Purinergic signalling in liver diseases: Pathological functions and therapeutic opportunities

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Summary
Extracellular nucleotides, including ATP, are essential regulators of liver function and serve as danger signals that trigger inflammation upon injury. Ectonucleotidases, which are expressed by liver-resident cells and recruited immune cells sequentially hydrolyse nucleotides to adenosine. The nucleotide/nucleoside balance orchestrates liver homeostasis, tissue repair, and functional restoration by regulating the crosstalk between liver-resident cells and recruited immune cells. In this review, we discuss our current knowledge on the role of purinergic signals in liver homeostasis, restriction of inflammation, stimulation of liver regeneration, modulation of fibrogenesis, and regulation of carcinogenesis. Moreover, we discuss potential targeted therapeutic strategies for liver diseases based on purinergic signals involving blockade of nucleotide receptors, enhancement of ectonucleoside triphosphate diphosphohydrolase activity, and activation of adenosine receptors.

Introduction
Purines and purine nucleotides are the oldest multifunctional biological molecules in evolutionary history. In addition to acting as universal intracellular energy currencies for biological reactions, ATP and its hydrolytic products, including ADP, AMP, and adenosine, serve as essential extracellular signals involved in physiological processes and pathological conditions. The purinergic signalling system consists of 3 major steps: release of intracellular nucleotides into the extracellular space, activation of type 1 (P1) and type 2 (P2) purinergic receptor families with autocrine and paracrine effects, and regulation of relative extracellular nucleotide/adenosine levels by ectonucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5′-nucleotidase (CD73) to terminate nucleotide signals (Fig. 1).

Over the past 5 years, several reviews have extensively highlighted that purinergic signals regulate liver function and injury responses. Moreover, the roles of purinergic signals in liver inflammation and fibrosis have recently been summarised. In the current review, we focus on recent evidence supporting the effects of purinergic signals on liver function and homeostasis, restriction of inflammation, stimulation of liver regeneration, modulation of fibrogenesis, and regulation of carcinogenesis. Furthermore, we discuss the potential therapeutic applications of adapting purinergic signalling for liver diseases of varying aetiology.

Functions of purine signalling in liver physiology and pathology
Liver-resident cells (Table 1) and immune cells recruited upon injury (Table 2) express their own purinoceptor subtypes and ectonucleotidases that respond to and regulate extracellular purinergic signals (Fig. 2). Two families of purinoceptors have been identified: purinergic 1 (P1) and purinergic 2 (P2) receptors. P1 receptors are activated by adenosine and include 4 receptor types: adenosine receptor A (Adora)-1 (A1), Adora-2A (A2A), Adora-2B (A2B), and Adora-3 (A3). P2 receptors are divided into 2 subgroups, with 1 subgroup consisting of 7 subtypes of ATP-gated channel P2X receptors (P2X1–7), which only bind to ATP, and the other subgroup consisting of 8 subtypes of Ca2+/phosphatidylinositol-coupled P2Y receptors (P2Y1,2,4,6,11–14), which can be activated by both ATP and other nucleotides, including ADP for P2Y1,11,13, nicotinic acid adenine dinucleotide phosphate for P2Y1,2,4,6,11–14, uridine triphosphate (UTP) for P2Y2,4,6,11, uridine diphosphate (UDP) for P2Y6, and UDP-glucose for P2Y4,6, thus serving as agonists of these P2Y receptors.

The NTPDase family is composed of 8 proteins (NTPDases 1–8), 4 of which (NTPDases 1, 2, 3 and 8) face the extracellular space. In the liver, NTPDase1 (also called CD39) is mainly expressed by endothelial cells and recruited immune cells, wherein it hydrolyses ATP and ADP to AMP; NTPDase2 is expressed by activated hepatic stellate cells (HSCs) and myofibroblasts and only

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hydrolyses ATP to ADP; NTPDase8 is restricted to the bile canaliculi of hepatocytes and ATP is its preferred substrate. CD39 is the rate-limiting enzyme for generation of AMP, which is ultimately converted into adenosine by CD73.

Under physiological conditions, nanomolar levels of ATP in the extracellular space maintain liver function and homeostasis (Fig. 1). Upon liver injury, millimolar levels of ATP can be detected in the extracellular space, as a consequence of tissue damage-related cell stress or cell death. After hydrolysis of ATP by NTPDases and CD73, the extracellular adenosine concentration increases from the nanomolar range (100 to 500 nM) to the micromolar range under physiological conditions, in response to inflammation, hypoxia, and ischaemia (Fig. 1). During the reconstitution process in the liver, purinergic signals exert their effects on liver-resident cells and recruit immune cells to restrict inflammation, stimulate regeneration, modulate fibrogenesis, and regulate carcinogenesis (Fig. 3).

**Homeostasis maintenance**

Under physiological conditions, hepatocytes and cholangiocytes continuously release ATP into the bile at a concentration of approximately 5 μM in humans for functional preservation and hepatocyte-cholangiocyte communication. Extracellular ATP controls multiple essential functions of hepatocytes, including glucose metabolism, cholesterol transport, and bile secretion, which are mediated by functional P2X4 and P2Y1,2,4,6,13 receptors on the basolateral and canalicular domains (Fig. 2).

In cholangiocytes, ATP is involved in bile secretion through Cl– channel activation and Na+/H+ exchange. As the hydrolytic product of ATP, adenosine activates the P1 receptors on hepatocytes to mediate Ca2+-dependent or cAMP-dependent ureagenesis, glycogenolysis, and gluconeogenesis. NTPDase8 and CD73, co-expressed in bile canaliculi, are involved in the salvage of nucleosides from bile and the secretion of biliary electrolytes/fluid.

