evacuation	IBD [29]
$A_{3A}R^{-/-}$KO (DSS)	Mucosa	Moderate protection against colitis; impaired innate immune response	IBD [30]
CD73 $^{-/-}$ KO (DSS), Colon	Epithelial Cells	High susceptibility to DSS-induced colitis. Increase TLR9 (mRNA), IL-IB, TNFα (ELISA), constitutive activation of NF-κB	IBD [213]
CD73 $^{-/-}$ KO (TNBS)	Mucosa	Increase severity of colitis (weight loss, colon shortening) Downregulation of INFα	IBD [64]
P2ry1 $^{-/-}$KO	Circular Muscle Cells	fIJP completely absent and delayed colonic transit	Analysis of phenotype [4]
$P2ry1^{-/-}$ KO	Circular Muscle Cells	Absent purinergic IJP Absence of spontaneous IJP	Analysis of phenotype [214]

DSS; Dextran Sodium Sulfate, **DNBS**; 2,4-Dinitrobenzene Sulfonic Acid, **fIJP;** Fast Inhibitory Junction Potential, **IBD**; Inflammatory Bowel Disease, **IBS**; Irritable Bowel Syndrome, **TNBS**; 2,4,6-Trinitrobenzenesulfonic acid, **VMR to CRD**; Visceromotor Reflex to Colorectal distension, ***S typhimurium;*** *Salmonella typhimurium*, ***T spiralis;*** *Trichinella spiralis*

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Table 2

Abnormalities of purinergic signaling in disease

Model	Target	Δ Gene expression	Functional Consequence	Disease
Mouse Colon (Zymosan)	Colonic DRG	↑$P2X_3R$ (Increased Function)	Increased excitability and enhanced purinergic signaling	IBS [215]
Rat (Acetic acid)	DRG from LS but not TL	↑$P2X_3R$	In association with visceral hypersensitivity	IBS [166]
Rat colon and Spinal Cord (CRD)	Whole Colon, Neurons	↑$P2X_4R$ (Immunoreactivity)	In association with visceral hypersensitivity	IBS [216]
Mouse Small Intestine (*T spiralis*)	Visceral Afferents	↑Purinergic Component (PPADS Sensitive)	Increased afferent sensitivity small intestine.	IBS [164]
Rat (STZ)	DRG L4-6	↑$P2X_2R$, ↑$P2X_3R$ (mRNA)	In association with Neuropathic Pain	Diabetic Neuropathy [217]
Rat (STZ)	DRG L4-6 (hind-paw labeled)	↑$P2X_3R$ (Protein Trafficking)	In association with Neuropathic Pain	Diabetic Neuropathy [218]
Rat (STZ)	Microglia from Spinal Cord	↑$P2X_4R$	In association with Neuropathic Pain	Diabetic Neuropathy reviewed in [219]
Human biopsies (ganglionic & aganglionic regions)	Myenteric and Submucose and Nerve Fiber in Muscle Layers	↓$P2Y_1R$ ↓$P2Y_2R$	Decrease in immunoreactivity for $P2Y_1$/$P2Y_2$ occurs in the aganglionic segment	Hirschprung's Disease [220]
Mouse Ileum (*S mansoni*)	Longitudinal Muscle	↓A_1AR	Impaired inhibitory adenosinergic modulation of cholinergic transmission	Chronic Inflammation [221]
Rat colon (DNBS)	LMMP	↑A_3AR ↑ADA (mRNA)	Decreased cholinergic contraction in nflamed tissue	IBD [145]
Mouse Colon (DSS)	Epithelium	↑$P2Y_2R$ ↑$P2Y_6$ R (mRNA)	Proinflammatory effect	IBD [53]
Rat (DNBS)	LMMP	↑$A_{2A}AR$ ↑CD73 (mRNA) A_1AR (Not Affected)	Inhibitory control of motor function converted from a predominant A_1 to A_{2A} dependent regulation	IBD [149]
Mouse (DSS and IEC-6)	Epithelial Cells	↑$P2Y_2R$ (mRNA)	Exacerbate inflammation	IBD [144,222]
Guinea pig (LMMP) (TNBS)	Smooth Muscle	Augmented Release of ATP, ADP, AMP, ADO, β-NAD (by HPLC)	Impaired purinergic fIJP via $P2Y_1R$ receptors	IBD [223]
Mouse(DSS), Guinea pig (TNBS)	Smooth Muscle	↓ATP & ADP Release Stimulus-Induced	Reduced IJP and propulsive motility	IBD [223]
OVX Female rats (TNBS-EtOH)	DRG neurons	↓$P2X_3R$ in association with ↓VMR	P2X3 and hyposensitivity in OVX rats reverted after Estrogens treatment	IBD [161]
Mouse Colon (DSS)	Macrophages (F4/80 +) Submucosal Arterioles	↑CD39 (ENPD1)(Protein & mRNA)	Linked to impaired arterioles-constriction	IBD [75]

