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REVIEW ARTICLE

Reviewing the role of P2Y receptors in specific gastrointestinal cancers

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Abstract

Extracellular nucleotides are important intercellular signaling molecules that were found enriched in the tumor microenvironment. In fact, interfering with G protein-coupled P2Y receptor signaling has emerged as a promising therapeutic alternative to treat aggressive and difficult-to-manage cancers such as those affecting the gastrointestinal system. In this review, we will discuss the functions of P2Y receptors in gastrointestinal cancers with an emphasis on colorectal, hepatic, and pancreatic cancers. We will show that $P2Y_2$ receptor up-regulation increases cancer cell proliferation, tumor growth, and metastasis in almost all studied gastrointestinal cancers. In contrast, we will present $P2Y_6$ receptor as having opposing roles in colorectal cancer vs. gastric cancer. In colorectal cancer, the $P2Y_6$ receptor induces carcinogenesis by inhibiting apoptosis, whereas $P2Y_6$ suppresses gastric cancer tumor growth by reducing β-catenin transcriptional activity. The contribution of the $P2Y_{11}$ receptor in the migration of liver and pancreatic cancer cells will be compared to its normal inhibitory function on this cellular process in ciliated cholangiocytes. Hence, we will demonstrate that the selective inhibition of the $P2Y_{12}$ receptor activity in platelets was associated to a reduction in the risk of developing colorectal cancer and metastasis formation. We will succinctly review the role of $P2Y_1$, $P2Y_4$, $P2Y_{13}$, and $P2Y_{14}$ receptors as the knowledge for these receptors in gastrointestinal cancers is sparse. Finally, redundant ligand selectivity, nucleotide high lability, cell context, and antibody reliability will be presented as the main difficulties in defining P2Y receptor functions in gastrointestinal cancers.

Keywords P2Y receptors · GI cancer · Tumor microenvironment · Cell proliferation · Metastasis · Drug resistance

Purinergic signaling

The idea that nucleotides, the building blocks of DNA and source of cellular energy, could also act as extracellular signaling molecules was perceived as a heresy. From the first description of the concept of "purinergic signaling" in 1972 by Burnstock, it took nearly 20 years before gaining scientific acceptance, which came after the cloning and characterization of the first purinoreceptors [1, 2]. During the 1990s, the expression of different receptor subtypes was reported in all systems of the human body, and extracellular nucleotides and nucleosides were accordingly found to modulate the physiology of all organs [2, 3]. Hence, purinergic signaling was associated with numerous pathological conditions such as neurological disorders, cystic fibrosis, diabetes, inflammatory bowel diseases, and cancer, as illustrated for gastrointestinal (GI) cancers (Fig. 1) [4]. Studies are now focusing on the development of selective purinergic receptor ligands as therapeutic tools and on the characterization of the signaling events downstream of receptor activation [4].

The purinergic system is composed of three distinct families of receptors, namely P0, P1, and P2 receptors that are selectively activated by adenine, adenosine, and tri- and diphosphonucleotides, respectively [5]. P2 receptors are further subdivided in two distinct families namely P2X and P2Y. P2X receptors comprise seven members (P2X1–7), which are all ATP-gated ion channels that trimerize to enable the influx of Ca^{2+}/Na^+ and efflux of K^+ [6]. The eight P2Y receptor

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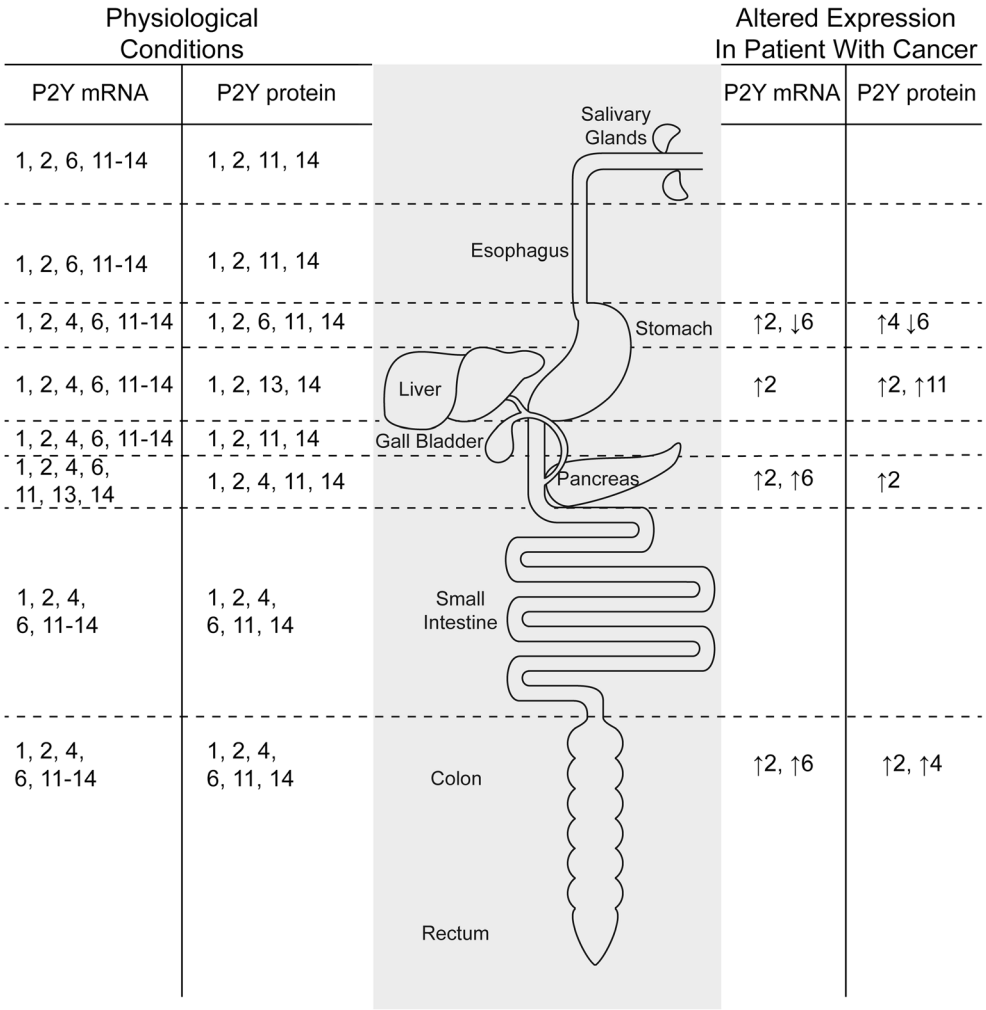