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**Key points**

- Purinergic signalling plays an essential role in liver function and homeostasis by mediating hepatocyte-cholangiocyte communication.
- Injury-induced release of ATP into the extracellular area serves as a danger alert signal for immune cell recruitment, and CD39 and CD73 expressed by the recruited cells scavenge extracellular ATP to generate immunosuppressive adenosine.
- Purinergic signalling stimulates liver regeneration by modulating the interaction of hepatocytes, sinusoidal endothelial cells, natural killer cells, and recruited haematopoietic stem cells.
- Nucleotide receptors exert different effects on biliary and non-biliary fibrosis, and adenosine and its receptors have discrepant effects on liver fibrosis in vivo.
- Nucleotides induce hepatocyte maltransformation and stimulate hepatic carcinoma cell proliferation and metastasis, while the effects of adenosine on carcinogenesis are still conflicting.
- Based on purinergic signals, blockade of nucleotide receptors, enhancement of NTPDase activity, and activation of adenosine receptors have shown promising therapeutic effects in liver diseases.
Table 1. Expression and functions of purinergic receptors and ectonucleotidases in liver-resident cells.

| Nucleotide receptors | NTPDases and CD73 | Adenosine receptors |
|----------------------|-------------------|-------------------|
| Hepatocytes          | P2X4: Na+ and Ca2+ transport; glycogen metabolism; and cell volume regulation; P2X7: Cytotoxicity and apoptosis induction; P2Y1: Glycogen metabolism; P2Y2: Glycogen metabolism and cell proliferation; P2Y4: Cholesterol transport; P1 and HDL endocytosis | NTPDase8: nucleoside salvage and bile secretion; CD73: Mallory-Denk body formation | A1 and A3: Ca2+-mediated ureagenesis; A2A: cAMP-mediated ureagenesis; A2B: cAMP-mediated ureagenesis; glycogenolysis and gluconeogenesis |
| Cholangiocytes       | P2X4: Cl- channel activation and bile secretion; P2Y2: Na+H+ exchange for bile secretion; downregulation of IL-6 transcription; P2Y4 and P2Y2: downregulation of IL-6 transcription; P2Y1: cAMP/Ca2+/IL-6 mediated to promote bile duct ligation | NTPDase8 and CD73: expressed on bile canaliculi | A2B: Ca2+-mediated IL-6 expression and protective effects for BDL motility |
| Hepatic stellate cells and myofibroblasts | P2X4, PKC/GSK3β-dependent proliferation, activation and synthesis of collagen I | NTPDase2: promotes cholangiocyte proliferation and protects against CCl4-induced liver injury | A2A: cAMP/PKA-regulated loss of contraction, proliferation promotion and apoptosis reduction; PKA/Src/MAPK/ERK-regulated collagen I production, and p38 MAPK-regulated collagen III production |
| Endothelial cells    | P2X4, P2Y1,2,14: release of IL-1β, PGE2, IL-6, and HDGMB1 to promote inflammation; CD39 and CD73: expression on endothelial cells to promote liver regeneration and limit inflammation | | A2A, A2B: enhancement of barrier protection and prevention of tissue leukocyte accumulation via cAMP |
| Kupffer cells        | P2X4: release of IL-1β, PGE2, IL-6, and HDGMB1 to promote inflammation; CD39 and CD73: few data available | A2: suppression of LPS-induced inflammatory cytokine secretion via cAMP/DUSP1 |

A1, adenosine receptor A1; A2A, adenosine receptor A2A; A2B, adenosine receptor A2B; A3, adenosine receptor A3; BDL, bile duct ligation; DC(s), dendritic cells; IFN, interferon; IL-1, interleukin-1; LPS, lipopolysaccharide; NK, natural killer; NKT, natural killer T; NTPDases, ectonucleoside triphosphate diphosphohydrolases; P1, purinergic type 1; P2, purinergic type 2; PGE2, reactive oxygen species; TNF, tumour necrosis factor; Treg(s), regulatory T cells.

Table 2. Functions of purinergic receptors in immune cells recruited to the liver.

| Nucleotide receptor | CD39 and CD73 expression | Adenosine receptor |
|---------------------|--------------------------|-------------------|
| Neutrophils         | P2X2, P2Y2: control of the chemotaxis effects of ATP for guiding of neutrophils to injured sites; P2Y2: liver infiltration | >90% express CD39; CD73 expressed to some extent | A1, A3: promotion of chemotaxis and phagocytosis; A2A, A2B: inhibition of adhesion to endothelia, trafficking and effector functions |
| Macrophages         | P2X4, P2Y2: release of HMGB1 and IL1β via ROS production; P2Y2: release of HMGB1 and IL1β via ROS production and upregulation of the activity and gene expression of CD39 | Double-positive for CD39 and CD73 | A2A: termination of macrophage activation and promotion of IL10 production; A2B: promotion of IL10 production and inhibition of GCSF-dependent proliferation |
| DCs                 | P2X5: inflammatory cytokine secretion and antigen presentation of DCs; P2Y1: DC maturation | Highly express CD39 and CD73 | A2: limitation of DC activation and performance of immunosuppressive functions |
| NK cells            | P2X5, P2Y1,2,14: inhibition of NK cell secretion of IFN-γ; suppression of NK cell cytotoxicity, and promotion of liver regeneration | 5% of CD8+ T cells express CD39, 50% of CD8+ T cells express CD73 | A2: inhibition of cytotoxic activity and cytokine production |
| T helper cells      | P2X5: promotion of the conversion to Th17 and inhibition of Treg function; P2Y1: stimulation of T cell activation | 20–30% of CD4+ T cells express CD39, 10% of CD4+ T cells express CD73 | A2A: inhibition of Th cell development and proinflammatory cytokine production |
| Cytotoxic T cells   | P2X5: blockade of the development of IL-10-producing CD8+ cells | 5% of CD8+ T cells express CD39, 50% of CD8+ T cells express CD73 | A2A: interference with initial activation, cytokine production, metabolic activity, and effector differentiation |
| Tregs               | P2X5: inhibition of Treg generation and reduction of Treg suppressive functions and cell stability | CD39 and CD73: mouse Tregs highly express CD73, while 1–5% of human Tregs express CD73 | A2A: Promotion of expansion of Tregs and enforcement of immunosuppressive functions; upregulation of CD39 and CD73 expression |
| NKT cells           | P2X5: enhancement of cytokine production and exaggeration of liver injury; induction of apoptosis | CD39 and CD73: affects cytokine secretion and cell survival | A2A: inhibition of NKT cell activation and suppression of NKT cell-triggered inflammatory responses |
| B lymphocytes       | Undefined | 90% of human B lymphocytes are CD39+CD73+ with immunosuppressive functions | A3: suppression of B cell expansion and functional activation |