Model	Target	Δ Gene expression	Functional Consequence	Disease
Rat Ileum/jejunum (TNBS)	Whole Tissue	↓A_1AR ↓A_{2A}AR ↓A_3AR (mRNA)	Altered nerve mediated cholinergic contractions	IBD [191]
Rat (TNBS)	Longitudinal Muscle	↑A_{2A}AR	Facilitate inhibition of cholinergic transmission	IBD [224]
Mouse Mast cell Deficient, Colon (TNBS, DSS)	Mononuclear Cells	↓$P2X_7R$ ↓ATP	Amelioration of colitis (↑$P2X_7R$ & ATP in WT)	IBD [54]
Guinea pig (TNBS)	Submucosal Neurons	↑P2X function (fEPSP)	Submucosal synapses	IBD [225]
Human mucosa & Rat (DSS)	Mucosa & EC	↑A_{2B}AR ↑HIF-1a (mRNA, protein)	Exacerbation of inflammation by increased 5HT release via A_{2B}AR	CD [129]
Human PBMC	Mononuclear cells	↑A_3AR (Protein)	Biomarker of disease and therapeutic target	CD [45]
Human biopsies	Epithelial Cells	↑$P2Y_2R$ ↑$P2Y_6R$ (mRNA)	Increased Neutrophils infiltration	CD & UC [53]
Human whole colonic biopsies	Colon	↑CD39 (Inactive CD vs UC) Variable in animal models	Exacerbation of inflammation	CD [52]
Human whole colonic biopsies	Colon	≈CD39 (Inactive UC)	Not difference	UC [52]
Human Serum/Neutrohil	Neutrophils	↑tADA, ↑ADA2 (Serum), ↑ADA1(Netrophils)	Increased inflammatory response (Possible biomarker of inflammation - Active Disease-)	CD [50]
Human colonic biopsies	Myenteric Neurons	↑$P2X_3R$ (Protein)	Dysmotility and Pain	CD & UC [162]
Human UC (colon) vs control	EC (5-HT^+ cells)	↓$P2X_3^+$/5-HT^+ cells from 15% to <1%	Expected to alter fast purinergic regulation of 5-HT release	UC [130]
Human mucosal biopsies	Mucosa	↓ADORA3, ↑ADORA2A, ↓AMPD3, ↑ADAR, ↓P2RY13, ↓P2RY14, ↑NT5E, etc	Unique purine dysregulation profile for CD/ distinguishes between CD and UC (biomarker of disease)	CD (biomarker of disease) [47]
Human mucosal biopsies	Mucosa	↑ADORA3, ↑AMPD3, ↑P2RY13, ↑P2RY14, ↑DPP4, ↓P2RY6, ↑NT5E, etc	Unique purine dysregulation profile for UC	UC (biomarker of disease) [47]
Human PBMC	Mononuclear Cells	↑ADORA2B, ↑ADORA2A, ↑AMPD3, ↓ADAR, ↓DPP4, ↓P2RX5, ↑P3RY5, ↑AMPD2, etc	Unique purine dysregulation profile for CD/ distinguishes between CD and UC	CD (biomarker of disease) [47]
Human PBMC	Mononuclear Cells	↓ADORA2B(w), ↓ADORA2A(w), ↑AMPD2↑ADAR, ↑DPP4, ↑P2RX5, ↓P2RX1, ↓P2RX2, ↓P2RX3, etc	Unique purine dysregulation profile for UC	UC (biomarker of disease) [47]
Human samples	Peripheral Blood	$P2X_7R$ (loss of function Arg307Gln, P=0.06)	Polymorphism is not a susceptibility factor for CD	CD [19]
Human colon and ileum	Epithelial Cells	↓$P2X_7R$ (Protein) ↑$P2X_7R$ (mRNA)	Increased PMNL transepithelial migration. Amplified inflammatory loop.	CD [51]
Human colonic biopsies and TNBS & DSS mice	Mast Cells	↑$P2X_7R$	Associated with aggravation of intestinal inflammation	CD (biomarker of disease) [54]

