Review

Understanding the roles of the P2X7 receptor in solid tumour progression and therapeutic perspectives☆

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Sébastien Roger [a,b,*], Bilel Jelassi [a], Isabelle Couillin [c], Pablo Pelegrin [d], Pierre Besson [a], Lin-Hua Jiang [e]

[a] Inserm UMR1069 Nutrition, Croissance et Cancer, Université François-Rabelais de Tours, 10 Boulevard Tonnellé, 37032 Tours, France
[b] Département de Physiologie Animale, UFR Sciences et Techniques, Université François-Rabelais de Tours, Avenue Monge, 37200 Tours, France
[c] UMR CNRS 7355 Experimental and Molecular Immunology and Neurogenetics, Université d'Orléans, 3B rue de la Ferollerie, F-45071 Orléans, France
[d] Inflammation and Experimental Surgery Research Unit, CIBERehd, Clinical University Hospital "Virgen de la Arrixaca", Murcia's BioHealth Research Institute IMIB-Arrixaca, Carretera Cartagena-Madrid s/n, 30120 Murcia, Spain
[e] School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom

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ABSTRACT

P2X7 is an intriguing ionotropic receptor for which the activation by extracellular ATP induces rapid inward cationic currents and intracellular signalling pathways associated with numerous physiological processes such as the induction of the inflammatory cascade, the survival and proliferation of cells. In contrast, long-term stimulation of P2X7 is generally associated with membrane permeabilisation and cell death. Recently, P2X7 has attracted great attention in the cancer field, and particularly in the neoplastic transformation and the progression of solid tumours. A growing number of studies were published; however they often appeared contradictory in their results and conclusions. As such, the involvement of P2X7 in the oncogenic process remains unclear so far. The present review aims to discuss the current knowledge and hypotheses on the involvement of the P2X7 receptor in the development and progression of solid tumours, and highlight the different aspects that require further clarification in order to decipher whether P2X7 could be considered as a cancer biomarker or as a target for pharmacological intervention. This article is part of a Special Issue entitled: Membrane channels and transporters in cancers.

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1. Introduction

The purinergic signalling, mediated by multiple receptors called P1 and P2 purinoreceptors upon extracellular stimulation by adenine nucleoside and nucleotide di- or triphosphates respectively, represents very dynamic and plastic mechanisms for controlling a diversity of crucial biological functions of cells and tissues such as cell-to-cell communications, secretory activities, membrane excitability, cell proliferation, cell differentiation and maturation, cell adhesion and migration, and cell death. P1 and P2 receptors are expressed in a large number of eukaryotes and particularly at the plasma membrane of virtually every mammalian cell [1]. P1 receptors are seven-transmembrane segments (7TM) G-protein coupled receptors and commonly termed adenosine receptors because they are physiologically activated by nanomolar levels of extracellular adenosine. P2 receptors can be divided into two subfamilies, P2X and P2Y receptors, based on their pharmacology and the different downstream signalling pathways they are associated with. The P2Y receptors are also 7TM G-protein coupled receptors, for which the activating ligands include ATP and some other purine or pyrimidine di- or tri-phosphate nucleotides such as ADP, UTP or UDP, and activation initiates signalling pathways dependent on the G-proteins they are coupled to [2]. There are eight P2Y subtypes in human cells, which can be further subdivided into two groups; P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 are coupled to G_q and activation of these receptors stimulates the phospholipase C (PLC)-IP_3-Ca^{2+} signalling pathways, and P2Y12, P2Y13, and P2Y14 are G_i-coupled receptors and their activation leads to inhibition of adenylyl cyclase (AC). P2Y11 receptor is also coupled to AC stimulation. [3]. By contrast, P2X receptors are ionotropic receptors which are physiologically and exclusively activated by extracellular ATP [4]. The P2X receptor family is composed of seven members with a membrane topology of two-transmembrane segments (TM1 and TM2) separated by a large extracellular loop for the binding of ATP. P2X receptors were proposed to be homo- or hetero-trimeric [5]. This has been confirmed in recent studies performed using X-ray crystallography to resolve the three-dimensional structure of the zebrafish P2X4 receptor [6,7]. P2X receptors constitute functional non-selective cation channels and their activation mediates very rapid cellular effects, generally resulting in a depolarizing inward current due to a major influx of Na^+ and Ca^{2+} into the cytosol at physiological membrane potential, while a minor but concomitant efflux of K^+ is generated. Besides this direct effect on the transmembrane polarity, P2X receptors further contribute to important increases in the intracellular Ca^{2+} concentration by activation of voltage-gated Ca^{2+} channels as a result of membrane depolarisation [8] and therefore activate intracellular Ca^{2+}-dependent signalling pathways that can have longer-term effects [9], and there are possibly other signalling pathways following K^+ efflux [10]. Some P2X receptors have also been identified in intracellular compartments, such as the P2X4 which is also localised in the lysosomes of some cells such as macrophages, microglia and endothelial cells [11,12].

Having a clear overview on all the signalling pathways activated, in a tissue, as a function of time is important to better understand the very complex resulting biological responses to extracellular ATP exposure. Indeed, all cells generally express numerous P1 and P2Y receptor subtypes and many excitable and non-excitable cells are also known to express P2X receptors. It has to be considered that P2 receptors are activated in specific ranges of purines in experimental conditions when agonists are exogenously applied; some receptors such as P2Y2 receptor and P2X1 receptor are highly sensitive to nanomolar or submicromolar concentrations of ATP, where some others, like P2X7 receptor, are much less sensitive and need more than 100 micromolar concentrations to record their activation in vitro [2,5,13]. However, considering a tissue in physiological conditions, there is no specific range of ATP concentration, even a very small one that would only activate a sole receptor subtype in a particular cell type.

A question naturally arises when one thinks on the purinergic signalling: what is the biological origin for these nucleotide ligands? Nucleotides can be released by cells constitutively or in response to either mechanical or chemical stimulations. When only considering ATP, mechanisms for its release into the extracellular compartment are multiple [14] and can be classified into *i*) transmembrane diffusion, *ii*) vesicular exocytosis and *iii*) cell lysis-related release. Depending on the considered release mechanism, extracellular ATP can reach different concentrations and therefore activates different combinations of P2 receptors, at least at the initial time. The vesicular release is a physiological cell-to-cell interaction mechanism that is classically associated with neurotransmission and leads to the exocytosis of micromolar concentrations of ATP in restricted domains and the activation of post-synaptic and pre-synaptic P2 receptors in close vicinity. However, the vesicular release of ATP could occur from many non-neuronal cell types such as endothelial cells [15], urothelial cells [16], fibroblasts and epithelial lung cancer cells [17], astrocytes [18], osteoblasts [19]

The transmembrane diffusion of ATP through channels, such as pannexins- or connexins-composed hemichannels, maxi-anion or volume-regulated anion channels, P2X7 receptors or ATP-Binding Cassettes (ABC) transporters, could result in a pericellular halo of low micromolar ATP concentration that is involved in the regulation of cell functions via an autocrine and/or paracrine fashion. In comparison, cell damage is responsible for much higher extracellular concentrations and the establishment of an ATP gradient possibly activating P2 receptors in multiple cells being in different distances from release site. Furthermore, ATP can also be synthesised extracellularly by ecto-adenylate kinases or nucleotide diphosphate kinase [20]. The life time of extracellular ATP is tightly controlled by multiple ecto-enzymes such as ectonucleotidases and nucleotide diphosphokinases that convert ATP to ADP and adenosine [21]. The presence and activity of such ectonucleotidases can terminate ATP-evoked signalling but can initiate distinctive signalling pathways by the stimulation of ADP-sensitive P2 receptors and adenosine receptors. The adenosinergic pathway plays an important role in cancer progression since extracellular adenosine

generated after extracellular ATP hydrolysis by the ectonucleotidases CD39 and CD73 is immunosuppressive, resulting in tumour progression, metastases appearance, and poor disease outcomes. Indeed adenosine is an autocrine or paracrine factor with potent immunoregulatory activity which facilitates tumour escape from the immune control. Targeted therapies toward the adenosinergic pathway, such as antibodies recognizing CD73 and CD39, have proven efficacy in mouse tumour models [22,23]. Therefore, the activation of P2 receptors depends on the concentration and identity of nucleotides in the extracellular compartment. In such conditions it is obvious that extracellular ATP can physiologically and successively activate numerous receptors and their associated intracellular signalling pathways. Each of these signalling pathways is tightly regulated so that cells and tissues evolve homogeneously. Numerous pathologies appeared to be dependent on the deregulations of one or multiple elements of the purinergic signalling cascades. One of these pathologies, which recently has drawn increasing attention, is the cancer disease, arising from altered purinergic signalling in both cancerous and non-cancerous cells in the tumour. Several excellent reviews on this topic were published, some of them being listed here [24–28].