A1, adenosine receptor A1; A2A, adenosine receptor A2A; A2B, adenosine receptor A2B; A3, adenosine receptor A3; DC(s), dendritic cells; GCSC, granulocyte colony-stimulating factor; IFN, interferon; IL-1, interleukin-1; NK, natural killer; NKT, natural killer T; P1, purinergic type 1; P2, purinergic type 2; ROS, reactive oxygen species; TNF, tumour necrosis factor; Treg(s), regulatory T cells.
Restriction of inflammation

Upon liver injury, the increased levels of extracellular ATP exert cytotoxic effects on hepatocytes via P2X7-mediated Ca^{2+}-dependent mechanisms. Extracellular ATP also binds to P2X7 receptors on the endothelium to produce chemokines and present adhesion molecules, including integrin αMβ2 and...
endothelial ligand intercellular adhesion molecule-1, in order to draw neutrophils out of the bloodstream. The chemokines and cytokines produced by neutrophils further recruit other immune cells to the site of injury.

Recruited immune cells expressing high levels of CD39 and CD73 scavenge extracellular ATP to generate immunosuppressive adenosine (Fig. 2). CD39 is expressed by approximately 90% of neutrophils, 90% of monocytes/macrophages, and natural killer (NK) cells, whereas CD73 is only expressed by small portions of these cells; these molecules hydrolyse ATP/ADP to AMP upon injury. CD39 and CD73 are co-expressed by sinusoidal endothelial cells, dendritic cells (DCs), regulatory T cells (Tregs), NK T (NKT) cells, and B lymphocytes, which are responsible for ATP/ADP hydrolysis and subsequent adenosine production.

Adenosine, the hydrolytic product of ATP, has negative feedback effects on endothelial cells and immune cells, reducing leukocyte recruitment, enhancing Treg activity, and suppressing T lymphocyte activation (Fig. 3). Activation of the adenosine receptors on endothelial cells increases intracellular cAMP levels to reseal endothelial junctions and enhance barrier protection, thus preventing the accumulation of immune cells in tissue. Adenosine functions as an anti-adhesive signal for neutrophil binding to microvascular endothelia and inhibits neutrophil trafficking and effector functions, including granule release, oxidative bursts, and cytokine production. With regard to Tregs, adenosine promotes the generation and expansion of Tregs, induces immunosuppressive functions, and upregulates CD39 and CD73 expression.

Adenosine terminates macrophage activation via A2A/NF-κB-induced reduction of tumour necrosis factor (TNF-α) expression and inhibits macrophage colony-stimulating factor-dependent proliferation of macrophages by inducing A2B/cAMP/p27kip-1 expression. Moreover, adenosine also inhibits the cytotoxic activity and cytokine production of NK cells and exerts immunosuppressive effects on DCs, including reducing interleukin (IL)-12, TNF-α, C-X-C motif chemokine ligand (CXCL)-10, C-C motif chemokine ligand (CCL)-2, and CCL-12 secretion, as well as decreasing major histocompatibility complex (MHC) class II expression, and impairing allogenic T cell proliferation via the cAMP/protein kinase A (PKA) signalling pathway. Furthermore, adenosine impairs the maturation of naïve T cells into Th cells and reduces T cell receptor-stimulated proinflammatory cytokine production by CD4+ Th cells. In addition, adenosine activates A2A receptors to inhibit NKT cell activation and suppresses NKT cell–triggered inflammatory responses.

**Stimulation of liver regeneration**

Regeneration is crucial for the restoration of hepatocyte volume and liver function. Purinergic signals participate in this process mainly by stimulating proliferation and protecting against injury. Partial
hepatectomy (PH) is the most extensively studied method for modelling liver regeneration. PH induces rapid release of ATP into the extracellular space in response to the mechanical stress, leading to hepatocyte cell cycle entry.42 P2X4 promotes liver regeneration following PH in mice by regulating biliary homeostasis, and P2X4 knockout increases hepatocyte necrosis and liver cholestasis, resulting in delayed regeneration.43 P2Y2 receptors are involved in the modulation of sinusoidal endothelial cells expressing hepatocyte growth factor (HGF) and IL-6 via phosphorylation of vascular endothelial growth factor (VEGF) receptor 2 (Fig. 3).44

PH increases CD39 expression in vascular and sinusoidal endothelial cells in response to VEGF and induces HGF secretion which stimulates liver regeneration.44 Depletion of CD39 induces endothelial cell apoptosis, impairs hepatocyte regeneration, and decreases overall survival post PH.45 NK cells, which are essential for hepatic parenchymal and non-parenchymal cellular crosstalk, help promote liver regeneration by hydrolysing ATP and increasing cytotoxicity.45 Administration of apyrase, an exogenous ATPase, can enhance hepatocyte proliferation and reduce liver injury by affecting P2X2- and P2Y1-activated NK cells.45 PH also leads to the mobilisation and recruitment of CD39+ haematopoietic stem cells from the bone marrow to the liver, promoting regeneration through CD39-dependent ATP hydrolysis and A2A receptor signalling.46

Modulation of fibrosis
Fibrosis and its end stage, cirrhosis, are common hepatopathologic conditions with different underlying aetiologies. Myofibroblasts (derived from HSCs) and portal fibroblasts are involved in extracellular matrix production in response to liver injury and play a central role in liver fibrosis.47