Model	Target	Δ Gene expression	Functional Consequence	Disease
Human Colonic biopsies and TNBS & DSS mice	Mast Cells	No change in $P2X_7R$ vs control	Not difference	UC [54]

ADA1 and 2; ADA Izoenzimes 1 and 2, **CFA;** Complete Freud's Adjuvant, **CRD**; Colorectal Distension, **DNBS**; 2,4-Dinitrobenzene Sulfonic Acid, **DRG;** Dorsal Root Ganglia**, DSS;** Dextran Sodium Sulfate, **GERD**; Gastroesophageal Reflux Disease, ***H felis;*** *Helicobacter felis*, ***H pylori:*** *Helicobacter pylori*, **HIF-1a**; Hypoxia-inducible factor 1-alpha, induces transcription and increases the activity of 59ecto-nucleotidase (CD73), the enzyme that converts AMP to adenosine. CD73 also regulates transcription of the ADORA2B receptor while suppressing transcription of the adenosine re-uptake transporters (ENT1 and 2), **IBD**; Inflammatory Bowel Disease, **IBS**; Irritable Bowel Syndrome, **LS**; Lumbosacral, **NCI;** Nerve Chronic Constriction Injury, **PBMC**; Peripheral blood mononuclear cells, ***S mansoni;*** *Schistosoma mansoni*, **STZ,** Streptozotocin (Model of diabetes and neuropathic pain), ***S typhimurium;*** *Salmonella typhimurium*, **tADA;** (CD26) total Adenosine deaminase, **TNBS**; 2,4,6-Trinitrobenzenesulfonic Acid, **TL**; Toracolumbar, ***T spiralis;*** *Trichinella spiralis*, **W;** Woman

Table 3

Affinities of orthosteric adenosine receptor ligands that are commonly used as pharmacological probes. Affinity is shown at the human adenosine receptors, unless noted. Structures are shown in Figures 1A and 2A.

No.	Compound	Affinity, pK_i[a]			
		A_1	A_{2A}	A_{2B}[b]	A_3
Adenosine receptor agonists					
1	adenosine[b]	~7 7.14 (r)	6.49 6.82 (r)	4.82 5.29 (r)	6.54 5.19 (r)
A_1-selective					
2	*R*-PIA	8.69 8.92 (r)	6.66 (r)	3.82	7.48 6.80 (r)
3	CPA	8.64	6.10	4.73	7.14
4	CCPA	9.08 8.89 (r)	5.64 6.02 (r)	4.73	7.42 6.63 (r)
5	5′-Cl-5′-deoxy-ENBA	9.29	5.89	5.87	5,56
A_{2A}-selective					
6	CGS21680	6.54 5.74 (r)	7.57 7.72 (r)	<5 <5 (r)	7.17 6.23 (r)
7	UK432,097	ND	8.4	ND	ND
A_{2B}-selective					
8	BAY 60-6583	<5[b]	<5[b]	8–8.5	<5[b]
A_3-selective					
9	IB-MECA (CF101)	7.29	5.54	4.96	8.74
10	Cl-IB-MECA (CF102)	6.66 6.55 (r)	5.27 6.33 (r)	<5	8.85 9.48 (r)
11	thio-Cl-IB-MECA	6.71	6.63	ND	9.42
12	MRS5698	<5	<5	<5	8.5
Adenosine receptor antagonists					
Non-selective					
13	Caffeine	4.97 4.39 (r)	5.02 4.32 (r)	4.98 4.52 (r)	4.88 <4 (r)