When focusing on solid tumours, several P2 receptors appear to be critical in the promotion and/or progression of the cancer [24,25,29]. It seems clear from numerous studies that some receptors have pro-cancerous activities and that their pharmacological inhibition, once potent and specific tools will become available, could represent new opportunities to treat the disease. This is certainly the case of the highly ATP- and UTP-sensitive P2Y2 receptor. There is compelling evidence to indicate a critical role for this receptor in cancer cell proliferation [30–32] and resistance to apoptosis [33], thus supporting tumour growth, and in cancer cell migration [34], invasion [35] and dissemination, therefore promoting tumour metastasis [36,37]. P2X5, and probably also P2Y12 receptors, are also strongly expressed by tumour cells and seem to mediate cell differentiation, resulting in an anti-proliferative effect in these cells (see [25]).

P2X7 is one of the purinergic receptors which have attracted much of the attention during the last five years in the context of cancer disease. However, while a growing number of studies show the involvement of this particular receptor in the oncogenic process, multiple aspects remain unclear so far such as the cells in the tumour which express the P2X7, the conditions for its activation in tumour sites, its functionality and the cell properties it is regulating. As such, many questions still remain unanswered that limit consideration of the P2X7 as a cancer biomarker or as a target for pharmacological intervention. The present review aims to discuss the current knowledge and hypotheses on the involvement of P2X7 in the development and progression of solid tumours, and highlight the different aspects that will have to be clarified with further investigations.

2. ATP in tumour sites

ATP is present in the range of 3–10 mM in intracellular compartments of cells. The extracellular concentration of ATP is much lower. It was estimated that the physiological concentration of ATP in blood is in the range of 20–100 nM and is increased after muscular exercise [38]. However, the concentration of ATP in tissues is hard to measure or estimate. The team of Francesco Di Virgilio (Ferrara, Italy) has developed a bioluminescent probe, called pmeLUC, for the measurement of extracellular ATP both in vitro and in vivo. This probe is a plasma membrane luciferase, with the ATP catalytic site facing the extracellular side, which can be transfected in reporter cells that are subsequently inoculated into tissues [39] or directly into cancer cells [40]. This was used to show that the extracellular microenvironment of tumours contains relatively high concentrations of ATP, in hundreds of micromolar range, whereas it is almost undetectable in healthy tissues [41]. While ATP was thought to be secreted by cancer cells [41,42], it has been postulated that the

main source for ATP could be dependent on dying cells [43] such as those in the perilesional hypoxic regions of solid tumours [44].

While exogenously applied ATP was demonstrated to have anti-neoplastic effects [45–50], it is also proposed that endogenous extracellular ATP could contribute to cancer progression and promote resistance to anticancer treatments [25]. Endogenously released extracellular ATP could also play a beneficial role during anti-cancer therapy by stimulating the immune system through immunogenic cell death [51]. The beneficial or detrimental role of ATP may depend on the mechanisms of cell death, on the type of ATP release, on the concentrations of ATP and obviously on the purinergic receptor signalling pathways involved. ATP can derive from different compartments of the cell, coming from mitochondria, endoplasmic reticulum or cytoplasm [52]. Tumour cells can undergo different types of cell death with both passive release or active secretion of ATP. Several mechanisms of ATP secretion in dying, dead or stressed cells have been reported [53]. Different programmed cell deaths such as apoptosis, autophagic cell death and pyroptosis promote ATP release by dying cells. Moreover, the non-programmed cell death called necrosis also leads to ATP release. These four ways to die promote ATP release via different mechanisms, and can be beneficial with immunogenic properties or detrimental with increased proliferation and/or migration of tumour cells. Anti-cancer therapies may reorient these beneficial versus detrimental potential of ATP release. In particular, the use of immunogenic cell death inducers such as chemotherapy agents in cancer therapy or infection by oncolytic viruses may favour immunogenic cell death. Indeed, oncolytic viruses were shown to induce secretion of ATP from human cancer cells [54]. Extracellular ATP, besides acting as a danger-associated molecular pattern (DAMP) that can stimulate anti-tumour immunity, also functions as a "find me" signal through P2Y2 receptors, facilitating rapid attraction of antigen presenting cells to sites of extensive apoptotic cell death.

2.1. Necrosis

ATP may be released by dying cells in response to non-programmed cues, such as the necrosis produced by pressure disruption, hypoxic injury, toxins, cancer, infection and trauma. Necrosis is characterised by cellular swelling and rupture of the plasma contents [55]. These processes activate the innate immune system and can promote a strong sterile inflammation through the NLRP3 inflammasome that activates caspase-1 leading to the secretion of the mature pro-inflammatory cytokine interleukin (IL)-1β and IL-18. This activation may be triggered in part by ATP produced by mitochondria and released from damaged cells [56].

2.2. Apoptosis and autophagy

Cells which die as part of physiological responses, such as apoptotic or autophagic cells, are removed from tissues to prevent immune reactions and maintain tissue homeostasis. Although apoptotic cell death has been considered to be non-immunogenic, recent studies unravelled that several anti-neoplastic agents and irradiations can trigger immunogenic apoptosis in particular through the active secretion of ATP [57]. Moreover, cells dying via autophagy can trigger pro-inflammatory responses through the release of ATP driving the inflammasome activation [40,51,58]. Indeed, autophagy has a dual role: it is a survival mechanism responding to poor nutritional or stress conditions to avoid cell death (and suppress apoptosis) by degradation of long-lived proteins and damaged organelles [59,60]. However, under certain circumstances, autophagy constitutes an alternative way for cell to dye through the autophago-lysosomal pathway [58]. Autophagic cell death induced by nutrient deprivation, hypoxia and infectious pathogens is a non-lytic process, with cell budding and characterised by caspase-independent autophagosome formation and action of lysosomal proteases. The DAMPs released are ATP and the nuclear protein chromatin-binding high mobility group box 1 protein (HMGB1). Phagocytosis of human

cancer cells dying through autophagy was shown to trigger activation of the NLRP3 inflammasome and secretion of mature IL-1β in human macrophages via an ATP-dependent mechanism [61]. Blockade of K^+ efflux during phagocytosis, addition of P2X7 antagonist, incubation in the presence of apyrase, or silencing NLRP3 protein expression, inhibit IL-1β secretion response [61]. Moreover, in mouse, the NLRP3 inflammasome was activated after macrophage engulfment of autophagic dying cells [62]. In autophagic dying cells, ATP was released via pannexin-1 channels. Moreover, in phagocytosis of autophagic dying cells, both P2X7 activation and subsequent K^+ efflux in macrophages are obligatory steps for inflammasome activation [62]. A recent report from G. Kroemer's group has shown that in response to immunological cell death inducers, ATP redistributes from lysosomes to autolysosomes and is secreted by a mechanism that requires the lysosomal protein LAMP1, which translocates to the plasma membrane in a strictly caspase-dependent manner. These findings suggest that caspase- and pannexin-1 channel-dependent lysosomal exocytosis has an essential role in ATP release in immunological cell death triggered by immunogenic chemotherapy [63]. In vivo, chemotherapeutic agents induce tumour autophagic cell death which is immunogenic through recruitment of dendritic cells and T cells into the tumour via the secretion [40,51,61].

In conclusion, it appears particularly important to understand the factors that determine the beneficial or pathogenic responses to extracellular ATP on cancer evolution. Extracellular ATP concentrations might be a determinant of the final outcome. Indeed it was shown that 250 nM extracellular ATP, which activates P2Y receptor signalling, does not modulate T regulatory cells, whereas 1 mM ATP which more likely activates P2X7, stimulates T regulatory cells, enhancing their ability to proliferate and to be immunosuppressive [64]. In addition, prolonged release of low amounts of ATP diminishes the capacity of mature dendritic cells to initiate type 1 immune responses [65,66].