Quiescent rat HSCs express functional P2Y2,4 receptors in response to UTP and ATP but switch to expressing P2Y6 after activation, in order to mediate pre-collagen transcription and cell contraction via UDP- and ATP-mediated inositol phosphate-3/4 signalling.48 Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS), a P2 receptor antagonist, can attenuate carbon tetrachloride (CCL4)- and dimethylnitrosamine (DMN)-induced liver fibrosis by blocking P2Y2/Ca2+ signalling-mediated extracellular matrix transcription and HSC proliferation.49 With regard to HSC activation, the expression of P2X2 receptors is significantly increased, promoting protein kinase C (PKC)/glycogen synthase kinase-3β (GSK3β)-dependent HSC proliferation and collagen production;50 the increased expression of P2X4 stimulates calcium entry and lysosomal exocytosis for ATP release, myofibroblast activation, and profibrogenic secretion.51 In CCL4-treated mouse liver fibrosis, P2X7 expression is enhanced, and treatment with A438079, a specific P2X7 antagonist, relieves liver injury, inflammatory responses, and collagen accumulation.51 In contrast to those subjected to CCL4-induced liver fibrosis, mice subjected to bile duct ligation (BDL) or fed a methionine- and choline-deficient diet (MCDD) exhibit increased expression of P2X4. In addition, P2X4 gene deficiency or treatment with the P2X4 antagonist 5-BBBB protects mice from BDL- or MCDD-induced liver fibrosis but not CCL4-induced liver fibrosis.52 These data reveal that purinergic signals have different impacts on biliary and non-biliary fibrosis, and the underlying mechanisms remain unclear.

The expression of NTPDase2 is increased in activated HSCs and portal fibroblasts after CCL4- or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-mediated induction of liver fibrosis. NTPDase2-deficient mice display CCL4-induced liver fibrosis faster than wild-type mice, suggesting that ATP hydrolysis by myofibroblasts has protective effects.52 CD73 is weakly expressed in quiescent HSCs and portal fibroblasts, but its expression is markedly induced in activated HSCs and portal fibroblasts after CCL4 intoxication and BDL.53 In contrast to NTPDase2-deficient mice, mice deficient in CD73 are protected against CCL4- and thiocetamide (TAA)-induced liver fibrosis.54 This may be due to low NTPDase2 expression in the myofibroblasts, but high CD73 expression in immune cells, endothelial cells, and myofibroblasts. Therefore, CD73 deficiency results in reduced adenosine production and low P2 receptor-mediated HSC proliferation and collagen expression.

In vitro studies have revealed that adenosine activates A2A receptors to inhibit cAMP/PKA/Rho-mediated HSC contraction;55 promote cAMP-PKA/Rac1/p38 mitogen-activating protein kinase (MAPK)-mediated HSC proliferation;56 stimulate PKA/Src- and MAPK/ERK-mediated collagen type I expression;57 and increase p38 MAPK-mediated collagen type III expression.58 However, the in vivo therapeutic effects of adenosine, its derivatives, and its receptor antagonists on liver fibrosis/cirrhosis remain controversial.

Adenosine A2A expression is increased in HSCs in TAA-induced murine liver fibrosis, and A2A depletion or treatment with the A2A-specific antagonist ZM241385 protects against TAA- or CCL4-induced liver fibrosis/cirrhosis, which is consistent with previous data on CD73 deficiency. Moreover, adenosine can attenuate CCL4-induced rat liver fibrosis by reducing collagen deposition59 and reverse CCL4-induced rat cirrhosis by enhancing fibrolytic activity and hepatocyte proliferation.60 Consistent with these findings, the aspartate salt of adenosine (IFC-305) was shown to reverse CCL4-induced cirrhosis in rats by reducing the M1/M2 macrophage ratio and inflammatory cytokine production.61 The potential therapeutic targets for liver fibrosis are summarised in Fig. 4; the discrepancies in these data might be attributable to the ubiquitous expression of P1 receptors and the different effects of adenosine on different cell types, such as myofibroblasts, hepatocytes, and immune cells. However, this requires further investigation.

Regulation of carcinogenesis
Carcinogenesis is closely associated with chronic inflammation of many aetiologies and often occurs on a background of cirrhosis. Large amounts of ATP are released into the extracellular space of the inflammatory tumour microenvironment and the fast-growing tumour centre. Hepatoma cells escape extracellular ATP-induced cytotoxicity via autophagy when the ATP concentration is less than 1 mM; however, apoptosis and cell death are induced when the concentration of ATP reaches 2.5 mM.62

In response to extracellular ATP, the expression of at least one of the P2X (P2X1, P2X2, P2X3, P2X4, or P2X6) or P2Y receptors (P2Y2, P2Y4, P2Y6, P2Y11, or P2Y14) is elevated in liver tumour tissue compared to uninvolved areas or normal liver tissue.63 Among these receptors, P2X4 is differentially expressed in HCV-induced hepatocellular carcinoma (HCC) and non-HCV HCC,63 and P2X7 is associated with poor recurrence-free survival in patients with HCV-induced HCC.64 Treatment with the P2X7 antagonist AF-353 (PubChem CID: 15953802) or A317491 reduces ATP-induced cell proliferation and impairs cell cycle progression. Among the P2Y receptors, P2Y11 is highly expressed in
HCC tissues but rarely detected in non-cancerous liver tissues.65 Moreover, P2Y11 contributes to ATP-induced Ca\textsuperscript{2+} signalling and HCC cell migration, and treatment with its antagonist (NF340) can attenuate the effects of ATP on HCC.66 Emerging evidence shows that P2Y2 is another type of P2Y receptor involved in ATP-induced HCC cell proliferation and migration.67 In addition, selective pharmacological inhibition of P2Y2 using MRS2312 can reduce the viability of HepG2, SK-Hep1, SNU449, Huh7, and Hep3B cells in a dose-dependent manner.68,69

In hepatocytes, CD39-induced purinergic signals regulate cell metabolism and proliferation, which in turn regulate carcinogenesis. In line with this notion, CD39 deficiency increases extracellular ATP concentration, thereby activating the MAPK and mTOR pathways to stimulate aerobic glycolysis and hepatocyte proliferation, leading to maltransformation.68 Over-expression of CD39 in Tregs and increased numbers of CD39\textsuperscript{+}Foxp3\textsuperscript{+} Tregs have prognostic value in HCC.69