No.	Compound	Affinity, pK_i[a]			
		A_1	A_{2A}	A_{2B}[b]	A_3
14	Theophylline	5.17 5.06 (r)	5.17 25,300 (r)	5.04 4.82 (r)	4.65 4.07 (r)
15	CGS15943	8.46 8.19 (r)	8.92	7.49	7.46
16	XAC	7.54 8.92 (r)	9.0 7.20 (r)	7.91	7.04
A_1-selective					
17	DPCPX (CPX)	8.52 9.0 (r)	7.22 6.30 (r)	7.29 6.73 (r)	6.61 4.37 (r)
18	PSB-36	9.2 9.91 (r)	6.01 6.26 (r)	6.73	5.64 5.19 (r)
A_{2A}-selective					
19	KW6002	6.08[b] 6.64(r)[b]	7.92 8.66 (r)	<5[b]	5.35[b]
20	CSC[d]	4.55 (r)	7.27 (r)	5.09	<5 (r)
21	ZM241,385	6.11	8.80	7.12	6.13
22	SCH442,416	5.95	8.39	<5	<5
A_{2B}-selective					
23	MRS1754	6.39 7.77 (r)	6.30 6.21 (r)	8.70 7.89 (r)	6.24
24	MRS1706	6.80	6.91	8.86	6.64
25	MRE2029-F20	6.70	<6	8.26	<6
26	PSB-603	<5 <5 (r)	<5 <5 (r)	9.26	<5
27	PSB-1115	<5 5.66 (r)	4.62 (r)	7.27	<5
A_3-selective					
28	MRS1523	<5 4.81 (r)	5.44 5.69 (r)	<5	7.72 6.95 (r)
29	PSB-10	5.77 6.09 (r)	5.57 5.22 (r)	ND	9.36
30	VUF5574	≤5 (r)	≤5 (r)	ND	8.39

No.	Compound	Affinity, pK_i[a]			
		A_1	A_{2A}	A_{2B}[b]	A_3
31	MRS1191	<5 4.40 (r)	<5 <5 (r)	<5	7.50 5.73 (r)
32	MRS1334	<5 (r)	<5 (r)	ND	8.57

[a] Data from binding assays, unless noted (for more details see [211] and references cited therein). Human, unless noted; r = rat.

[b] From functional studies

[c] ND = no data available

[d] K_i at monoamine oxidase-B = 80.6 nM [226]