Fig. 1 Normal and cancer altered P2Y receptor expression in the GI tract. Normal expression was determined according to the Human Protein Atlas database (https://www.proteinatlas.org/) and from reference [3]. Reported altered expression in cancer was based on the discussion presented in this review

members ($P2Y_{1,2,4,6,11–14}$) are G protein-coupled receptors (GPCR) that recognize a wide range of endogenous nucleotides as ligands (Table 1). It is well accepted that $P2Y_{1,2,4,6}$ receptors are generally $G\alpha_q$-coupled, and $P2Y_{12–14}$ receptors are $G\alpha_{i/o}$-coupled, although $P2Y_{2,4,6}$ receptors were reported to also recruit $G\alpha_o$ and $G\alpha_{12/13}$ proteins (Table 1) [7]. The $P2Y_{11}$ receptor couples to both $G\alpha_q$ and $G\alpha_s$ and thus generates, upon activation, a simultaneous increase in intracellular calcium ($[Ca^{2+}]_i$) and cAMP levels. The presence of multiple purinoceptors on one cell membrane, common endogenous ligands, and the competition for the same G protein pool clearly illustrate the complex signaling network triggered by extracellular nucleotides. The presence of ectonucleoside triphosphate diphosphohydrolases (NTPDases) and the ecto-5′-nucleotidase (5′-NT) adds another layer of complexity [9]. NTPDases hydrolyze nucleoside tri-and diphosphates to nucleoside monophosphate, whereas the 5′-NT produces extracellular adenosine from AMP [5]. Consequently, a single dose of ATP with its hydrolysis products will activate all three families of purinergic receptors and subsequently an array of cellular responses.

Table 1 P2Y receptor-associated Gα proteins and their preferred natural ligands [3, 7, 8]

Receptors	Gα protein subunits	Ligands
$P2Y_1$	$G\alpha_q$	ADP
$P2Y_2$	$G\alpha_q$ ($G\alpha_o$, $G\alpha_{12}$)	ATP, UTP
$P2Y_4$	$G\alpha_q$ ($G\alpha_o$)	UTP
$P2Y_6$	$G\alpha_q$ ($G\alpha_{12/13}$)	UDP
$P2Y_{11}$	$G\alpha_q$, $G\alpha_s$	ATP
$P2Y_{12}$	$G\alpha_{i/o}$	ADP
$P2Y_{13}$	$G\alpha_{i/o}$	ADP
$P2Y_{14}$	$G\alpha_{i/o}$	UDP-glucose

The tumor microenvironment (TME) is composed of an intricate network of interactions initiated by a population of cancer cells that mold functions of normal mesenchymal and immune cells to favor cancer cell immune evasion, proliferation, and dissemination [10, 11]. Disruption of heterotypic cell-cell communication in TME is an important strategy against cancer [12, 13]. This particular environment is rich

in numerous growth factors and cytokines as well as extracellular ATP, adenosine, and other tri- and diphosphate nucleotides such as UDP [8, 14]. In this context, purinergic receptors are receiving heightened attention as they are perceived as promising drug targets in adjuvant therapy for most cancers [8]. GI cancers represent some of the most prevalent and deadliest forms of cancer worldwide. Indeed, colorectal cancer (CRC) is the third most common cancer worldwide [15]. Pancreatic cancer, and most predominantly pancreatic ductal adenocarcinoma (PDAC), with a 5-year survival rate of less than 5%, the absence a reliable biomarker for early screening and ineffective treatments, represents one of the most, if not the most, lethal GI cancer [16]. Thus, investigation of purinergic receptor functions in GI cancers could reveal novel targets for the development of urgently needed therapeutics for these cancers. More particularly, P2Y receptor-derived anti-cancer therapies could be used to disrupt heterotypic cell-cell communication in TME, a key element of cancer biology as pointed out above.

In this review, we will focus on the role of metabotropic P2Y receptors in GI cancers, with an emphasis on CRC, hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), and PDAC. Involvement of $P2Y_2$, $P2Y_6$, and $P2Y_{11}$ receptors in cancer cell proliferation and migration, apoptosis, and drug resistance will be covered in greater details, since functions for the other P2Y receptors in GI cancers are poorly documented. We will also present an interesting new therapeutic avenue that target $P2Y_{12}$ receptor functions in platelets as a mean to reduce the progression of CRC. Throughout this review, we will present limitations that, somehow, hinder the precise characterization of P2Y receptor functions in cancer and physiological conditions.

The $P2Y_2$ receptor generally promotes GI cancer carcinogenesis

In CRC, the $P2Y_2$ receptor stimulates cancer cell proliferation and increases resistance to apoptosis and cancer cell survival to chemotherapeutic drugs

The $P2Y_2$ receptor was the first P2Y receptor associated to GI cancers [17]. These initial observations were realized in primary CRC cell cultures obtained from seven independent resected colorectal tumors [17]. In fact, among all the tested nucleotides, ATP and UTP equipotently elicited the greatest increase in intracellular calcium ($[Ca^{2+}]_i$). This pharmacological profile was associated to *P2RY2* transcript expression, which was confirmed by RT-PCR analysis and further validated in the CRC cell line HT-29 [17]. Finally, these authors reported that prolonged stimulation with 100 to 500 μM of the nucleotidase-resistant ATP-γ-S inhibited cell proliferation and stimulated apoptosis. Similar conclusions were reached years later in Colo320 DM cells [18]. In Kyse-140 oesophageal cancer cell line, the addition of high ATP concentrations arrested cells in S-phase while increasing caspase-3 activity [19]. Again, these antiproliferative and proapoptotic effects were linked to $P2Y_2$ activation that was found expressed both in Kyse-140 cells and in primary oesophageal cancer cells. Paradoxically, these first attempts at characterizing the roles of $P2Y_2$ in GI cancers were, with another 2010 study in Caco-2 cells, the only reports concluding that $P2Y_2$ acted as an antiproliferative and proapoptotic agent [17–20].