3. Functional particularities of the P2X7 receptor and relevance to the cancer disease

Among the members of the P2X receptors family, the P2X7 receptor [67,68] is very unique by multiple features, from its molecular structure, to its biophysical and pharmacological properties [69]. Even the cells and tissues expressing P2X7 in physiological conditions are different from those observed for other P2X. Indeed, P2X1–P2X6 receptors are expressed widely, even if not exclusively, in the nervous system [70] where they underlie the neurotransmission at post-synaptic sites generating action potentials or at pre-synaptic sites modulating neurotransmitter release [9]. In contrast, while the expression of P2X7 receptors in glial cells is well accepted [71,72], its expression in neurons remains a topic of debate because of the use of poorly selective antibodies [73,74]. The well accepted distribution of P2X7 receptor is in immune cells coming from the hematopoeitic lineage, such as monocytes, macrophages, dendritic cells, B and T lymphocytes, mast cells and epidermal Langerhans cells, in which participation of the receptor in the immune response has been extensively documented. Particularly, the participation of P2X7 is critical in the inflammation by inducing the activation of the inflammasome and caspase-1 with the subsequent release of the pro-inflammatory IL-1β from Pathogens Associated Molecular Patterns (PAMPs)-primed macrophages and microglial cells [75,76]. The P2X7 receptor is expressed in bone cells, in both osteoblasts [77,78] and osteoclasts [79], and seems to be critical in the balance maintained between osteoblast and osteoclast activities in normal bone [80] as well as deregulations occurring in bone-related cancers [81]. Furthermore, P2X7 is expressed in some epithelial cells, and especially from exocrine glands. Its expression in submandibular gland acini and ducts, as well as its involvement in the salivary secretion in rodents is well documented [82,83]. P2X7 is also expressed in kidney cells [84], and specifically in basolateral membranes [85], however its physiological role remains elusive. Taken together, these different studies suggest

that P2X7 receptors seem to be mainly involved in the response to extracellular ATP in non-excitable cells.

As introduced, P2X7 also demonstrates structural, such as the carboxy-terminal end, and functional properties, such as the sensitivity to ATP or the membrane permeabilisation for cationic dyes induced by its stimulation, that are very unique among all P2X. Some of these functional particularities have been considered as being hallmarks of the P2X7 receptor, and were often used to characterise P2X7 expression or functionality in native cells or in heterologous expression systems. More than being solely hallmarks, we believe that they could be of biological relevance, and particularly in the context of the cancer progression. Furthermore the gene encoding for P2X7 is highly polymorphic and give many splice variants exhibiting both structural and functional particularities. These aspects are commented in the following sections.

3.1. Gene, splicing variants and single nucleotide polymorphisms (SNPs)

The human P2X7 is encoded by the *P2RX7* (purinergic receptor P2X, ligand-gated ion channel, 7) gene located on the locus 12q24.31. The *P2RX7* gene comprises 13 coding exons. Ten naturally occurring alternative splicing variants have been identified in humans and have been named P2X7A to J [86,87]. The P2X7A variant is the well-characterised full-length receptor [68]. Among the ten, five splice variants (P2X7B, P2X7C, P2X7E, P2X7G and P2X7J) are lacking the C-terminal intracellular tail of the receptor. The truncated P2X7B seems to display the same pharmacological properties, towards both agonists and antagonists, as the P2X7A and to be functional as an ion channel but it is unable to trigger membrane permeabilisation for large cationic molecules [86], a property referred to as being the pore formation and often associated with cell apoptosis. The P2X7I variant is due to a 5′-intronic splice site and is responsible for a null allele, that is ineffective in inducing membrane permeabilisation to fluorescent dyes such as ethidium [88].

The P2X7J variant is a polypeptide composed of 258 amino acid residues and compared to the full-length variant it lacks the distal 337 amino acids that correspond to the entire intracellular carboxyterminal end, the second transmembrane domain and the distal part of the extracellular loop. This variant, which is unable to induce pore formation, has been proposed to oligomerise with the full-length P2X7A receptor and to act as in dominant negative fashion [87]. This variant was identified in cervical cancer cells and was proposed to represent a new marker for cervical cancer or of defective apoptosis by preventing the cell death due to the stimulation of P2X7A by the ATP analogue 3′-O-(4-benzoyl)benzoyl-ATP (BzATP) [89].

Splice variants have also been identified in rodents. The first identified was termed P2X7k and is results from using alternative exon 1 in the rodent *P2XR7* gene. This variant is fully functional and bears a different N-terminal extremity and first transmembrane domain conferring an increased sensitivity to agonists, and slower deactivation kinetics compared to the human variants [90]. There are also two variants, P2X7 13B and P2X7 13C, that are truncated in the C-terminus, due to an alternative splicing in the exon 13. These two variants displayed a low membrane addressing, low channel function and no membrane permeabilisation [91].

Therefore, depending on the splice variants considered the biophysical properties of the receptor, its surface expression, as well as interacting molecules and downstream signalling pathways could be highly different, and this might be of importance in the cancer.

The human P2X7 receptor is highly polymorphic, and numerous single nucleotide polymorphisms (SNPs) have been identified in the last years. While a vast majority of these SNPs are located into intronic sequences, about 150 non-synonymous SNPs (NS-SNPs) have been reported. Some of these variants have drawn a lot of attention and were particularly studied following genetic association studies that proposed them as important genetic factors altering the susceptibility of individuals to various diseases [92,93]. Some of these NS-SNPs are responsible for alterations in functional properties of the human receptor, which

were mostly assessed by recording inward currents and/or dye uptake. The V76A, R117W, L191P, T357S, E496A or G150R, E186K, R276H, R307Q, I568N polymorphisms are responsible for partial or complete loss-of-function receptors, respectively, while the mutations H155Y, H270R and A348T are responsible for gain-of-functions [94–100].

Many of these polymorphisms were postulated to be involved in the aetiology of different pathologies [92], however, only the 1513A > C polymorphism (rs3751143), resulting in the loss-of-function E496A substitution was further studied in the context of the cancerous disease. It was initially proposed to be associated with familial B-cell chronic lymphocytic leukaemia (CLL) and to contribute to the pathology by inhibiting the P2X7-dependent apoptotic signalling pathway [101, 102]. Authors of these studies found that the 1513C allele frequency was significantly higher in familial CLL patients than in patients with sporadic CLL or in normal subjects coming from the Australian population [102] and proposed that the presence of this polymorphism could have a prognostic significance. However, such an association has not been supported by subsequent studies performed in Swedish [103] or British cohorts [104,105], and no correlation was found between the existence of this polymorphism in the *P2RX7* gene in CLL patients and their clinical outcome, characterised by the time between the first diagnosis of the disease and the initiation of chemotherapy [106]. Besides, the frequency of this polymorphism studied in samples coming from patients with multiple myeloma was not significantly different from that found in normal healthy subjects. There was no effect of the 1513A > C polymorphism on the clinical prognostic markers and survival in multiple myeloma [107].

Only two studies were performed to study the prevalence of SNPs in the P2X7 gene in solid tumours. The first one was performed in papillary thyroid cancer (PTC) in which the expression of two P2X7 polymorphisms was analyzed in lymphocytes from 121 patients. While the heterozygous expression of the 1513A > C polymorphism was not significantly different in the PTC patient group versus healthy subjects, it was found a very strong increase of the homozygous expression in patients suffering from the follicular histological variant of PTC. In detail, 13.6% of patients with follicular PTC were homozygous for the 1513C polymorphism compared with 2.6% of patients with the classical variant of PTC and 2% of healthy subjects. Furthermore, a positive correlation was identified between the 1513A > C polymorphism and TNM stage and overall tumour aggressiveness [108]. This would suggest that a loss-of-function of the receptor could be favourable for cancer progression.

The second study was very recently performed in the context of prostate cancer, and led to an opposite hypothesis. Indeed a genetic analysis was performed in the P2X7 gene for studying well characterised SNPs, and it was found a significant association of the 1513A > C polymorphism leading to a loss-of-function of the receptor in low grades of prostate cancers while the major allele seemed to be associated with aggressive stages [109].

3.2. General structure of the P2X7 receptor

The P2X7 receptor, like other P2X, is functional under a multimeric assembly of three subunits [110,111]. Each subunit bears a dolphin-like three-dimensional topology as predicted by the X-ray crystallography analyses performed on the zebrafish P2X4 receptor in the closed and ATP-bound open states [6,7] and subsequent structural modelling [69]. In line with other P2X, the P2X7 subunit consists of a large, glycosylated and cysteine-rich extracellular domain composed of 285 amino acids, two transmembrane-spanning helices called TM1 and TM2 domains, a short intracellular N-terminal domain and an intracellular C-terminal domain. The TM1 and TM2 domains in the human P2X7 comprise residues T28-S47 and N332-L354, respectively. One structural particularity of P2X7, being responsible for multiple functional specificities, is the fact that with 595 amino acid residues the P2X7 protein is significantly bigger than any other P2X subunit. This difference in the protein size is solely due to the C-terminal domain which is longer than those of all other P2X subunits by containing from 70 to 220 more residues [5].