CD73 is highly expressed in approximately 50% of all HCC tissues compared with paired adjacent normal tissues, and is a negative prognostic indicator for recurrence and overall survival.70 CD73 not only promotes the proliferation, migration, and invasion of HCC cells in vitro but also enhances HCC growth and metastasis in vivo.70,71 Mechanistically, the enzymatic activity of CD73 is required to mediate its effects in HCC, and treatment with α,β-methylene ADP (APCP), an inhibitor of CD73 activity, can partially suppress tumour growth.70

In an HCC cell line with high CD73 expression, A2A receptors are involved in the activation of PI3K and Akt phosphorylation, which induces HCC cell proliferation and invasion.72 Similar to CD73 inhibition, blocking the A2A receptor with istradefylline (KW6002) inhibits tumour growth. Interestingly, co-targeting CD73 and A2A receptors with ACP and KW6002 exerts synergistic suppressive effects on HCC cells.73 A3 receptors are also reported to be highly expressed in the tumour tissues and peripheral blood mononuclear cells of patients with HCC. In addition, treatment with CF102, an A3 receptor agonist, inhibits PI3K-NF-κB-mediated HCC cell growth and induces apoptosis in a dose-dependent manner.72,73 A phase I/II open-label dose escalation study revealed that CF102 therapy was safe and well
Table 3. Clinical studies of drugs targeting purinergic signals or receptors.

| Disease                  | Drugs                  | Target                  | NCT number       | Phase | Status     | Results                                      |
|--------------------------|------------------------|-------------------------|------------------|-------|------------|----------------------------------------------|
| Chronic Hepatitis C      | CF-102                 | A3 receptor agonist     | NCT00790673      | Phase I-II | Complete  | Non-serious adverse events include palpitations, fatigue, and headache |
| Hepatic impairment       | KW6002                 | A2A receptor antagonist | NCT02256033      | Phase I  | Complete   | No result posted                             |
| NASH                     | CF-102                 | A3 receptor agonist     | NCT02927314      | Phase II | Complete   | No result posted                             |
| HCC                      | CF-102                 | A3 receptor agonist     | NCT00790218      | Phase I-II | Complete  | Safe and well tolerated with favourable PK characteristics in Child Pugh A and B HCC patients |

Table 4. Potential therapeutic targets for liver diseases based on purinergic signals.

| Animal model    | Therapeutic targets based on purinergic signals                                                                 |
|-----------------|---------------------------------------------------------------------------------------------------------------|
| HBV hepatitis   | - P2X7 antagonists (suramin, PPADS, BBG) reduce HBV entry into hepatocytes. A P2Y11 antagonist (NF157) attenuates mitochondrial dysfunction, oxidative stress, and cytokine production of hepatocytes. | |
| Alcohol-induced | - A P2X2 receptor antagonists (suramin) attenuate inflammation, lipid accumulation and liver injury. An A1 receptor antagonist (DPCPX) and an A2B receptor antagonist (enprofylline) reduce hepatic triglyceride levels. |
| Drug-induced liver injury | - A P2X2 antagonist (A438079) reduces APAP-induced hepatocyte necrosis and proinflammatory cytokine production of Kupffer cells. An exogenous ATPase (apryrase) attenuates APAP-induced hepatocyte necrosis and neutrophil infiltration. |
| Cholestatic injury | - A P2X4 antagonist (5-BBD) attenuates BDL-liver fibrosis; an A1 receptor antagonist (DPCPX) stimulates bile acid and bilirubin elimination. |
| IR injury        | - Apyrase and 5'-nucleotidase have hepatoprotective effects. An A1 agonist (CCPA), A2 receptor agonists (CGS-21680, ATL146e), and an A3 receptor agonist (CF102) attenuate hepatocyte and sinusoidal endothelial cell apoptosis. |
| Liver transplantation | - Apyrase and an A2B receptor agonist (CGS21680) have inflammatory-attenuating effects. |
| HCC             | - P2X3 antagonists (AF-353 or A317491), a P2Y11 antagonist (NF340), a P2Y2 antagonist (MR52312), a CD73 inhibitor (APC1), and an A2A receptor antagonist (K60602) inhibit HCC growth. |

A2A, adenosine receptor A2A; A3, adenosine receptor A3; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; PK, pharmacokinetic.

There are no suitable explanations for the conflicting findings regarding the roles of adenosine in carcinogenesis (Fig. 4), and further studies are needed to reveal the therapeutic effects of adenosine receptors on hepatoma cells.

**Viral hepatitis**

Hepatotropic viruses, which can infect and replicate in hepatocytes and destroy infected cells, are major causes of chronic liver diseases, leading to fibrosis, cirrhosis, and HCC.

Purinergic receptors are necessary for the entry of HBV and HDV into primary human hepatocytes, as evidenced by previous findings, which showed that blocking one or more of these receptors with suramin, PPADS, or brilliant blue G markedly reduces infection of cells. When human MIHA hepatocytes are transfected with HBx-encoding plasmids, the expression of the P2Y11 receptor is increased, resulting in mitochondrial dysfunction, oxidative stress, cytokine and chemokine production, and activation of the p38/MAPK and NF-κB pathways. However, treatment with NF157, a specific antagonist of P2Y11, can attenuate the effects of HBx in hepatocytes. With regard to immune cells, the percentage of CD39+ Tregs in peripheral blood is higher in asymptomatic HBV carriers, but lower in patients with chronic active hepatitis B or HBV-associated acute-on-chronic liver failure than in healthy controls. Furthermore, the proportions of circulating CD39+ Tregs are positively correlated with serum HBV copy numbers but negatively correlated with serum alanine aminotransferase (ALT) levels. In addition, CD39+ Tregs accumulate in the portal areas of liver tissues in patients with chronic HBV, but their functions remain unclear.

HCV RNA replication consumes ATP and reduces cytoplasmic ATP levels, but the extracellular ATP concentration is approximately 5 mM at the replication sites and 1 mM at the periphery sites without HCV replication. The NS3 helicase of HCV binds ATP, and targeting this ATP-binding site may serve as a potential therapeutic strategy for HCV. Stable expression of the HCV structural protein E1E2 in Huh7 cells results in markedly increased P2X4 expression, and P2X4 expression is significantly tolerated, with a median overall survival of 7.8 months in patients, including those who had previously received sorafenib treatment (67%) (Table 3).
higher in HCV-induced HCC than in non-HCV HCC. With regard to peripheral blood mononuclear cells, P2X7 expression is increased in treatment-naive patients with chronic HCV, as well as in patients achieving a sustained virologic response, but it remains unaltered in treatment non-responders. CD73 mRNA levels in the livers of patients with HCV-related fibrosis are less than 20% of those in normal controls. However, therapeutic strategies based on these findings have not yet been developed.