An important change of view in the function of the $P2Y_2$ receptor emanated following the demonstration by Coutinho-Silva and colleagues that lower concentrations of ATP, as well as UTP, stimulated proliferation of colorectal cancer cell lines Caco-2 and HCT8 [21]. Interestingly, blocking adenosine uptake by dipyridamole prior to cell stimulation with a high concentration of ATP (1.5 mM) considerably decreased the number of apototic cells, while the broad-spectrum P1 receptor inhibitor 8-(p-sulfophenyl)theophylline had no effect [21]. The authors proposed that adenosine generated from the hydrolysis of ATP by ectonucleotidases had a P1 receptor-independent cytoxicity effect that was partly responsible for the observed apoptosis [21]. It was suggested that the P2X7 receptor, and other unidentified purinergic receptors, mediated the apoptotic effect in response to high ATP concentrations [21]. In fact, pretreatment of Caco-2 and HCT8 cells with the irreversible P2X7 receptor antagonist periodate-oxidized ATP before stimulation with 2 mM ATP inhibited the proapototic effect by more than 50%. It was concluded that low concentration of ATP activated the $P2Y_2$ receptor and induced the proliferative response. On the other hand, in the presence of high ATP concentrations, the activation of P2X7 promoted apoptosis along with the cytotoxic effect resulting from the accumulation of adenosine [21]. In light of these results, the antiproliferative and apoptotic responses observed in HT-29, Colo320, and Kyse-140 cells are most probably mediated by the P2X7 receptor and/or cytotoxic effect of adenosine as proposed by the Coutinho-Silva study [21]. This exemplifies two potential limitations in characterizing the functions of P2Y receptors. First, purinoreceptors ligand selectivity is redundant and they have a wide range of affinities, which goes from the nanomolar (e.g., $P2Y_2$) to hundreds of micromolar (e.g., P2X7) [22]. Second, nucleotides are highly labile as a result of ectonucleotidase activities.

The association between $P2Y_2$ and CRC was further validated by two independent studies reporting modulations of $P2Y_2$ expression in human patients [23, 24]. However, the conclusion to these studies was contradictory, probably as a result of differences in methodology. In fact, the first study by Nylund and colleagues compared resected colon tumors with adjacent non-cancerous margins and observed an increase in $P2Y_2$ protein expression in tumors [23]. In contrast, the second study reported a decrease in $P2Y_2$ mRNA and protein

expression in CRC samples after comparing colorectal specimens obtained from healthy individuals to those of patients with CRC [24]. Thus, $P2Y_2$ appeared to be differentially regulated between CRC patients and healthy individuals, but also within colorectal tumors and adjacent non-cancerous margins. Concomitantly, a retroviral expression screening assay identified the *P2RY2* gene as a potential transforming factor [25]. In these experiments, a retroviral cDNA expression library was generated from the human CRC cell line RKO and expressed in BOSC23 cells to produce viral particles. Normal mouse fibroblasts, NIH 3T3, were then infected and focus formation assays revealed that $P2Y_2$ acted as an oncogene. These findings were confirmed in anchorage-independent growth and tumor formation assays in nude mice [25]. While this $P2Y_2$ overexpression model certainly leads to receptor expression levels greater than those observed in cancerous cells, there are now strong experimental evidences that $P2Y_2$ promotes prostate, ovarian, and breast cancer cell invasion and metastasis [26–28]. These effects were associated with the up-regulation of epithelial-mesenchymal transition-related gene expression such as vimentin, snail family transcriptional repressor 1 (SNAI1), and cadherin 1 (E-cadherin) [26–28]. Despite these findings, the $P2Y_2$ receptor is still not recognized as a bona fide oncogene. However, accepting this possibility gives an interesting angle to its functions in most cancers.

Mechanistically, stimulation of the mitogen-activated protein kinase (MAPK) pathway by $P2Y_2$, and probably also by $P2Y_4$ and $P2Y_6$, was associated to the proliferation of colorectal-derived Caco-2 cells (Fig. 2) [29, 30]. Indeed, stimulation of Caco-2 cells with 10 μM ATP, UTP, or UDP caused a rapid phosphorylation of extracellular-regulated kinases 1/2 (ERK1/2), c-jun-N-terminal kinases 1/2 (JNK1/2), and p38 MAPK [29]. Once phosphorylated, ERK1/2 and JNK1/2 translocate to the nucleus where they can phosphorylate the activating transcription factor 1 and 2 (ATF-1 and ATF-2) and JunD [30]. These transcription factors are subunits of the activator protein-1 (AP-1) complexes that regulate multiple cell responses, including proliferation, differentiation, and apoptosis [35]. Finally, addition of MAPK inhibitors suppressed the proliferative response induced by ATP, confirming the activation of this cell signaling pathway by $P2Y_2$ [30]. While the phosphorylation of the different MAPK was calcium- and protein kinase C (PKC)-dependent, the transactivation of the epidermal growth factor receptor (EGFR) appeared as a key element in the $P2Y_2$-dependent stimulation of cancerous cell proliferation [29]. The transactivation of EGFR, and of other receptor tyrosine kinases, by $P2Y_2$ requires the selective recruitment and activation of Src family kinases (SFKs) to the two Src homology 3 domains (SH3) found in the $P2Y_2$ receptor C-terminal domain [29, 36].

Intrinsic or acquired resistance to apoptosis and resistance to drug-induced apoptosis are hallmarks of human cancer cells. In CRC, resistance to ursolic acid (UA) was associated with $P2Y_2$ stimulation of cyclooxygenase-2 (COX-2) via the SFK/p38 pathway [31]. UA is a pentacyclic triterpenoid carboxylic acid naturally found in many dietary plants that was shown to have potent anti-cancer properties [37, 38]. Briefly, it was reported that HT-29 cells treated with UA reacted with a rapid increase in the concentration of intracellular ATP, as well as by a 4-fold increase in *P2RY2* transcript levels. The increase in the concentration of intracellular ATP was paralleled to its release in the extracellular environment and to the stimulation of $P2Y_2$, which then recruited and activated SFKs [31]. Curiously, the inhibition of phospholipase C beta (PLCβ), one of $P2Y_2$ downstream effectors, with U73122 did not block p38 phosphorylation nor did it prevent UA-induced apoptosis [31]. Nonetheless, PKC was still required for p38 phosphorylation and UA-induced apoptosis. These apparent discrepancies suggested that calcium- and diacylglycerol-independent atypical PKCs might be involved [31]. It would also be of interest to determine if the contribution of $P2Y_2$ to UA-induced apoptosis might involve a cross-talk with EGFR via SFKs, followed by the downstream phosphorylation of p38 MAPK (Fig. 2). Anyhow, the signaling endpoint was the up-regulation of COX-2 protein expression and increase resistance to apoptosis by a still unidentified mechanism that was apparently not related to the production of prostaglandin E2 [39].

Another means by which cancer cells can resist to chemotherapy is by exporting drugs out of their cytoplasm. This efflux mechanism is mediated by transporters of the ATP-binding cassette (ABC) superfamily, of which the multidrug resistance-associated protein 2 (MRP2) expression was found to be upregulated in CRC [40]. In Caco-2 cells, $P2Y_2$ was shown to confer resistance to etoposide, cisplatin and doxorubicin, conventional chemotherapeutic drugs, by increasing MRP2 expression [32]. MRP2 up-regulation was dependent on the MAPK/ERK pathway and was abolished by suramin. This inhibitory profile was associated to $P2Y_2$ activity that leads to the increase expression of MRP2 [32]. However, it was not excluded that other purinoreceptors could also be involved [32]. Regardless, stimulation of Caco-2 cells with ATP significantly increased the IC_{50} value of etoposide necessary to induce apoptosis vs. control cells [32].