The C-terminus of P2X7 has been implicated in regulating receptor function and sub-cellular localisation, but also in protein–protein interactions and the initiation of intracellular signalling cascades [112]. Of importance, it was demonstrated that the P2X7 C-terminus possesses a microdomain (P582-Y595) containing a canonical motif Yxxϕ (Y588-S589-G590-F591). This motif was critical for the receptor targeting to basolateral membranes of epithelial cells, and that its deletion or mutations of key amino acid residues induced the targeting to the apical membrane [85]. Since P2X7B and P2X7J variants are truncated in the C-terminus, they are devoid of this basolateral motif, and changes in their specific expression in epithelial cancer cells could interfere with epithelial cell physiology and could be responsible or associated with neoplastic transformation.

3.3. Sensitivity to ATP and allosteric modulation of P2X7

While the mammalian P2X7 orthologs exhibit important differences in their agonist sensitivity, the rat P2X7 being 3–10-fold and 30–100-fold more sensitive to ATP and BzATP, respectively, than the human [68,113] and mouse receptors [114], they all constitute the less sensitive P2 receptors to extracellular ATP. Residues and motifs involved in P2X7 sensitivity to both ATP and BzATP in the different mammalian orthologs were recently reviewed [69]. Experimentally, when acutely activated by exogenous ATP, hundreds of micromolars are required to record P2X7-mediated currents, Ca^{2+} increases or P2X7-related dye uptake [5] and there are only few biological conditions for which such high concentrations can be reached, the more likely being due to the local release of ATP content by dying cells or when ectonucleotidases are down-regulated [115]. Thus P2X7 was thought to mostly be activated in threatening situations such as infections and tissue injuries.

However, it has been proposed that P2X7 could also be allosterically modulated in different manners. In such conditions, the receptor could display a higher sensitivity and could potentially be activated in vivo in lower concentrations of ATP in the extracellular compartment. As an example, the mouse P2X7 can be activated by extracellular nicotinamide adenine dinucleotide (NAD). This activation of the receptor can be independent of, or in synergy with, ATP binding via ADP-ribosylation of the residue R125, catalyzed by the activity of ADP-ribosyltransferase 2 ecto-enzymes [116,117]. Residue R125 is proposed to be located in close vicinity with the putative ATP-binding site and ADP-ribosylation would favour the binding of the ADP moiety to this site and thereby activate the receptor [117]. The corresponding residue in the human P2X7 is also predicted to project towards the ATP-binding site supporting the hypothesis that ADP-ribose binds to the same site as ATP [118].

The antimicrobial peptide LL-37 coming from the maturation of cathelicidin hCAP18, the only cathelicidin found in humans, was also postulated to activate the P2X7 receptor through a mechanism that is not fully understood. Indeed, LL-37 was initially demonstrated to promote the processing and release of IL-1β from monocytes [119] and to prevent the apoptosis of neutrophils through the activation of P2X7 [120]. Later on, it was shown that LL-37 could activate human P2X7 receptors, as assessed as P2X7-dependent dye uptake and intracellular calcium increase, heterologously expressed in HEK293 cells and native receptors expressed in murine NIH3T3 fibroblasts and this way leading to an increase in cell proliferation [121]. Importantly, LL-37 exhibits a helix-forming propensity due to the specific distribution of positively and negatively charged residues allowing for the formation of intramolecular salt bridges [122]. Thanks to this helicoidal structure, LL-37 is postulated to insert deeply into, and to affect eukaryotic membrane integrity [123]. It was therefore suggested that the aggregation of LL-37 in the plasma membrane may be involved in the activation of P2X7 [121]. Interestingly, it has been shown that while LL-37 is present at the

surface of normal epithelial cells, it is highly over-expressed in both mRNA and proteins in different tumours such as breast, lung, prostate, ovary cancers and melanoma. Furthermore, it seems that it is synthesised by cancer cells and not by stromal cells, and that its expression is significantly higher in biopsies of high than in low histological grades of tumours [124–128]. LL-37 was shown to increase metastatic development in immunodeficient SCID mice inoculated with human cancer cells [129]. Conversely, LL-37 could participate in the activation of immune cells and thereby the anticancerous response of the organism [130,131]. These different properties have been postulated to be under the regulation of P2X7 receptors (see Section 5), either expressed by cancer or immune cells of the tumours. Thus, it can be postulated that LL-37 exerts its cancerous effects through the allosteric modulation of P2X7 receptors. Alternatively, it is possible that other allosteric modulators from the tumour microenvironment, not yet identified, could act on P2X7 to increase its sensitivity to ATP in vivo.

3.4. P2X7 receptor function: ion channel and pore dilatation

3.4.1. P2X7 receptor ion channel properties

Activation of P2X7, like all other members of the P2X receptor family, opens within milliseconds a transmembrane ion-conducting pathway that is selectively permeable to small cations such Ca^{2+}, Na^+ and K^+ [67,68]. As mentioned above, opening of the P2X7 receptor ion channel can mediate extracellular Ca^{2+} entry into the cell to elevate the intracellular Ca^{2+} concentrations, and also intracellular K^+ efflux or movement out of the cell. For ligand-gated Ca^{2+}-permeable ion channels like the P2X receptors including the P2X7, the ion channel functional properties can be studied using the patch-clamp recording technique to directly measure agonist-induced ionic currents through open channels (e.g., [113]). An alternative and widely-used means is using fluorescent Ca^{2+} indicators, such as Fura-2 or Fluo-4, to monitor agonist-induced increases in the intracellular Ca^{2+} concentrations resulting from extracellular Ca^{2+} influx and thereby indirectly characterise the Ca^{2+}-permeable ion channel functions (e.g., [132]). The ion channel properties of P2X7 have several biophysical properties that contrast with the other P2X receptors. A unique functional property of the P2X7 receptor ion channels is that they display no or little desensitisation in the presence of agonist even for several tens of seconds or minutes, in contrast with the complete desensitisation of the P2X1 and P2X3 receptors within a few seconds and the strong desensitisation of P2X2, P2X4 and P2X5 receptors during tens of seconds [5,132]. Also, the P2X7 receptor ion channels exhibit no discernible rectification, that is, the open channels permit ions to move into or out of the cell with similar easiness (e.g., [133]).

The most striking particularity of P2X7-mediated currents, compared to all other members of the P2X family [5] and to all other ligand-gated ionotropic receptors, is the absence of current desensitisation to agonist application. Instead, P2X7-mediated currents are incredibly increasing in amplitude upon repeated brief applications or sustained application of agonists, and this is associated with an increase in the sensitivity to the agonist and was called facilitation [133–135]. As detailed previously, a structural particularity of P2X7 compared to all other P2X members, is the existence of a 200 residues-long intracellular carboxyterminal end that is involved in both receptor functional regulations, by post-translational modifications, cellular localisation and protein interactions, and signalling pathway activation [112]. From the initial studies describing P2X7, it was demonstrated that the C-terminus was critical in regulating gating properties [67,68]. The offset (deactivation) kinetics of P2X7-mediated currents were also known, from the initial characterisation of the receptor, to be dependent on the C-terminus and to differ from rat to human orthologs [68]. It was also known for long that the P2X7 was the only P2X receptor to show such incredible current amplitude and onset kinetics increases during both prolonged and repeated agonist stimulations [67,68,136]. These increases in current amplitudes and activation