Therapeutic strategies for HBV and HCV have been successfully developed. A phase I/II clinical trial testing the A3 receptor agonist CF102 for HCV revealed no serious adverse effects, including palpitations, fatigue, and headache (Table 4). Based on the disease-specific expression of P2Y1 for HBV and P2Y4 for HCV, antagonists for these receptors may be used to treat patients who have progressive fibrosis despite suppression of viral replication.

**Alcohol-related liver disease**

Alcohol is metabolised into large amounts of toxic intermediate substances by hepatocytes. As a result, endogenous sterile danger signals (including ATP) are released from damaged hepatocytes, leading to the recruitment of macrophages and neutrophils to the liver and exacerbating liver injury and inflammation.

Alcohol significantly increases serum and liver ATP levels by directly damaging hepatocytes and inducing IL-1β production. Mice deficient in P2X7 are protected from alcohol-induced liver damage. Treatment with gentiopicroside, the main active secoiridoid glycoside of Gentiana manshurica Kitag, reduces P2X7 receptor-mediated IL-1β maturation and release in ATP/lipopolysaccharide (LPS)-stimulated macrophages, and suppresses P2X7-NOD-like receptor protein 3 (NLPR3) activation in mouse models of acute and chronic alcohol-induced steatohepatitis. Alcohol also increases P2Y2 expression in an alcohol-induced steatohepatitis mouse model, and blockade of P2Y2 receptors with suramin attenuates inflammation, lipid accumulation, and liver injury, while concomitantly downregulating CD39 expression.

CD39 exerts protective effects against alcohol-induced steatohepatitis by hydrolysing extracellular ATP and indirectly regulating P2Y2 expression. Mice deficient in CD73 are protected from ethanol-induced fatty liver after being fed an ethanol-containing liquid Lieber-DeCarli diet due to the reduced adenosine-mediated extracellular matrix deposition by HSCs. In accordance with this finding, treatment with the A1 receptor antagonist DPCPX and the A2B receptor antagonist enprofylline can reduce hepatic triglyceride levels.

**Autoimmune liver diseases**

Autoimmune liver diseases are progressive inflammatory liver diseases caused by the breakdown of self-tolerance; they are characterised by high serum levels of autoantibodies and interface hepatitis on liver biopsy. Concanavalin A (ConA)-induced liver injury models are most commonly used to study autoimmune hepatitis (AIH), and significant ATP release can be detected as early as 2 hours post ConA injection. P2Y2 is the only P2 isoform whose expression is increased after ConA injection, while the expression of P2X4,7 and P2Y4,6,14 is reduced. Moreover, treatment with the A2A receptor antagonist KW6002 and a triazine derivative (IMT) can suppress ethanol-induced hepatic steatohepatitis activity.

DDC treatment reduces CD73 activity by 60% in detergent-soluble liver fractions. CD73+ mice have fewer Mallory-Denk bodies and display less cellular ballooning and steatosis after DDC feeding than wild-type mice, suggesting that CD73 antagonists may have protective effects against the development of fatty liver.

A2A expression in liver cells, including hepatocytes, is higher in HFD-fed mice than in low-fat diet-fed mice. Compared to normal diet feeding, HFD feeding induces severe hepatic steatosis and inflammation when A2A receptors are totally disrupted or A2A expression is specifically depleted in myeloid cells. Stimulation of A2A receptors by CGS21680 prevents hepatocyte lipotoxicity, increases the immunosuppressive activity of Tregs, and inhibits cytokine and chemokine production for proinflammatory cell recruitment and expansion, thereby ameliorating MCDD-induced NASH in rodents.

Besides weight loss, there is no effective treatment available for patients with NASH. A phase II clinical trial testing the A3 receptor agonist CF102 for NASH was completed recently, but the results have not been published (Table 3).

Antagonists of P2X7 or CD73 and agonists of A2A receptors may serve as new therapeutic options for NASH.

**Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis**

Globally, non-alcoholic fatty liver disease (NAFLD) and its progressive form, non-alcoholic steatohepatitis (NASH), are having an increasing impact on public health.

In NASH models of MCDD or upon co-administration of a high-fat diet (HFD) and a low dose of the environmental toxin bromodichloromethane, P2X7 expression is increased in hepatocytes, Kupffer cells, and liver sinusoidal endothelial cells, which promotes oxidative stress-induced autophagy, inflammation, and disease progression. ATP activates the P2X7 receptors on Kupffer cells to enhance antigen presentation as well as TNF-α and monocyte chemotactic protein-2 production in CCL4-treated HFD-fed obese mice. P2X7 receptors also participate in HSC proliferation via the glucose transporter-4 (GLUT4)/protein kinase B (Akt)/hexokinase 2 (HK2) pathway in HFD-induced NAFLD. Moreover, P2X7 deficiency protects against leucocyte infiltration and hepatocyte apoptosis, decreasing inflammation and fibrosis in HFD-fed mice treated with CCL4. This suggests that P2X7 antagonists are potential therapeutic agents for the treatment of NAFLD/NASH.

DDC treatment reduces CD73 activity by 60% in detergent-soluble liver fractions. CD73+ mice have fewer Mallory-Denk bodies and display less cellular ballooning and steatosis after DDC feeding than wild-type mice, suggesting that CD73 antagonists may have protective effects against the development of fatty liver.