While $P2Y_2$ promotes the proliferation and survival of colorectal cancerous cells, analyses in hepatic and pancreatic cancers revealed that this receptor also favor the dissemination of cancer cells and invasion of adjacent tissues, all processes linked to the formation of metastasis.

$P2Y_2$ receptor activation contributes to cell dissemination and survival to hypoxia in hepatocellular carcinoma

The formation of metastatic foci in distant organs often involves the transformation of cancer cells to highly motile

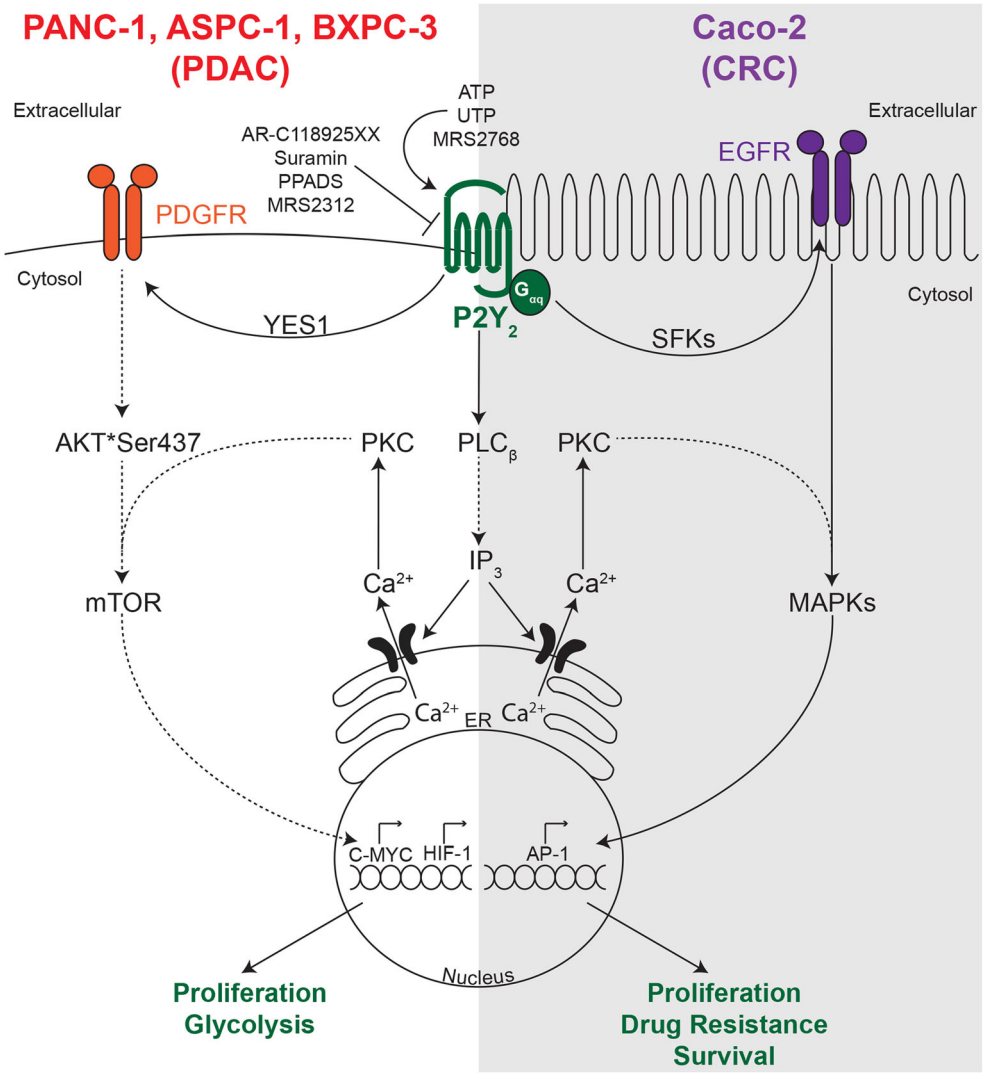

Fig. 2 Suggested $P2Y_2$ receptor signaling in pancreatic ductal adenocarcinoma (PDAC) and colorectal cancer (CRC) [29–34]

and invasive cells through a process known as epithelial-to-mesenchymal transition [26–28]. In HCC, activation of $P2Y_2$ was shown to increase cancer cell proliferation and tumor growth, while promoting migration [41]. Comparison of primary culture of human HCC and HepG2 cells to normal hepatocytes or the human normal hepatocyte cell line LO2, revealed that $P2Y_2$ was overexpressed in HCC [41]. As a result, stimulation of HCC cells with ATP or UTP significantly increased the $[Ca^{2+}]_i$ when compared to levels measured in normal cells. The increased receptor expression and activity in HepG2 cells correlated with the ATP stimulatory effects on cell proliferation and migration. These effects were abolished when cells were treated with suramin but foremost following the expression of shRNA targeting the *P2RY2* gene. Interestingly, inhibition of plasma membrane store-operated calcium channels (SOCs) and the down-regulation of the ER calcium sensor stromal interaction molecule 1 (STIM1) expression by shRNA also prevented the stimulation of cell proliferation and migration that was induced by $P2Y_2$. These findings revealed that Ca^{2+} signaling via the $P2Y_2$ receptor not only requires Ca^{2+} release from the ER, but that it was also dependent upon extracellular calcium entry. Finally, the addition of exogenous ATP to nude mice bearing HepG2 xenografts markedly increased the tumor size when compared to non-stimulated controls or mice xenografted with HepG2 expressing shRNA targeting either *P2RY2* or *STIM1* [41].

Pathological hypoxia is a common feature of the TME for most cancers, including HCC. This hypoxic environment positively alters cancer cell metabolism to favor cell survival while contributing to aberrant tumor vascularization and increasing metastatic potential [42]. One way that cancer cells trigger these survival responses is via the expression of the transcription factor hypoxia-induced factor 1-alpha (HIF-1α), as observed in multiple HCC cell lines [42, 43]. In this context, the up-regulation of HIF-1α expression in response to hypoxic conditions resulted in an increase of $P2Y_2$ protein expression in normal hepatocyte cell line HepaRG and in cancerous HepG2 cells [43]. As a result, overexpression of $P2Y_2$

under hypoxic conditions promoted cell survival of the five HCC cell lines HepG2, SK-Hep1, SNU449, Huh7, and Hep3B, an effect that was inhibited by MRS2312, a $P2Y_2$ selective antagonist, or through down-regulation of receptor expression by shRNA [43]. It was then concluded that HIF-1α promoted survival of HCC cells to hypoxia by increasing $P2Y_2$ expression [43].