kinetics are also accompanied by an increase in the agonist sensitivity during the facilitation process [135]. However, insights into the molecular mechanism supporting such biophysical properties appeared quite recently and remain so far incomplete. It was demonstrated that the rat P2X7 presented both Ca^{2+}-dependent and Ca^{2+}-independent facilitation processes. Indeed the Ca^{2+}-dependent facilitation was due to the binding of Ca^{2+}-calmodulin (CaM) to a 17-residues-long 1-5-16 calmodulin binding motif between I541 and R557. Such a motif is different from the well-known "IQ" or "IQ-like" motifs as it is characterised as being Ca^{2+}-dependent, meaning that CaM needs to coordinate four Ca^{2+} ions at each of its EF-hands, before to bind to the motif. The combined substitutions I541T and S552C in the C-terminus of the rat P2X7 resulted in the complete loss of CaM binding to the receptor and the Ca^{2+}-dependent facilitation, without affecting the Ca^{2+}-independent facilitation or the increase in agonist sensitivity [135]. Interestingly, only the rat receptor, among all mammalian and non-mammalian P2X7 cloned up to now, seems to bear such a CaM-binding site. Indeed, the human receptor was shown to only possess an important current facilitation process that was Ca^{2+}-independent. However, when substituting three residues of the human C-terminus, T541I, C552S and G559V, a CaM-binding motif and a Ca^{2+}-dependent facilitation, similar to what is observed with the rat receptor, were introduced. This indicates that the change in human receptor conformation, following Ca^{2+}-CaM binding and responsible for the increase in current amplitude, was also possible [134]. The molecular process responsible for the Ca^{2+}-independent facilitation is still unknown but seems to be independent on the receptor addressing to the membrane. However, another domain of the C-terminus, close to the TM2 and composed of 18 amino acids (from C372 to V389) and containing 6 cysteine residues, was described to be determinant. This cysteine-rich domain is highly conserved between species, and its deletion in the rat sequence resulted into the complete loss of facilitation. This mutant also showed higher agonist sensitivity and slower deactivation kinetics compared to the WT receptor [134]. Thus, it is speculated that the increase in agonist sensitivity during current facilitation is correlated to the Ca^{2+}-independent process, found in all orthologs. It could also be postulated that this cystein-rich domain acts as a hinge region for the control of conformational changes of the receptor during both Ca^{2+}-dependent and Ca^{2+}-independent facilitation. This same region was also demonstrated to be critical in receptor dilatation and permeability to N-methyl-D-glucamine (NMDG), but not in the dye uptake [137]. A separate study describes such increased current responses as sensitisation, and proposes that it results from the difference in the conductive state of the ion channel arising from the different number of ATP molecules bound to the P2X7 receptor [138].

While the molecular mechanisms involved in P2X7 facilitation/sensitisation are not fully characterised, such a process, along with the absence of desensitisation, must be taken into account when thinking on the biological functions of P2X7 in vivo. Indeed, this could explain an activity of the receptor in vivo under low but constant concentrations of extracellular ATP that could be released through an autocrine/paracrine mechanism, and not only it would maintain but exacerbate plasma membrane depolarisation and signalling pathways dependent on P2X7 activation.

3.4.2. P2X7 receptor activation-induced pore formation

P2X7 is perhaps best known for its capacity to induce a phenomenon called membrane permeabilisation that refers to the formation of large pores that allow passage of large cationic molecules up to 900 Da across the plasma membrane [67,68]. Due to this unique functional property, this receptor was initially named P2Z receptor to separate from other P2X receptors before molecular cloning [139]. The large pores appear after the activation of P2X7 for tens of seconds to minutes. The time-course or kinetics of the large pore formation or dilation can be determined using patch-clamp recording in bi-ionic extracellular and intracellular solutions; upon prolonged application of agonist, there is a

progressive increase in the permeability of the plasma membrane to organic cations, such as NMDG [67,68,137], which are much larger in size than the physiological cations such as Ca^{2+}, Na^+ and K^+. The large pore formation can be demonstrated under more physiological conditions by monitoring progressive efflux of preloaded fluorescent dyes, for example, calceine [140], and more commonly, accumulation of extracellular fluorescent dyes, such as YO-PRO-1 (e.g., [141]) and ethidium (e.g., [98]), inside the cells during receptor activation. The latter is widely referred to as fluorescent dye uptake.

Two fundamentally distinctive pore-forming mechanisms have been proposed to explain how the large pore forms following P2X7 receptor activation [5]. The first and widely recognised mechanism is that separate protein(s) constitute the NMDG-permeable or dye uptake pores and the pores open upon activation of P2X7 via physical interactions or conformational coupling. There is evidence to support the hemichannel composed of pannexin-1 proteins as being the pore-forming unit [140,142–144]. The second mechanism entails that the large pore is an intrinsic functional property of the P2X7 receptor, resulting from progressive dilatation of the small ion-conducting pathway. Such a mechanism offers single and satisfactory interpretations of the remarkable effects on the large pore-forming capacity and dynamics induced by diverse structural difference or disturbances [67, 90,145,146]. This is also supported by a recent study showing that P2X7 receptor ion channels carrying mutations in the ion-conducting pathway are able to pass molecules with sizes up to 14 Å [133]. Therefore, both mechanisms have gained strong support by various studies so far. It is worth pointing out that these mechanisms are mutually exclusive and in fact, a multiple of pore-forming mechanisms have been proposed [147]. It is also worth mentioning that formation of large pores occurs after activation of some other homomeric or heteromeric P2X receptors, including P2X2, P2X4, P2X2/3 and P2X2/5 [148–150] and via different pore-forming mechanisms. For example, there is evidence to suggest no major role of pannexin-1 channel in the pore formation induced by activation of the recombinant P2X2 receptor in HEK293 cells [151] or endogenous P2X4 receptor in microglia [152].

3.5. P2X7 pharmacology and ligands: potential cancer therapeutic strategies?

The first three mammalian P2X7 receptors were molecularly identified in 1996–1997 [67,68,136]. Compelling evidence emerged from subsequent studies using transgenic P2X7 deficient mice and derived cells supports an important role for the P2X7 receptor in mediating ATP-induced generation of IL-1β, a key proinflammatory cytokine, and in the pathogenesis of arthritis and chronic pain [153–155]. These findings prompted huge interest in search for potent and selective P2X7 receptor ligands. ATP and BzATP still remain the agonists available up to date. As mentioned above, BzATP, an ATP synthetic analogue, exhibits a greater potency at the P2X7 receptor than ATP with an opposite order of potency at the other P2X receptors [13]; such a unique agonist profile has been widely used to indicate functional expression of P2X7 receptors, but both ATP and BzATP are not useful therapeutic agents, because of their rapid degradation by ubiquitously expressed and membrane-delimited enzymes, such as ecto-nucleoside triphosphate diphosphohydrolases. In contrast, several potent P2X7 antagonists have been discovered over the past decade, some of which show striking selectivity among P2X7 receptors of different mammalian species [69]. KN-62, initially identified as a Calmodulin-kinase II inhibitor [156], was also characterised as being the first potent human P2X7 selective antagonist versus other P2X receptors with an IC_{50} of 40–100 nM, to which the rat P2X7 receptor is largely insensitive [157]. Brilliant blue G (BBG) is the first potent rat P2X7 selective antagonist with an IC_{50} of 10 nM, which is slightly less potent at the P2X7 receptor of other species origins [158]. Systemic administration of BBG in rodents reduced tissue loss and improved motor function following spinal cord injury [159], and prevented development of morphine tolerance [160],

suggesting that pharmacological intervention of P2X7 receptor is a promising therapeutic approach. A number of medicinal chemistry programmes have led to discovery of several series of drug-like compounds with distinctive structural properties as highly selective human P2X7 antagonists with nanomolar potency. Representative examples shown in Fig. 1 include A438079 and A83997 (tetrazole or trizole-based compounds); A740003, A759029 and A804598 (cyanoguanidine derivatives); AZ10606120 (an adamantane amide); AZ11645373 (a cyclic imide); and CE-224535 (an azauracil derivative).

As discussed above, prolonged activation of P2X7 receptors causes cell death and there is some evidence suggesting such a cytolytic effect may bring therapeutic benefits to certain cancerous conditions such as human hematopoietic malignancies including chronic lymphocytic leukaemia (CLL) [161]. Heterologous expression in K562 cells of the N187D mutant P2X7 receptor, identified in human J6-1 leukaemia cells, accelerated cell proliferation and reduced BzATP-induced cell death [162]. Moreover, the tumours developed faster, with the size and weight becoming larger and heavier, in immune-deficient mice implanted with cells expressing the mutant receptor. These observations suggest enhancing P2X7 receptor activity may slow down the pathogenesis and progression of CLL and thus be beneficial.