A2A expression in liver cells, including hepatocytes, is higher in HFD-fed mice than in low-fat diet-fed mice. Compared to normal diet feeding, HFD feeding induces severe hepatic steatosis and inflammation when A2A receptors are totally disrupted or A2A expression is specifically depleted in myeloid cells. Stimulation of A2A receptors by CGS21680 prevents hepatocyte lipotoxicity, increases the immunosuppressive activity of Tregs, and inhibits cytokine and chemokine production for proinflammatory cell recruitment and expansion, thereby ameliorating MCDD-induced NASH in rodents. Besides weight loss, there is no effective treatment available for patients with NASH. A phase II clinical trial testing the A3 receptor agonist CF102 for NASH was completed recently, but the results have not been published (Table 3).

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proportions of CD73⁺, IL-10⁺, and TGF-β1⁺ cells but lower frequencies of TNF-α⁺ and interferon (IFN)−γ⁺ cells than Th17⁺CD39⁻ cells. Low levels of CD39 and A2A expression may contribute to the perpetuation of Th17 cell effector properties and thereby the pathogenesis of juvenile autoimmune liver diseases.

Blocking A2A receptors with ZM241385 aggravates ConA-induced liver injury, whereas activating A2A receptors with ATL-146e markedly attenuates ConA-induced liver injury. Similarly, administration of the A3 agonist CFI02 prevents hepatocyte necrosis by downregulating proapoptotic protein expression and limits inflammation by reducing the phosphorylation of GSK-3β and the expression of NF-κB and TNF-α, thus protecting liver tissues from ConA-induced injury.

According to the guidelines, immunosuppressive medication is recommended for AIH treatment. However, studies based on purinergic signalling reveal new therapeutic targets for AIH, including P2Y₂ antagonists or A2A and A3 agonists, with different underlying mechanisms.

**Drug-induced liver injury**

Acetaminophen (APAP) overdose is the most frequent cause of acute liver failure in the United States and in European countries. Purine signalling plays an essential role in APAP-induced liver damage since large amounts of ATP are released into the extracellular area by APAP-injured necrotic hepatocytes. ATP not only directly induces cytotoxic effects on hepatocytes via P2 receptor-dependent increases in Ca²⁺ concentration, but also activates the P2X₇ receptors on Kupffer cells to secrete the proinflammatory cytokine IL-1β. Genetic deletion of P2X₇ significantly reduces serum ALT levels, liver necrosis scores, and neutrophil infiltration in APAP-induced liver injury. Accordingly, treatment with the specific P2X₇ antagonist A438079 markedly downregulates the APAP-induced cell death pathway of hepatocytes and/or damage-associated molecular pattern (DAMP)-mediated proinflammatory cytokine production by Kupffer cells.

Deficiency of CD39 aggravates APAP-induced liver haemorrhage and mortality, and administration of apyrase reduces neutrophil infiltration and serum ALT, as well as proinflammatory cytokine levels, thus attenuating APAP-induced liver injury. However, treatment with the non-specific P1 receptor agonist theophylline further increases serum ALT levels and worsens APAP-mediated liver injury, while treatment with the A2A receptor agonist ATL-146e significantly inhibits D-galactosamine (GalN)− and LPS-induced acute liver injury, partially by inhibiting TNF-α production.

To date, N-acetylcysteine is the only therapy proven to be effective for drug-induced liver injury. Since P2X₇ antagonists or A2A agonists are effective in experimental animal models of drug-induced liver injury, clinical trials could be carried out to compare their efficacy with N-acetylcysteine.

**Cholestatic liver injury**

Cholestasis is induced by impaired bile flow in the liver, which results in accumulation of bile constituents in the liver and blood. A lack of P2X₇ receptors protects mice from BDL-induced fibrosis by reducing the reactivity of bile ductules and myofibroblasts without affecting immune cells; pharmacological inhibition of P2X₇ with 5-BDBD can attenuate myofibroblast activation and collagen accumulation.

Although NTPDase 2 expression is increased in CCl₄-treated rat livers, its expression is reduced in the portal fibroblasts of BDL rats and absent in patients with cirrhotic PBC. CD39 expression is relatively low in the livers of normal individuals and patients with primary sclerosing cholangitis, but high in the immune cells of the colon; depletion of CD39 enhances biliary injury and fibrosis by affecting gut-imprinted CD8⁺ T cells. Mice deficient in CD39 display more severe biliary fibrosis after exposure to DDC than wild-type mice, mainly due to loss of CD39 expression on myeloid cells. Hence, administration of CD39-expressing myeloid cells may serve as a therapeutic strategy for cholestatic diseases.

Alpha-naphthyl isothiocyanate, a hepatotoxin that induces intrahepatic cholestasis by damaging cholangiocytes and hepatocytes, increases adenosine A1 receptor expression. Deficiency of A1 receptors or treatment with the A1 antagonist DPCPX stimulates bile acid and bilirubin elimination, thus attenuating α-naphthyl isothiocyanate-induced cholestasis. This suggests that A1 antagonists may exert therapeutic effects in cholestatic diseases.

For cholestatic diseases, therapies range from ursodeoxycholic acid and farnesoid X receptor agonists to peroxisome proliferator-activated receptor agonists. Strategies based on purinergic signalling, including P2X₄ antagonists, CD39-expressing myeloid cells, and A1 antagonists have shown therapeutic effects in experimental animal models of cholestatic disease, though clinical trials are needed to compare their effects with those of other available therapies.

**Ischaemia-reperfusion injury**

Ischaemia-reperfusion (IR) injury occurs in many clinical settings, including liver resections, haemorrhagic shock, and liver transplantation, wherein ATP content is reduced in mitochondria isolated from liver tissue, resulting in reactive oxygen species (ROS) generation and Ca²⁺-induced mitochondrial swelling. Surgical application of ischaemic preconditioning (IPC), a brief period of portal triad occlusion and reperfusion before sustained IR, induces specificity protein 1 (Sp1)-dependent CD39 expression in hepatocytes. However, pharmacological inhibition of CD39 with sodium polyoxotungstate (POM-1) in wild-type mice or CD39 deficiency in mice abolishes the hepatoprotective effects of IPC. Similarly, pharmacologic inhibition of CD37 with APOC or deficiency of CD37 in mice increases hepatocyte necrosis after IPC. Consistent with these findings, administration of apyrase or 5′-nucleotidase to wild-type mice can protect against IR-induced liver injury.