$P2Y_2$ promotes nuclear Ca^{2+} signaling in cholangiocarcinoma

Cholangiocytes, the epithelial cells of the biliary tracts, play a crucial role in bile final formulation and secretion [44]. Extracellular ATP is present in bile, and activation of P2 receptors plays a central role in the regulation of normal biliary epithelial cell functions [45, 46]. While the roles of P2 receptors in CCA are still vague, RT-PCR and Western blot analyses along with pharmacological profiling revealed that the $P2Y_2$ receptor was the predominant purinoreceptor in the human biliary epithelial cell line Mz-Cha-1 [47]. Interestingly, it was discovered that activation of $P2Y_2$ with ATP not only stimulated the classical Ca^{2+} release from the ER, but also Ca^{2+} influx to the nucleus [47]. In fact, it was reported that nuclear Ca^{2+} transients were not abolished by thapsigargin, a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor (SERCA) that depletes inositol triphosphate (IP_3)-sensitive Ca^{2+} stores, suggesting that nuclear Ca^{2+} entry was independent of endoplasmic Ca^{2+}. This finding could be of significant importance as a previous study in HCC cell lines SK-HEP-1 and HepG2 showed that nuclear rather than cytoplasmic Ca^{2+} stimulated cell proliferation [48]. Consequently, it would have been pertinent to determine if the activation of $P2Y_2$ in Mz-Cha-1 cells also leads to nuclear Ca^{2+} influx and thus providing a growth advantage for cancer cells vs normal cholangiocytes. Interestingly, the modulation of nuclear Ca^{2+} levels by $P2Y_2$, and potentially other P2Y receptors, is an appealing idea that could dictate the regulation of gene expression through Ca^{2+}-sensitive transcription factors such as nuclear factor of activated T cells (NFAT) as an example [49].

$P2Y_2$ receptor promotes pancreatic cancer progression by enhancing cell glycolysis

Similarly to CRC and HCC, the increased expression of $P2Y_2$ was the first observation that linked this receptor to human pancreatic cancers. More specifically, protein and mRNA expression levels of NTPDase1 and -2 and $P2Y_2$ were up-regulated in pancreatic tissues of 28 patients suffering from different stages of PDAC when compared to normal pancreas biopsies [50]. However, while high levels of NTPDases expression correlated with better clinical outcomes, *P2RY2*-increased expression was associated with poor prognosis [50]. Thus, NTPDases, strictly expressed by normal cells around malignant tissues, seemed to contain cancer progression by dampening the tumorigenic responses induced by $P2Y_2$ [50].

$P2Y_2$-dependent signaling pathways in PDAC were first analyzed in the pancreatic ductal adenocarcinoma cell line, PANC-1 [33]. As described for colorectal and liver cancer cell lines, $P2Y_2$ was the main purinoreceptor expressed in PANC-1. Stimulation with UTP or with the selective $P2Y_2$ agonist MRS2768 increased PANC-1 proliferation, an effect that was blocked by suramin and shRNA targeting *P2RY2*. The proliferative effect was dependent on the downstream activation of PLC, the mobilization of intracellular calcium and the subsequent stimulation of PKC. In contrast to Caco-2 cells, the selective inhibition of ERK1/2 and JNK signaling pathways did not prevent the proliferation of PANC-1 cells in response to $P2Y_2$ activation. Instead, it was found that PANC-1 proliferation was dependent on the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or AKT) pathway. Hence, the activating phosphorylation of AKT on Ser473 was dependent on PKC, SFK, Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), and PI3K activities, thus underpinning a complex signaling network triggered by $P2Y_2$ activation in PANC-1 (Fig. 2).

Details of this network and its cellular consequences in cancer were untangled by the discovery that $P2Y_2$ enhanced glycolysis in PDAC, through PI3K/AKT and mammalian target of rapamycin (mTOR) pathways [34]. It was found, by screening The Cancer Genome Atlas database and analyzing 264 new PDAC specimens, that high *P2RY2* expression correlated with poor prognosis. Receptor mRNA transcripts and protein overexpression were also confirmed in six PDAC cell lines, compared to normal pancreatic duct HPNE cells. The highest levels of expression were measured in AsPC-1 and BxPC-3 cell lines, in which the activation of $P2Y_2$ with ATP and UTP stimulated cell proliferation, while UDP and ADP had no effect. The $P2Y_2$ proliferative effect was abolished in AsPC-1 and BxPC-3 stably expressing shRNA against *P2RY2*, thus confirming that this response was specific to $P2Y_2R$ [34]. The authors then compared AsPC-1 sh*P2RY2* cell line and AsPC-1 control shRNA (AsPC-1 shNC) after ATP treatment in a high-throughput differential gene analysis. This experiment revealed that the expression of multiple genes associated with glycolysis, PI3K-AKT-mTOR signaling, and C-MYC was activated downstream of $P2Y_2$ stimulation. Supporting these results, an up-regulation of glycolysis, as measured by extracellular acidification rates (ECAR), was measured in the AsPC-1 shNC cells treated with ATP in comparison to non-treated control and ATP-treated AsPC-1 sh*P2RY2* cells. Treatment with a selective $P2Y_2$ antagonist or PI3K and mTOR inhibitors confirmed that the increase in glycolytic rates seen in ATP-treated AsPC-1 and BxPC-3 cells were dependent on the PI3K/AKT-mTOR pathway. Activation of this pathway elevated C-MYC expression levels and, interestingly, also the expression of HIF-1α. Thus, it

appears from what was observed in HepG2 cells that *P2RY2* is not only a HIF-1α gene target, but that the receptor can also stimulate HIF-1α expression. In contrast to Caco-2 cells, $P2Y_2$ transactivation of the platelet-derived growth factor receptor (PDGFR), and not EGFR, was required to activate the PIK3/AKT pathway in AsPC-1 and BxPC-3 cells. Moreover, YES1 mediated the cross-talk between $P2Y_2$ and PDGFR [34]. Of note, YES1 is part of the SKFs family that was previously shown to be involved in $P2Y_2$/EGFR-dependent signaling in Caco-2 cells (Fig. 2) [29]. Finally, the inhibition of $P2Y_2$ activation with AR-C118925XX, a selective $P2Y_2$ antagonist, suppressed tumor growth in PDAC mouse models. In fact, the combination of AR-C118925XX and gemcitabine, a chemotherapeutic agent, was much more effective at inhibiting tumor growth and increased the survival time as compared to monotherapy with either molecule [34]. It would be of great interest to validate if the $P2Y_2$ receptor can be targeted to modulate cancer cell glycolysis to reduce the tumor load and increase survival for other GI cancers. In fact, this potential therapeutic avenue could well be applicable to CRC since ECAR measurements in colorectal cancer cell line HT-29 were increased in response to $P2Y_2$ stimulation [51].