Table 4

Drugs in clinical trials targeting purinergic receptors

ID #	Drug	Target	Disease/Symptom	Sponsor	Phase	Status	Efficacy
NCT00376454	**GW493838 (66)**	**A_1AR Agonist**	Neuropathic Pain	GlaxoSmithKline	II	Completed	Results not available
NCT01123772	**INO-8875 (67)**	**A_1AR Agonist**	Dose escalation (HV)	Inotek Pharmaceuticals Corporation	I	Completed	Results not available
NCT00354458	**KW-3902IV (18)**	**A_1AR Antag**	AHF with RI	NovaCardia, Inc.	III	Completed [88]	No (SE, ↑ Stroke)
NCT00160134	**SLV320 (74)**	**A_1AR Antag**	AHF with RI	Solvay Pharmaceuticals	II	Completed [227]	Yes
NCT00744341	**SLV320 (74)**	**A_1AR Antag**	AHF with RI	Solvay Pharmaceuticals	II	Completed [228]	Not conclusive
NCT00452777	**BVT.115959**	**A_{2A}AR Agonist**	Diabetic Neuropathic Pain	Swedish Orphan Biovitrum	II	Complete	Results not available
NCT00208312	**Regadenoson (68)**	**A_{2A}AR Agonist**	Stress Agent*	Astellas Pharma US, Inc.	III	Completed [229]	Yes
NCT00862641	**Regadenoson (68)**	**A_{2A}AR Agonist**	Stress Agent *	Astellas Pharma Inc	IV	Completed [230]	Yes
NCT00312364	**MRE0094 (70)**	**A_{2A}AR Agonist**	Diabetic Complications	Pfizer	II	Completed	Results not available
NCT01940848	**STW5, Iberogast, BAY98-7411**	**A_{2A}AR Antag**	IBS	BAYER	III	Recruiting	Results not available
N/A	**STW5/II**	**A_{2A}AR Antag**	IBS	Steigerwald Arzneimittelwerke GmbH	II	Completed [189]	Yes
N/A	**STW5**	**A_{2A}AR Antag**	Functional Dyspepsia	Steigerwald Arzneimittelwerke GmbH	II	Completed [188]	Yes
NCT01190735	**Caffeine (13)**	**A_{2A}AR Antag**	Parkinson's Disease	McGill University Health Center	II	Completed	Results not available
NCT01190735	**Caffeine (13)**	**A_{2A}AR Antag**	Parkinson's Disease	McGill University Health Center	II	Completed	Results not available
NCT01155466	**Preladenant (76)**	**A_{2A}AR Antag**	Parkinson's Disease	Merck	III	Completed	Results not available
NCT00006337	**KW-6002 (73)**	**A_{2A}AR Antag**	Parkinson's Disease	NINDS	II	Completed	Results not available
NCT00783276	**SYN115 (77)**	**A_{2A}AR Antag**	Cocaine addiction	National Institute on Drug Abuse (NIDA)	0	Completed [231]	Yes
NCT01435486	**Caffeine Citrate**	**A_{2A}AR Antag**	Bronchiolitis	Maastricht University Medical Center	N/A	Recruiting	Results not available
NCT01034306	**CF101 (9)**	**A_3AR Agonist**	RA	Can-Fite BioPharma	II	Recruiting	Results not available
NCT00837291	**CF101 (9)**	**A_3AR Agonist**	Osteoarthritis of the Knee	Can-Fite BioPharma	II	Not yet Recruiting	Results not available
NCT00428974	**CF101 (9)**	**A_3AR Agonist**	Plaque-type Psoriasis	Can-Fite BioPharma	II	Completed [35]	Yes
NCT00790673	**CF102 (10)**	**A_3AR Agonist**	Chronic Hepatitis C	Can-Fite BioPharma	I, II	Completed	Results not available
NCT00790218	**CF102 (10)**	**A_3AR Agonist**	Hepatocellular Carcinoma	Can-Fite BioPharma	I, II	Unknown	Results not available
NCT00349466	**CF101 (9)**	**A_3AR Agonist**	Dry Eye Syndrome	Can-Fite BioPharma	II	Completed [37]	Yes (SE, ↓ Intra ocular pressure)