Our recent studies, on the other hand, have shown that selective inhibition using KN-62 and A740003 of the human P2X7 receptor endogenously expressed in the highly invasive breast cancer MDA-MB-435 s cells and human lung cancer A549 cells prohibited ATP-induced cell migration and particularly cell invasiveness in vitro and in vivo [132,163]. In addition to synthetic chemicals developed by medicinal chemistry, active compounds from traditional remedies prepared using plants and other natural sources could be used in therapies. For example, emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an anthraquinone derivative, extracted from the rhubarb *Rheum officinale Baill* that has been used in Chinese traditional medicine for centuries, and is known to exhibit antitumour properties as well as anti-inflammatory and immunosuppressive properties, albeit the underlying mechanisms appearing multiple [132,164–166]. Our initial study showed that emodin is a potent P2X7 antagonist, inhibiting ATP-induced macrophage cell death [167]. In the following study, we have further demonstrated that emodin suppressed the invasiveness of breast MDA-MB-435 s and lung A549 cancer cells through antagonism of the human P2X7 receptor [132]. These studies provide clear evidences to indicate an important role for the P2X7 receptors in mediating cancer cell metastasis, the major cause for high mortality. These studies also showed proof of concept that pharmacological inhibition of the P2X7 receptors as a promising therapeutic strategy. P2X7 antagonists have been tested in clinical trials for treatment of rheumatoid arthritis, osteoarthritis, chronic obstructive pulmonary disease and inflammatory bowel disease. It is clear that it becomes interesting to test in pre-clinical studies and clinical trials P2X7 antagonists, which have proved to be clinically safe such as CE-224535 and emodin, in treatment of some epithelial cancers, such as breast cancer.

4. Expression and functional properties of P2X7 receptors in solid tumours

In most types of cells endogenously or heterogeneously expressing P2X7, the pharmacological activation of the receptor using high concentrations of ATP or BzATP was generally associated with dramatic cellular events such as membrane permeabilisation and blebbing, loss of asymmetric distribution in phosphatidyl serine, cell swelling, increase of internal Ca^{2+}, loss of mitochondrial potential [168,169]. These entire phenomena were demonstrated to be reversible when the agonist applications were brief, and were associated to what was called "pseudoapoptosis". However, these events were eventually leading to cell death upon a prolonged stimulation (more than 30 min) [169]. This latter aspect was reminiscent to the initial properties associated with the cytolytic function of extracellular ATP due to this same receptor
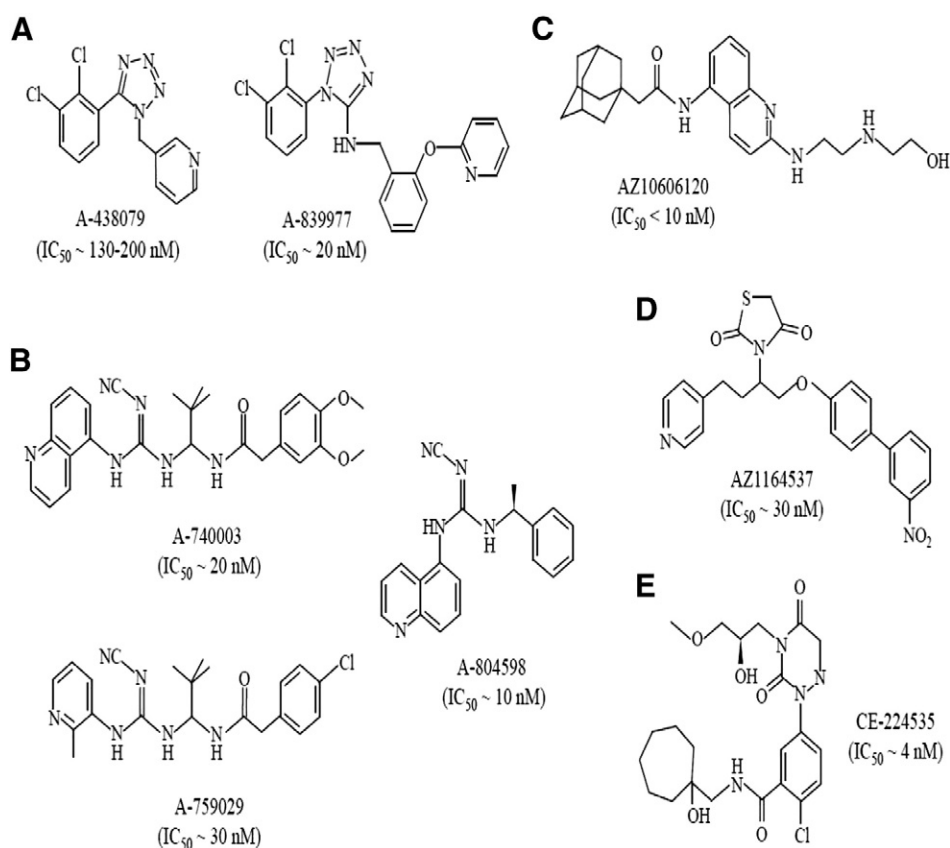
Fig. 1. Chemical structures of representative small molecular weight P2X7 receptor selective antagonists developed from medicine chemistry. (A), tetrazole/trizole-based compounds A-438079 and A-83997. (B), cyanoguanidine derivatives A-740003, A-759029 and A-804598. (C), adamantane amide AZ10606120. (D), cyclic imide AZ11645373. (E), azauracil derivative CE-224535. The concentration inhibiting by 50% agonist-induced human P2X7 receptor responses (increases in the cytosolic Ca^{2+}, ionic currents or fluorescent dye uptake) or IC_{50} value is shown in brackets.

[170–173]. These particularities initially led to formulate the hypothesis that P2X7 over-expression could be deleterious for cells [67,169,174, 175] and that stimulating its activity could represent new clinical therapies for treating cancers [50].

During the last decade, multiple studies assessed the expression levels of P2X7 in different cancer biopsies and cancer cell lines (See Table 1). P2X7 expression seemed to be decreased in some studies while others reported a very high increase of mRNA, proteins, or both in several tumours compared to corresponding normal tissues. However, the full functional characterisation of the receptor was very often questioned and rarely provided, thus limiting the hypotheses on its participation in oncogenic processes.

4.1. Expression levels in solid tumours and association with tumour grade/tumour progression

4.1.1. Breast cancer

There are contradictory results in assessing the expression of P2X7 in both biopsies and cell lines. An initial study on breast cancer was performed to identify the protein expression of P2X7 by immunohistochemistry (IHC) on 40 haematoxylin and eosin (H&E) stained samples of different histological categories including normal, hyperplastic tissues as well as lobular carcinoma in situ, ductal carcinoma in situ, invasive lobular and invasive ductal carcinoma [176]. Normal and hyperplastic breast epithelial samples were completely devoid of P2X7 expression, while all studied cases of in situ or invasive lobular or ductal carcinoma were intensely labelled. However, only cancer cells from invasive carcinomas had membrane labelling of P2X7, while cancer cells from in situ carcinoma seemed to only show intracellular immunoreactivity. While this initial study was very important, it was not specified

what molecular part of the receptor was used as an antigen for the antibody used. As such it is not clear whether the IHC signal obtained is referring to one or multiple P2X7 variants, and particularly if this antibody was also recognizing the C-terminus truncated variants. Although the functionality of P2X7 was not assessed in invasive carcinomas, authors were still under the influence of the cytolytic hypothesis on the cellular role of the receptor. They postulated that "an attempt was being made to induce apoptosis" [176], suggesting in a deterministic evolutionary conception, that following the carcinogenetic transformation cancer cells have initiated the apoptotic process, but unsuccessfully. However, authors proposed that P2X7 expression could be used in order to identify and differentiate early stages of breast cancers from hyperplastic lobules [176].

Correlatively to the initial IHC, some studies proposed that P2X7 could be over-expressed during the breast cancer initiation or progression and become functional at the plasma membrane of cancer cells. Indeed, the expression of both mRNA and proteins for P2X7 was identified in the highly invasive human breast cancer cell lines MDA-MB-435s, while it was very weakly or not expressed in non-cancerous mammary epithelial cells HMEC, MCF-10A and 184A1 [132,163]. Furthermore, P2X7 was found to be fully functional at the plasma membrane of MDA-MB-435 s cells, giving rise to ATP-induced inward currents, increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) and ethidium uptake, that were prevented by antagonists of P2X7 or by specific small interfering RNA [132,163]. It was indicated that the variant expressed was the full length P2X7A. However, the expression of other variants was not addressed. In line with this hypothesis, P2X7 proteins were also detected in highly invasive MDA-MB-231 breast cancer cells [177]. In weakly invasive MCF-7 cancer cells, messengers RNA were amplified and while proteins could be detected in Western

Table 1

P2X7 expression, functionality and postulated roles in solid tumours.