In this section, we have seen that the $P2Y_2$ receptor was involved at every stages of tumorigenesis in colorectal, liver, and pancreatic cancers. While there were some differences in the activated signaling cascades (Fig. 2), the cellular responses triggered by $P2Y_2$ were generally pro-tumorigenic in all studied cancers. Moreover, overexpression of $P2Y_2$ was associated with poor prognosis.

$P2Y_6$ receptor promotes pancreatic and colorectal cancer tumorigenesis, but suppresses growth of gastric cancer cells

Contrary to breast cancer in which $P2Y_6$ promotes breast cancer metastasis and is considered as an integral component of the TME [14], the role of this UDP-selective receptor in GI cancers is just emerging. As an example of this recent interest, it was observed in PDAC that *P2RY6* mRNA expression was increased in cancerous tissues vs. non-cancerous controls [50]. In vitro, the addition of UDP to PANC-1 cells stimulated proliferation through a mechanism involving PLC, an increase in $[Ca^{2+}]i$ and PKC [52].

Similarly to the PDAC observations, the expression of *P2RY6* transcripts was also increased in human CRC tumors when compared to match adjacent non-cancerous resection margins [53]. The proliferative effect of UDP was also observed in Caco-2 and HCT8 colorectal cancer cell lines [21]. Hence, our group reported that $P2Y_6$ promoted CRC tumorigenesis by protecting HT-29 colorectal cancer cells from apoptosis and by increasing the resistance of primary colorectal cancer cells to 5-fluorouracil (5-FU), a common chemotherapeutic agent used in the treatment of CRC [53]. In this study, *P2ry6* knockout mice ($P2ry6^{-/-}$) and WT littermates ($P2ry6^{+/+}$) received azoxymethane along with dextran sodium sulfate (AOM/DSS) to induce CRC in a model recapitulating human-colitis-associated CRC. $P2ry6^{-/-}$ mice displayed reduced tumor loads and decreased dysplastic grade when compared to $P2ry6^{+/+}$ mice, suggesting that invalidation of *P2ry6* protected cells from colorectal carcinogenesis [53]. Moreover, immunofluorescence staining for CD31, which marks vascular endothelial cells and thus vascularization, demonstrated that the dysplastic lesions of WT animals were more vascularized contrary to those found in knockout mice. Furthermore β-catenin staining was found in the cytosol but mostly in the perinuclear and nuclear regions in $P2ry6^{+/+}$ dysplastic areas, whereas β-catenin expression was mainly observed at the plasma membrane and in the cytosol of intestinal epithelial cells in AOM/DSS-treated $P2ry6^{-/-}$ animals [53]. Of note, aberrant nuclear accumulation of β-catenin is a hallmark of CRC [54]. Hence, the β-catenin nuclear localization in dysplastic regions found in $P2ry6^{+/+}$ mice correlated with the increased expression of C-MYC, one of β-catenin target genes, while C-MYC staining was undetected in $P2ry6^{-/-}$ cells (Fig. 3) [53]. Next, the mechanisms by which $P2Y_6$ receptor promoted colorectal carcinogenesis were elucidated. It was found that activation of the $P2Y_6$ with its selective agonist MRS2693 protected HT-29 cells from apoptosis. Interestingly, Western blot analysis of multiple pro- and anti-apoptotic proteins revealed that this protective effect appeared to be strictly mediated by the X-linked inhibitor of apoptosis protein (XIAP), which acts on caspase-3 to block its activity [56]. In fact, it was shown that $P2Y_6$ selective activation induced XIAP stabilization via the phosphorylation of Ser87. This phosphorylation was dependent on the PI3K-AKT pathway which was suppressed in HT-29 cells stably expressing shRNA against *P2RY6* (Fig. 3). These in vitro analyses translated in vivo, since AKT activating phosphorylation at Thr308 was markedly downregulated in colons of $P2ry6^{-/-}$ mice when compared to WT animals. Finally, to determine if $P2Y_6$-induced resistance to apoptosis could also protect CRC cells from chemotherapeutic drugs, CRC-derived tumoroids were treated with 5-FU in the presence or absence of MRS2693 and the number of living and dying tumoroids were evaluated. Results revealed a greater than 2-fold increase in the number of living tumoroids in the MRS-treated group, thus confirming that $P2Y_6$ contributed to chemoresistance. Taken together, these results suggest that the $P2Y_6$ receptor could be a novel target in CRC.

Surprisingly and contrary to CRC and PDAC, $P2Y_6$ activation in gastric cancer triggered an antiproliferative response through the SOCE/Ca^{2+}/β-catenin pathway [55]. While *P2RY2* mRNA and $P2Y_4$ protein were overexpressed in GC patients and GC cell lines MKN-45 and SGC-7901, both mRNA and $P2Y_6$ protein levels