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ID #	Drug	Target	Disease/Symptom	Sponsor	Phase	Status	Efficacy
NCT01235234	**CF101 (9)**	**A_3AR Agonist**	Dry Eye Syndrome	Can-Fite BioPharma	III	Completed [40]	Questionable efficacy
NCT01033422	**CF101 (9)**	**A_3AR Agonist**	Ocular Hypertension	Can-Fite BioPharma	II	Recruiting	Results not available
NCT01905124	**CF101 (9)**	**A_3AR Agonist**	Uveitis	Can-Fite BioPharma	II	Not yet Recruiting	Results not available
NCT00298636	**Adenosine (1)**	**AR Agonist**	Perioperative Pain	Xsira Pharmaceuticals	II	Completed	Results not available
NCT00881686	**Adenosine (1)**	**AR Agonist**	MRI	Xijing Hospital	I, II	Completed	Results not available
NCT01123525	**Adenosine (1)**	**AR Agonist**	Cardioplegia	University Hospital of North Norway	I, II	Completed [232]	Yes
NCT01022151	**Aminophylline (13)**	**AR Antag**	Recovery from Anesthesia	King Faisal University	II	Completed [233]	Yes
NCT01369745	**Dipyridamole (61)**	**ENT1/2 Inhib**	RA	Zalicus	II	Completed	Results not available
NCT01091571	**Dipyridamole (61)**	**ENT1/2 Inhib**	Endotoxemia	Radboud University	IV	Completed [82]	Yes
NCT01554579	**AF-219**	**$P2X_3R$ Antag**	Osteoarthritis of the Knee	Afferent Pharmaceuticals, Inc.	II	Recruiting	Results not available
NCT01569438	**AF-219**	**$P2X_3R$ Antag**	Bladder Pain Syndrome	Afferent Pharmaceuticals, Inc.	II	Recruiting	Results not available
NCT01432730	**AF-219**	**$P2X_3R$ Antag**	Chronic Cough	Afferent Pharmaceuticals, Inc.	II	Completed	Results not available
D8830C00002[&]	**AZD9056**	**$P2X_7R$ Antag**	CD	Astra Zeneca	II	Completed [234]	Yes
NCT00520572	**AZD9056**	**$P2X_7R$ Antag**	RA	Astra Zeneca	II	Completed[235]	No
NCT00628095	**CE-224,535 (84)**	**$P2X_7R$ Antag**	RA	Pfizer	II, III	Completed [187]	No
NCT00849134	**GSK1482160 (85)**	**$P2X_7R$ Antag**	Inflammatory Pain (HV)	GlaxoSmithKline	I	Completed [236]	Yes (↓ IL1B after LPS)
N/A	**ATP**	**P2X?**	Postoperative Orofacial Surgery Pain	Multicentre Study (Academic Institutions)	II	Completed [172]	Yes
NCT01107912	**Prasugrel (80) vs Clopidogrel (79)**	**$P2Y_{12}R$ Antag**	CAD	Eli Lilly and Company	I	Completed [237]	Yes
NCT00557921	**CGT-2168**	**$P2Y_{12}R$ Antag**	CAD	Cogentus Pharmaceticals	III	Completed [238]	Yes
NCT01099566	**Prasugrel (80)**	**$P2Y_{12}R$ Antag**	Sepsis (HV)	Medical University of Vienna	IV	Completed [239]	Yes

AHF with RI; Acute Congestive/Decompensate Heart Failure with Renal Impairment, **Antag**; Antagonist, **ENT1/2**; Extracellular Nucleoside Transporters 1/2, **CAD**; Coronary Artery Disease, **CD**; Crohn's Disease, **HV**; Healthy Volunteers, **IBS**; Irritable Bowel Syndrome, **Inhib**; Inhibitor, **MRI**; Myocardial Reperfusion Injury, **N/A**; Information not available, NINDS; National Institute of Neurological Disorders and Stroke, **RA**; Rheumatoid Arthritis, SE; Side-effects,

[*] Stress Agent for Myocardial Perfusion Imaging in Coronary Artery Disease/Asthma and Pulmonary Disease, **ID#**; ClinicalTrials.gov Identifier,

[&] astrazenecaclinicaltrials.com Identifier

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Table 5

Medicinal candidates with efficacy in preclinical models

Selected structures are shown in Figures 1 and 2, as indicated by compound numbers in parentheses.