IPC induces adenosine-mediated tissue protection against hepatic IR. IPC or treatment with the A1 agonist, 2-chloro-N6-cyclopentyladenosine (CCPA), preserves mitochondrial ATP content, reduces ROS generation, and increases the threshold of Ca²⁺-induced mitochondrial swelling by preserving oxidative phosphorylation efficiency and downregulating the Akt(Thr172)/GSK-3β(ThrSer) pathway. Moreover, treatment with the A2 receptor agonist CGS-21680 attenuates hepatocyte apoptosis by reducing caspase-3 activity and protects sinusoidal endothelial cells against storage/reperfusion injury by stimulating adenylate cyclase activity and cAMP formation. A proteome study on hepatocytes and sinusoidal endothelial cells isolated from IR mice revealed that A2A stimulation with CGS21680 rescues the pathways of carbohydrate, protein, and lipid supply and metabolism that are downregulated by IR, and increases the levels of antioxidant enzymes, including arginase, pyruvate kinase, and 3-ketoacyl-CoA thiolase, particularly in sinusoidal endothelial cells, protecting against IR injury.
NKT cells initiate the inflammatory cascade in hepatic IR injury by secreting IFN-γ and recruiting neutrophils. However, blockade of NKT cell activation with NK1.1 antibodies, CD1d antibodies, or A2A agonists, such as ATL146e inhibits IFN-γ production, attenuates hepatocyte necrosis, and reduces hepatic IR injury.119 Moreover, treatment with the adenosine A3 receptor agonist CF102 not only attenuates hepatocyte apoptosis after IR injury, but also stimulates liver regeneration.120

Attenuation of IR injury improves recovery from clinical surgery, and based on previous studies of purinergic signalling, clinical trials are needed to confirm whether apyrase, 5′-nucleotidase, and A2A or A3 agonists can prevent IR injury.

Liver transplantation
Liver transplantation is the most effective therapy for acute liver failure and end-stage liver diseases. Purinergic signals are extensively involved in host immunotolerance and donor graft injury. Donor livers from transgenic mice overexpressing CD39 are protected against IR injury after extended cold preservation.121 In contrast, donor livers from Cd39−/− mice are associated with more severe graft injury, higher proinflammatory cytokine production, and shorter survival times than those from wild-type mice after allograft transplantation.122,123 In the context of mouse MHC-mismatched orthotopic liver transplantation, CD39-deficient donor livers display severe immune-mediated liver injury and enhanced anti-donor T cell proliferation due to reduced Treg populations and enhanced IFN-γ expression on CD8+IFN-γ+ T cells. However, administration of apyrase partially reverses the acute rejection of Cd39−/− allograft livers and prolongs survival.124

Plasma ATP levels are only slightly elevated in non-tolerant patients along the continuum from before withdrawal of immunosuppression up to rejection, whereas tolerant patients have much higher levels of adenosine and higher frequencies of CD39+ Tregs than non-tolerant patients.124 The expression of the A2B receptor was found to be increased after reperfusion in a rat orthotopic small-for-size liver transplantation model, and activation of the A2B receptor with CGS21680 can attenuate inflammatory responses by activating NF-κB.125,126 Hence, administration of apyrase or A2B agonists may prevent acute rejection after liver transplantation, and further clinical investigations are needed to confirm the effects of new immunosuppressive strategies.

Conclusions
Purinergic signalling plays a crucial role in maintaining liver function under physiological conditions and serves as the central regulator of danger signals, injury minimisation, and liver function restoration upon liver injury.

Regarding liver-resident cells, specific P2 receptors have been shown to be involved in different liver diseases. For example, P2X7 is involved in alcohol-related liver disease, NASH, and drug-induced liver injury; P2Y2 is involved in ALD and AIH; and P2X3 and P2Y11 are involved in HCC. An in-depth understanding of disease-specific purinoreceptor expression and its functions in basic cell biology will help advance clinical pharmacotherapeutic research on different liver diseases.

Regarding immune cells, circulating Tregs have been implicated in viral hepatitis and AIH pathogenesis, rejection after liver transplantation, as well as HCC recurrence and overall survival. Hence, it is important to confirm the diagnostic and prognostic value of circulating Tregs and other immune cells for liver diseases and explore the feasibility of applying these cells to therapy.

There are still some controversial data related to the roles of purinergic signals, especially adenosine, in liver fibrosis and HCC. This may be because the cell-specific functions of purinergic signals in liver-resident cells and recruited immune cells have not been completely revealed. Although therapies based on purinergic signalling to treat animal models of liver diseases show promising results, clinical trials on these drugs are limited. Therefore, further clinical investigations on the in vivo effects of purinergic signals are crucial to develop novel therapies for liver diseases.

Abbreviations
A1, adenosine receptor A1; A2A, adenosine receptor A2A; A2B, adenosine receptor A2B; A3, adenosine receptor A3; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; APAP, acetaminophen; APCR, 2,3-oxido-4-oxo-4-phenylbutyric acid; BDL, bile duct ligation; CCl4, carbon tetrachloride; CD73, ecto-5′-nucleotidase; ConA, concanavalin A; DCs, dendritic cells; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; HD, high-fat diet; HGF, hepatocyte growth factor; HSCs, hepatic stellate cells; IFN, interferon; IL-, interleukin-1; IPC, ischaemic preconditioning; IR, ischaemia-reperfusion; MAPK, mitogen-activating protein kinase; MCDD, methionine- and choline-deficient diet; MHC, major histocompatibility complex; NAFLD, non-alcoholic fatty liver disease; NK, natural killer; NKT, natural killer T; NTPDases, ectonucleoside triphosphate diphosphohydrolases; PBC, primary biliary cholangitis; P1, purinergic type 1; P2, purinergic type 2; PH, partial hepatectomy; PKA, protein kinase A; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate; ROS, reactive oxygen species; TAA, thioacetamide; Tregs, regulatory T cells; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

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Conflicts of interest
P.W., J.J., and D.Z. declare no conflicts of interest. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions
P.W. and D.Z conceived and designed the study. J.D. developed the initial draft of the manuscript. All the authors worked together on subsequent drafts.

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