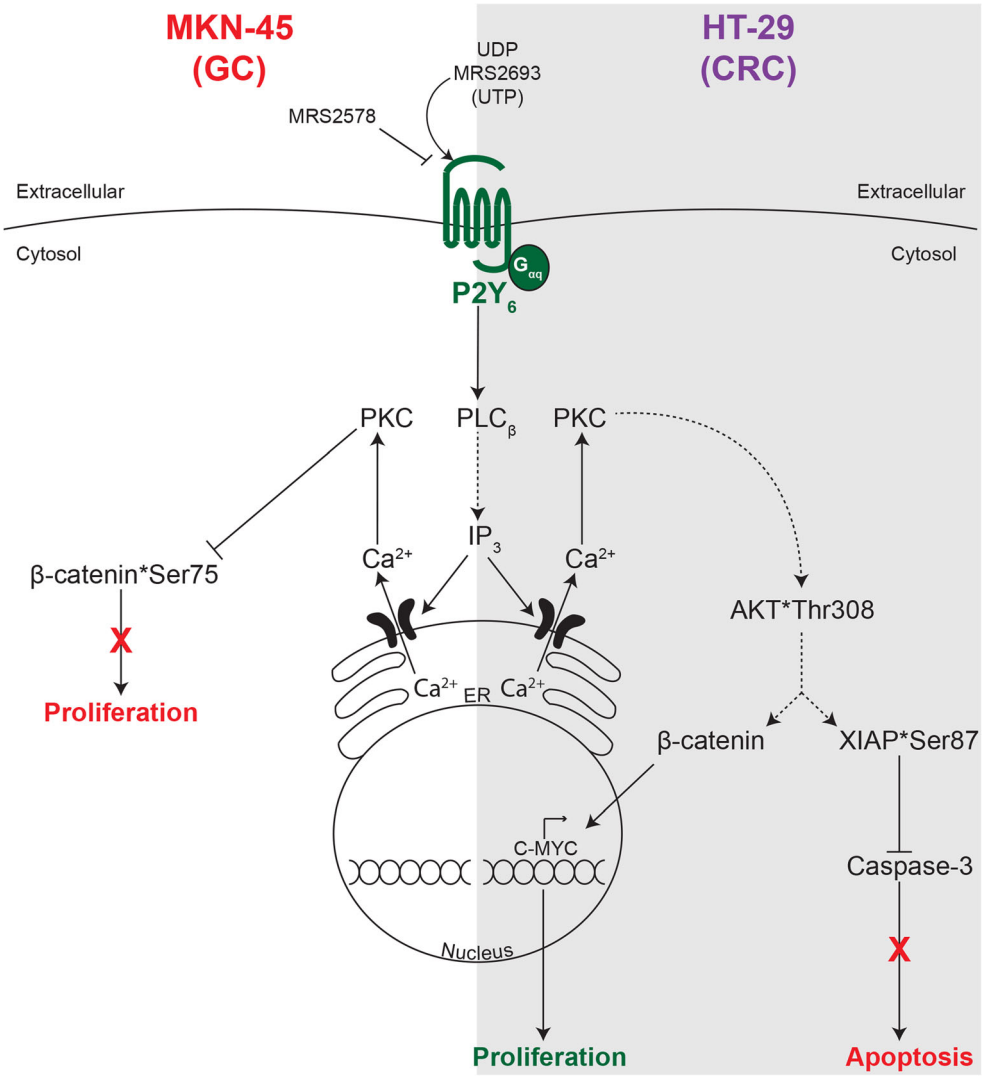

Fig. 3 Suggested $P2Y_6$ receptor signaling in gastric cancer (GC) and colorectal cancer (CRC). [53, 55]

were down-regulated. Low *P2RY6* expression further correlated with poor prognosis and was associated with poor differentiation, enhanced tumor size, and increased dissemination of cancer cells to lymph nodes. In MKN-45 and SGC-7901 cells, UDP and UTP repressed proliferation through $P2Y_6$-mediated Ca^{2+} release and suppression of β-catenin signaling. Even if $P2Y_6$ can bind UTP, albeit with a much lower affinity than UDP, it was curious that UTP did not induce a proliferative response by activating the $P2Y_2$ receptor [3]. To ensure that $P2Y_6$ was responsible for the antiproliferative effect of UTP, the authors confirmed that UTP-dependent repression of MKN-45 cell proliferation could be reversed by blocking $P2Y_6$ receptor activation with MRS2578, a $P2Y_6$ antagonist, or through the use of shRNA targeting *P2RY6*. $P2Y_6$ exerted its antiproliferative effect by repressing cyclin D1 expression, which is necessary for G1 progression and S-phase entry [55, 57]. The mechanism of β-catenin suppression was next investigated. It was reported that $G\alpha_q$-signaling could promote nuclear export of β-catenin and its subsequent degradation in the cytoplasm by calpain [58]. However, this mechanism was not involved in MKN-45 and SGC-7901 cells, since UDP and UTP did not alter β-catenin protein expression levels. In fact, the suppression of β-catenin signaling was mediated by the inhibition of its transcriptional activity, as demonstrated by reduction in the level of β-catenin phosphorylation on Ser675 (Fig. 3). The suggested role of $P2Y_6$ in tumor suppression was validated, in vivo, in an elegantly designed xenograft experiment. Briefly, SGC-7901 cells were xenografted in both armpits of nude mice and $P2Y_6$ agonists or saline were injected in the left and right armpit, respectively. Tumors in each armpit were compared and showed that tumor growth was indeed suppressed in the $P2Y_6$-agonist-treated armpit. Thus, from a therapeutic point of view, it appears that targeting $P2Y_6$ in GC would require a selective agonist, while an antagonist would be necessary for the treatment of CRC and PDAC.

$P2Y_{11}$ receptor stimulates the migration of pancreatic and liver cancer cells, but inhibits this response in normal ciliated cholangiocytes

Apart from previous mentions of transcript expression in Caco-2, HCT-8, and PANC-1 cell lines, the dual $G\alpha_q$- and $G\alpha_s$-coupled $P2Y_{11}$ receptor has just recently surfaced as an important regulator of cell migration in pancreatic and liver cancers [21, 52]. In PDAC cell lines BxPC-3 and Capan-2, the $P2Y_{11}$ receptor was shown to cooperate with protease-activated receptor 2 (PAR-2) in driving cancer cell migration [59]. In this study, the authors initially tested if PAR-2 promoted pancreatic cancer cell migration, as previously observed for breast and colon cancer cells [60–63]. This hypothesis was challenged when they observed that the selective activation of PAR-2 stimulated migration in wound scratch assays, while PAR-2 activation had no effect on migration in transwell assays. It was then discovered that ATP, only when added in combination with PAR-2 activating peptides, could promote BxPC-3 and Capan-2 cell migration in transwell assays. The selective inhibition of $P2Y_1$, $P2Y_{11}$, and $P2Y_{13}$ receptor activities revealed that $P2Y_{11}$ was the sole P2Y receptors potentiating the effect of PAR-2 on cell migration. However, this conclusion may need reconsideration as the authors did not take into account the possible participation of $P2Y_2$ in the cross-talk with PAR-2, despite demonstrations of $P2Y_2$ expression and activity in BxPC-3 cells [34]. Finally, using a panel of cell signaling inhibitors, it was shown that migration in PDAC cells in response to PAR-2 and $P2Y_{11}$ activation was mediated through an EGFR/Src/MAPK pathway [59].

Characterization of P2 receptor expression and function in the HCC cell line Huh-7 showed that $P2Y_{11}$ stimulated the migration of these cancer cells [64]. In fact, it was determined that $P2Y_{11}$, but not $P2Y_2$, was the main purinoreceptor involved in the Ca^{2+} response elicited by ATP stimulation of Huh-7 cells. In HepG2, $P2Y_{11}$ was partially contributing to the ATP-induced Ca^{2+} release, as demonstrated using selective-$P2Y_{11}$ antagonist or shRNA [64]. This result suggested that $P2Y_2$ was not the only receptor present in HepG2 cells and that part of the cellular responses triggered by ATP are mediated by the $P2Y_{11}$ in this cell line. From a clinical perspective, the $P2Y_{11}$ receptor could be an interesting HCC biomarker. Indeed, immunohistochemistry analyses showed abundant expression of $P2Y_{11}$ in HCC tissues, while no staining was detected in normal liver biopsies. It could also represent a promising drug target, since selective activation of $P2Y_{11}$ stimulated Huh-7 cell migration in transwell assays. However, additional experiments are needed since the ATP/$P2Y_{11}$-dependent migration of Huh-7 cells could also involve P1 receptors as a result of ATP hydrolysis to adenosine by nucleotidases [64].