Drug/Treatment	Receptor Target	Cellular Target	Disease	Model	Mechanism	Efficacy
Paeoniflorin (72)	A_1AR Agonist	Neurons	**IBS**	Rat (Maternal Separation-CRD)	Blocks visceral pain (Inhibition of CRD-Glutamate release and action in central structures of pain perception)	Yes [193]
FK352, DPCPX (17)	A_1AR Antagonist	Possibly Myenteric Neurons	**Post-Operative Ileus**	Anesthetized Rats (pentobarbital) or Surgical Trauma	Improve propulsive motility (Reversed the slowed colonic propulsion)	Yes [152]
STW5 (Iberogast)	A_{2A}AR Antagonist	Possibly Myenteric Neurons	**IBD**	RAT Ileum/Jejunum (TNBS)	↑A_{2A}AR (Inhibition of cholinergic transmission)	Yes [191]
ATL-313	A_{2A}AR Agonist	Mucosa	**GERD**	Mouse IL-10 Deficient (*H. Pylori*)	ATL-313 Reduce inflammation, bacterial load was increased	Yes [133]
Inosine	A_{2A}AR Agonist	Mucosal T-cells	**IBD**	Rat (TNBS)	Improved leukocyte infiltration and epithelium destruction. Partially Reverted by SCH-442416 (A2AR Antagonist)	Yes [62]
ATL-801 (78)	A_{2B}AR Antagonist	Epithelial Cells	**IBD**	Mouse (DSS), IL-$10^{-/-}$ KO (Piroxicam)	Ameliorate experimental colitis, ↓ Adenosine-mediated cAMP level, Inhibit secretion	Yes [96]
A-317491 (83)	$P2X_3$R Antagonist	DRG Neurons	**Neuropathic Pain**	Rat (CFA & NCI)	Blocks specifically $P2X_3$ & $P2X_{2/3}$R	Yes [240,241]
AF-353 (82)	$P2X_3$R, $P2X_{3/2}$R Antagonist	Neurons	**Pharmacokinetic Profile (rats)**	Recombinant Expression of Human and Rat P2X3 in CHOK-K1	Blocks specifically $P2X_3$ & $P2X_{2/3}$R	Yes [175]
Diaminopyrimidines	$P2X_3$R, $P2X_{3/2}$R Antagonist	DRG Neurons	**Inflammatory Pain**	Recombinant Expression of P2X3	Blocks specifically $P2X_3$ & $P2X_{2/3}$R	Yes [176]
AZ004	$P2X_3$R, $P2X_{3/2}$R Antagonist	Neurons	**Inflammatory & Neuropathic Pain**	Inflammatory Pain Model	Blocks specifically $P2X_3$ & $P2X_{2/3}$R	Yes [163]

Drug/Treatment	Receptor Target	Cellular Target	Disease	Model	Mechanism	Efficacy
Electroacupunture at He- Mu points	$P2X_4R$	Neurons from Colon and Spinal Cord	**IBS**	RAT (CRD)	↓$P2X_4R$: Decrease Visceral Hypersensitivity	Yes [216]

CFA; Complete Freud's Adjuvant, **CRD**; Colorectal Distension, **DRG**; Dorsal root ganglia, **GERD**; Gastroesophageal Reflux Disease, **IBD**; Inflammatory Bowel Disease, **IBS**; Irritable Bowel Syndrome, **NCI;** Nerve Chronic Constriction Injury, **TNBS**; 2,4,6 Trinitrobenzenesulfonic acid

Drug License: STW5; BAYER, **ATL-313**; Santen Pharmaceutical Co, **ATL-801**; Adenosine Therapeutics, **AF353**; Afferent Pharmaceuticals Inc, **AZ004**; AstraZeneca

Table 6

Purinergic drugs for treating inflammation-induced diarrhea in experimental models

Drug	Purinergic target	Model	References
ATL-801 (78)	A_{2B} antagonist	DSS colitis, IL-10 KO spontaneous colitis	Kolachala V et al, 2008 [96] (ameliorates colitis)
PSB-1115 (27)	A_{2B} antagonist	DSS colitis	Frick JS et al, 2009 [68] (exacerbates colitis)
IB-MECA (9)	A_3 agonist	IL-10 KO, DSS colitis	Gessi S et al, 2008 [242]
ATL-146e (69)	A_{2A} agonist	Rabbit colitis, spont. Ileitis in SAMP1/YitFc mice	Odashima M et al, 2005[132]
4-amino-2-(2-hydroxyl-1-decyl) pyrazole[3,4-d]pyrimidine (APP) (63)	AdoDase inhibitor	DNBS-induced colitis	Antonioli L et al, 2007 [49]
Pentostatin (62)	AdoDase inhibitor	Severe IL-10−/− colitis with piroxicam-induced colitis	Brown JB et al, 2008 [243]
Dipyridamole (61)	ENT1, ENT2 inhibitor	LPS/phytohaemagglutinin-induced gut mononuclear cells from CD patients	Poturoglu S et al, 2009 [83]
MRS2500 (51)	$P2Y_1$ antagonist	Neurogenic secretion model (in vitro)	Fang X et al, 2006 [103]