Expression of P2X7 mRNA was assessed by PCR, and P2X7 proteins by immunohistochemistry (IHC), western blotting (WB) or Flow cytometry (FC).

Cancer types	Samples: biopsies/cells (h, human; m, mouse; r, rat)	Expression (mRNA, proteins, variants)	Functionality of P2X7 at the plasma membrane	Cellular effects of P2X7 agonist/antagonists	Postulated role	References
Breast cancer	Human biopsies of in situ or invasive lobular or ductal breast carcinoma	Protein (IHC)	Not assessed	–	Early marker	[176]
	Human lobular or ductal breast carcinoma	Protein (IHC)	Not assessed	–	Apoptosis	[178]
	MDA-MB-435 s (h)	mRNA, proteins (WB), P2X7A.	Current, [Ca^{2+}]i, dye (WB), P2X7A.	Yes (ATP/KN62, A438079, A740003, emodin)	Cancer cell migration and invasiveness	[132,163]
	MDA-MB-231 (h)	Proteins (WB)	Not assessed	Not assessed	Apoptosis	[179]
		Proteins (WB)	Not assessed	Yes (BzATP /−)	Invasion	[177]
		mRNA, protein (WB)	Not assessed	Yes (ATPγS/oATP)	Inhibition of migration and growth	[219]
	MCF7 (h)	mRNA	No current	Not identified	Not identified	[34]
		Proteins, variant?	Not assessed	Not assessed	Apoptosis	[179]
	Py8119 (m)	mRNA	Not assessed	Yes (ATPγS /−)	Inhibition of migration and tumour growth	[219]
Breast cancer	Human cancer biopsies	Protein (IHC)	Not assessed	–	Early marker	[180] [181]
	Laser-capture microdissected biopsies	mRNA, proteins (IHC, WB)	Not assessed	–	Cancer marker	[182]
	PC-3 (h)	mRNA, proteins (WB)	Not assessed	Not assessed	Not identified	[212]
	PC-3 (h), DU-145(h)	–	Not assessed	Yes (ATP/KN-62)	Cancer cell invasiveness	[109]
	PC-3(h)	mRNA	No lucifer yellow uptake	Yes (ATP/−)	Not identified	[241]
Lung cancer	A549 (h), PC-9(h), H292 (h)	mRNA, protein (WB)	Ethidium uptake	Yes (ATP, BzATP/A438079, emodin)	Cancer cell migration and invasiveness	[132,183,184]
Pancreatic cancer	Human biopsies	mRNA, protein (IHC)	Not assessed	Not assessed	Not identified	[185]
Papilliary thyroid cancer (PTC)	Human PTC biopsies	mRNA, protein (IHC, WB)	Not assessed	–	Trophic activity through IL-6 release	[186]
	FB1 (h), FB2 (h)	Protein (IHC)	[Ca^{2+}]i	Not assessed	Cancer aggressiveness	[186]
	Human PTC biopsies	Protein (IHC)	Not assessed	(ATP, BzATP/oATP, KN-62)	Poor prognosis	[188]
	Human PTC biopsies	Full-length protein (IHC)	Not assessed	–	–	[189]
	Human PTC biopsies		Not assessed	–		[178]
Skin cancers	Nonnmelanoma					
	Necrotic/aptotic areas of basal cell and suqmous cell carcinomas	Protein (IHC)	Not assessed	–	Apoptosis	[190]

	A431 (h)	Protein (IHC)	Not assessed	Yes (ATP, ATPγS, BzATP)	Apoptosis	[190]
	Melanoma					
	Human biopsies	Protein (IHC)	Not assessed	–	Apoptosis	[191]
	Human biopsies	Protein (IHC), P2X7A?	Not assessed	–	Apoptosis	[30]
	A375 (h)	mRNA, protein (IHC), P2X7A?	YO-PRO-1 uptake	Yes (BzATP/KN-62)	Apoptosis	[30]
	Skmel28 (h), Bris (h), HBL (h), 518A2 (h)	mRNA	Not assessed	Not assessed	Not identified	Fig. 2, this review
	B16 (m)	mRNA, protein (WB)	Ethidium uptake	Yes (ATP/A438079, oATP)	m	[192] [204]
	B16 (m)	mRNA, P2X7a variant	Ethidium uptake	Yes (ATP/—)	Cell death	[193]
	B16 (m)	mRNA, protein (WB)	Not assessed	Yes (ATP,BzATP / KN-62)	Apoptosis	[218]
Brain cancers	*Neuroblastoma*					
	Human biopsies	Protein (IHC), P2X7A?	–	–	–	[42]
	GI-CA-N (h), HTLA-230 (h), GI-ME-N (h), LAN-5 (h), LAN-1 (h), SK-N-BE-2 (h) and SH-SY-5Y (h)	Protein (IHC), P2X7A?	Not assessed	Not assessed	–	[42]
	ACN (h)	Protein (IHC), P2X7A?	[Ca^{2+}]i, YO-PRO-1 uptake	Yes (ATP, BzATP / KN-62)	Substance P-dependent proliferation	[42]
	Neuro-2a (m)	mRNA, protein (WB)	[Ca^{2+}]i	Yes (ATP / oATP, BBG, A438079)	Cell survival	[205]
	Neuro-2a (m)	mRNA, protein (WB, IHC)	Current, [Ca^{2+}]i	Yes (ATP, BzATP /KN-62, BBG, A438079)	Undifferentiated proliferative phenotype	[206]
	Glioma					
	C6 (r)	mRNA, protein (WB, IHC)	[Ca^{2+}]i, ethidium uptake	Yes (BzATP/oATP)	Migration	[195]
	C6 (r)	mRNA, protein (WB, IHC)	[Ca^{2+}]i	Yes (BzATP/oATP)	Suppression of P2X7 promotes gowth through EGFR induction	[196]
	GL261 (m)	mRNA, protein (WB)	Propidium iodine uptake	Yes (ATP, BzATP / oATP)	Cell death	[197]
	U-138MG (h), U-251MG (h)	mRNA, protein (FC)	Ethidium uptake	Yes (ATP, BzATP / A740003)	Not identified	[194]
	M059J (h)	mRNA, protein (FC)	Ethidium uptake	Yes (ATP, BzATP / A740003)	Increase in radiosensitive cell death	[194]
Colorectal cancers	Human colon adenocarcinoma	Protein (IHC)	Not assessed	Not assessed	Not identified	[178]
	HCT8 (h), Caco-2 (h)	mRNA, protein (WB, IHC)	[Ca^{2+}]i	Yes (ATP, BzATP / oATP)	Apoptosis	[178]
	MCA38 (m)	mRNA, P2X7a variant	Ethidium uptake	Yes (ATP, BzATP / KN-62)	Cell death	[193]
	CRC MC-26 (m)	mRNA, proteins	Not assessed	Not assessed	–	[199]
Uterine cancers	Cervix and endometrial cancer biopsies	Reduced expression of mRNA, proteins (WB, IHC)	–	–	–	[178,201,202]
	Cervical biopsies, CaSki (h), HeLa (h), SiHa (h), HT3 (h)	Dominant-negative P2X7J	Not assessed	Not assessed	protection against apoptosis	[87,89]
Ovarian cancer	Human ovarian biopsies	Protein (IHC)	–	–	Cell survival	[200]
	SKOV-3 (h), CAOV-3 (h)	Protein (IHC)	[Ca^{2+}]i	Yes (ATP, BzATP / A438079, AZ10606120)	Cell survival	[200]

blotting experiments [177], the stimulation of cells with high concentrations of ATP was ineffective in inducing P2X7-related inward currents suggesting that the receptor could not be functional at the plasma membrane [34].

In contrast with these studies, an IHC study performed with 26 breast carcinoma samples, 13 ductal carcinomas and 3 lobular carcinomas indicated a tendency for a reduced expression of the full-length P2X7 in cancer biopsies [178]. Another recent study examined the protein expression of P2X7 by IHC, using an antibody recognizing the carboxyterminal end of the receptor, in 80 paired-samples breast biopsies and found that P2X7 immunostaining was reduced in cancer tissues compared to adjacent non tumour mammary tissues [179]. In the same study, P2X7 proteins were however identified in both weakly MCF-7 and highly aggressive MDA-MB-231 cancer cells, but its expression was proposed to be reduced, similarly as in cancer biopsies, by the over-expression of the mature oncogene miRNA miR-150 [179].