A study on $P2Y_{11}$ functions in cholangiocytes and CCA eloquently illustrated how a simple change in the cellular context, here the absence or presence of chemosensory cilia, can lead to opposing cell responses in the same cell type [65]. Cholangiocytes express primary cilia that act as mechano-, chemo-, and osmosensors [66]. However, these sensory organelles are missing in CCA cell lines and in human bile duct cancer samples, which suggested the involvement of cilia in the development and/or progression of this cancer [66]. In this context, Mansini and colleagues tested if nucleotide detection by cilia was involved in CCA cell migration and growth [65]. The effect of ATP on migration and invasion was first tested on ciliated cholangiocytes, experimentally deciliated cholangiocytes and the CCA cell line HUCCT1 [65]. Interestingly, ATP inhibited migration and invasion in ciliated cells, while it promoted migration and invasion in normal deciliated cholangiocytes and HUCCT1 cells. The ciliary-dependent inhibition of migration and invasion was linked to the activation of tumor suppressor gene *liver kinase B1* (*LKB1* or *STK11*) [65]. Interestingly, LKB1 was activated upon ATP stimulation in ciliated cholangiocytes, but not in deciliated cholangiocytes or HUCCT1 cells. In ciliated cholangiocytes, LKB1 phosphorylation activated the phosphatase and tensin homolog (PTEN) and inhibited AKT. In fact, introduction of a shRNA against the *P2RY11* gene confirmed that this receptor was responsible for the ATP-dependent phosphorylation of LKB1 by protein kinase A [65]. Thus, loss of chemosensory cilia in cholangiocytes is enough to switch $P2Y_{11}$ signalization from inducing an anti- to a pro-tumorigenic response.

Targeting $P2Y_{12}$ receptor in platelets reduces the risk of developing colorectal cancer

There are currently multiple evidences that cancer cells are capable of "hijacking" platelets as a mean to disseminate across the human body [67]. During their passage in the blood stream, platelets can be activated by tumor cells by a mechanism that involves the platelet Fcγ receptor IIa [68]. Once activated, platelets release a plethora of bioactive compounds such as lipids, microRNAs, and growth factors that further enhance platelet-cancer cell interactions and stimulate metastasis, angiogenesis, and drug resistance [67]. Hence, degranulation of tumor-activated platelets leads to the release of large quantity of ATP and ADP stored in platelet-dense granules [69]. Interestingly, the ATP-dependent activation of the $P2Y_2$ receptor promoted cancer cell extravasation by increasing vascular permeability [69]. These findings were the rationale behind the observed efficacy of antiplatelet agents, such as aspirin, in the prevention of solid cancer development and inhibition of metastasis [70]. Similarly, two netted-case studies reported that the antiplatelet agent clopidogrel, an irreversible $P2Y_{12}$ antagonist, offered protection against CRC in two

European populations [71, 72]. In fact, both studies concluded that clopidogrel alone or in combination with low-dose aspirin reduced the risk of developing CRC by 20 to 30%. Moreover, the protective effect was found to be effective only after 1 year of treatment and was not maintained after drug discontinuation [71]. Another study in mice showed that aspirin and ticagrelor, a reversible $P2Y_{12}$ antagonist, prevented CRC metastasis by disrupting crosstalk between platelets and tumor cells [73].

$P2Y_1$, $P2Y_4$, $P2Y_{13}$, and $P2Y_{14}$ receptors are missing links in GI cancers

Even do the $P2Y_1$ was the first P2 receptor to be cloned and extensively studied for nearly 25 years, its role in GI cancers remains enigmatic [74]. It was suggested that $P2Y_1$ could inhibit cell proliferation while inducing apoptosis in Caco-2 and HCT-8 cells [21]. Hence, the $P2Y_1$ receptor was associated to colon adenocarcinoma SW480 cell apoptosis in response to a treatment with the nitrite oxide donor, glyceryl trinitrate, and reactive oxygen species producer H89 [75]. In contrast, $P2Y_1$ increased proliferation of PANC-1 cells in a PLC, IP_3, and PKC-dependent manner [52]. In HCT rat hepatoma cell line, $P2Y_1$ signalization was required to counter osmotic swelling [76]. Finally, *P2RY1* transcripts were detected in the CCA cell line Mz-Cha-1, but the mRNA did not seem to be translated [47].

Expression data for the UTP-selective $P2Y_4$ receptor are the main evidences supporting its potential involvement in GI cancers. Indeed, *P2RY4* mRNA expression was reported in Caco-2, HCT-8 and in Mz-Cha-1 cells [21, 47]. $P2Y_4$ transcripts and protein expression were also detected in HCC cell lines HepG2 and BEL-7404 as well as in HCC tissues. However, the expression levels were similar to those measured in normal hepatocytes or normal hepatocyte cell line LO2 [41]. Western blot and immunohistochemistry analyses suggested that the $P2Y_4$ receptor might be expressed in HT-29 cells, while it also seemed to be overexpressed in CRC patients [23, 77]. However, these analyses must be interpreted with caution, as the reported Western blot signals were not in accordance with $P2Y_4$ expected molecular weight of 41 kDa [23, 77]. Furthermore, serious reliability issues in antibodies targeting P2Y receptors have been reported and were also observed by our group [78, 79]. In fact, antibody reliability is an important limitation in working with P2Y receptors, as it is with other GPCR. Regarding the $P2Y_{13}$ and $P2Y_{14}$ receptors, apart from reports mentioning their expression in some GI cancer cell lines [20, 52], nothing is known about their role in GI cancers.

Conclusion

In this review, we have highlighted the contribution of $P2Y_{2,6 \text{ and } 11}$ receptors in GI cancers. Given their involvement in cancer cell proliferation, metabolism, dissemination, apoptosis, and resistance to chemotherapeutic drugs, the modulation of their activities might have a direct impact on the development and/or progression of colorectal, liver, pancreatic, and gastric cancers. However, due to the complexity of the purinergic signaling network and P2Y receptor interacting patterns, it would be surprising that P2Y receptors act alone to promote tumorigenesis. We also highlighted that more fundamental studies are mandatory to define the role of $P2Y_1$, $P2Y_4$, $P2Y_{13}$, and $P2Y_{14}$ receptors to provide a broader perspective of P2Y receptor global activities in GI cancers. Finally, considering the positive benefits of regulating platelet functions in cancers, combinatorial therapy using classical chemotherapeutic agents and $P2Y_{12}$ receptor-selective antagonists, such as clopidogrel, represent a promising research avenue for GI cancers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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