4.1.2. Prostate cancer

An initial study of a cohort of 116 biopsies, obtained from men aged from 47 to 86 years, and confirmed to be cancerous in the range of the Gleason sum score 4–9, assessed the expression of P2X7 proteins by IHC [180]. It was found that all cancer biopsies were positive for P2X7 expression, with no correlation with the age of patients or with the Gleason score. P2X7 was also identified in non-cancerous epithelial cells adjacent to the margins of the tumour; however the labelling seemed to be distinct depending on the stage/vicinity of cells to the tumours. Indeed P2X7 appeared to be expressed in the nucleus of non-cancerous cells, then into the cytoplasm and finally on the apical membrane of cells progressing into the cancer phenotype. In comparison, P2X7 was totally absent from prostate tissues coming from patients with no evidence of cancer. These results raised the possibility that P2X7 appears during the cancerous progression [180]. In a subsequent study, the same team assessed by IHC the expression of P2X7 and compared this to levels of the prostate-specific antigen (PSA) in prostate cancer biopsies from 174 patients. They found a positive correlation between the P2X7 expression and increasing levels of PSA in patients who were later on diagnosed to be cancerous by H&E staining. These results further sustained the hypothesis by which the expression of P2X7 could represent an early marker for the diagnosis of prostate cancer [181]. In these two initial studies, it is not clear whether the P2X7 variant expressed is the full-length P2X7A or another alternative splicing variant, and its functionality was not assessed. While it is possible that P2X7 is functional at the plasma membrane of prostate cancer cells, the authors still characterised the receptor as being "apoptotic" and suggested that it might be non-functional.

Ravenna and collaborators assessed the mRNA and protein expression of different markers generally associated with inflammation, in both cancer and non-cancer prostate samples coming from laser-capture microdissection of biopsies and showing no or low leukocyte infiltrate. They found that P2X7 was highly up-regulated in cancer cells compared to non–cancer cells from the adjacent host tissue and that this was correlated to the over-expression of cancer proliferative receptors Epidermal Growth Factor Receptor (EGFR) and Estrogen Receptor (ER)α. Authors suggested that a whole pattern of proteins usually associated with inflammatory or reparative functions are up-regulated in cancer cells of transformed tissues, devoid of detectable leukocyte infiltrate, and could contribute to the cancer progression [182].

4.1.3. Lung cancer

To date there is no paper assessing the expression of P2X7 in cancer versus non-cancer lung biopsies with the aim of studying possible correlations with cancer progression and clinical grades. However, there are several in vitro studies relating the expression of P2X7 in human lung cancer cells, such as in A549, PC-9 and H292 but not in non-cancerous BEAS-2B bronchial epithelial cells [132,183,184], suggesting that P2X7 could be up-regulated during the malignant transformation of lung epithelial cells.

4.1.4. Pancreatic cancer

The mRNA expression of P2X7 and the presence of proteins were analysed in chronic pancreatitis (samples from 11 patients) and pancreatic cancer (samples from 28 patients) and compared to normal pancreas tissues. P2X7 was significantly up-regulated at the mRNA level in biopsies of chronic pancreatitis compared to normal pancreas and to pancreatic cancer. At the protein level, P2X7 was not significantly increased in both pancreatic cancer and chronic pancreatitis biopsies compared to normal pancreas. In conclusion, P2X7 was not significantly overexpressed in pancreatic cancer, and seemed to be mainly expressed by infiltrating leukocytes in the pancreas [185].

4.1.5. Papillary Thyroid Cancer (PTC)

In an initial study performed on the thyroid cancer, the mRNA for P2X7 was found to be about two times more abundant in 37 human papillary thyroid cancer samples when compared with contralateral non-cancer tissues. Furthermore, immunochemistry was also performed with anti-P2X7 antibodies and P2X7 proteins were shown to be strongly expressed in all cancerous tissues tested, both in classical and in follicular histological forms of the cancer, while they were not present in normal thyroid tissue of the same individual. Malignant thyrocytes showed a diffuse P2X7 labelling into the cytoplasm as well as intense labelling at the cell periphery. P2X7 mRNA and functional proteins were also expressed in FB1 and FB2 human thyroid cancer cell lines in which stimulation by both ATP and BzATP enhanced the release of the IL-6 [186]. IL-6 local expression is related to the aggressiveness of PTC [187] and P2X7 could therefore participate in the development of such a cancer phenotype. The over-expression of the full-length P2X7 in PTC was confirmed in another IHC study performed with 4 samples [178]. In a subsequent IHC study performed with 43 PTC samples compared to 19 samples coming from nodular goiter, it has been demonstrated that the expression of both the X-linked inhibitor of apoptosis (XIAP) and the P2X7 was increased in PTC samples. Furthermore, P2X7 expression was associated with an increased in the tumour size as well as with capsular infiltration and lymph node metastases. Authors of the study proposed that P2X7 may represent a predictor of the aggressiveness of PTC [188]. Recently, P2X7 protein expression was studied by IHC in 170 biopsies of PTC patients, 84 of which having the chronic autoimmune thyroid disease Hashimoto's thyroiditis (HT) and the other 86 without HT. The expression of P2X7 was significantly higher in the absence of HT and correlated with poor prognostic factors such as tumour multifocality, lymphovascular invasion and extra-thyroid extension [189]. In conclusion, P2X7 seems to be over-expressed in PTC and associated with the aggressiveness of the disease.

4.1.6. Skin cancers

Skin cancers are histologically subdivided into non-melanomas, which comprise basal cell and squamous-cell carcinomas, and melanomas. Squamous cell carcinomas are more frequent and more aggressive than basal cell carcinomas since they can give rise to distant metastases. Melanoma is the more dangerous and aggressive skin cancer, which initiates after the cancerous transformation of melanocytes and has a high potential for metastasis.

4.1.6.1. Basal-cell and squamous-cell carcinomas.
The expression of P2 receptors was assessed by IHC analysis in human biopsies of basal cell and squamous cell carcinomas, and P2X7 was found to be expressed, especially in the necrotic center of nodular basal cell carcinomas and in apoptotic cells in superficial multifocal and infiltrative basal cell carcinomas. P2X7 is also expressed in the human A431 squamous cell carcinoma cell line [190] and, while its functionality was not demonstrated, stimulation with high doses of ATP, ATPγS and BzATP reduced cell

numbers in vitro [190]. With these results, authors claimed that P2X7 was involved in the ATP-mediated apoptosis in cancer cells.

4.1.6.2. Melanoma. In an IHC study performed on 80 biopsies coming from patients with superficial spreading melanomas, the P2X7 protein was reported to be over-expressed in cancer versus non-cancer samples. This increase in P2X7 labelling was also extended to 2 μm layer of non-cancerous keratinocytes of the epidermis surrounding the tumour [191]. Another study performed in 14 specimens of human melanoma with an antibody recognizing a C-terminal epitope in P2X7, came to confirm these initial results. Indeed, it showed that all melanoma samples expressed P2X7, and over 75% of cancer cells in the biopsy were stained positively [30]. Again, these results could suggest that P2X7 is over-expressed during the neoplastic transformation of keratinocytes. Authors of these two separate studies indicated P2X7 as a potential melanoma marker. The receptor was associated with apoptosis [30] and was even proposed that "melanoma cell proliferation overwhelmed the apoptotic defence" [191].

Data obtained from our group indicated that mRNA expression of P2X7, among other P2X, can be detected by RT-PCR in Skmel28, Bris, HBL, 518A2 human melanoma cell lines (Fig. 2). The presence of proteins and their activity at the plasma membrane was not assessed so far. P2X7 mRNA and proteins were also identified in human melanoma cell lines such as the A375 [30], and in the murine B16 melanoma cell line [192] in which the variant expressed is P2X7a and not the P2X7k variant [193] that exhibits a higher ligand sensitivity [90].

4.1.7. Brain cancers

4.1.7.1. Neuroblastoma. Neuroblastomas are malignant tumours of the childhood that mainly originate from embryonic nerve cells in the sympathetic nervous system. In 2006, it was shown, by IHC using an antibody targeting the last 20 amino acids of the receptor, that P2X7 proteins were expressed in neuroblastoma primary tumours. The vast majority of cancer cells in the tumour displayed an intense labelling. The number of the samples analyzed was moderate but the majority of tumours showed high P2X7 expression irrespectively of the histological grade of the disease (localised versus metastatic). P2X7 proteins were also detected in the cytosol of numerous human neuroblastoma
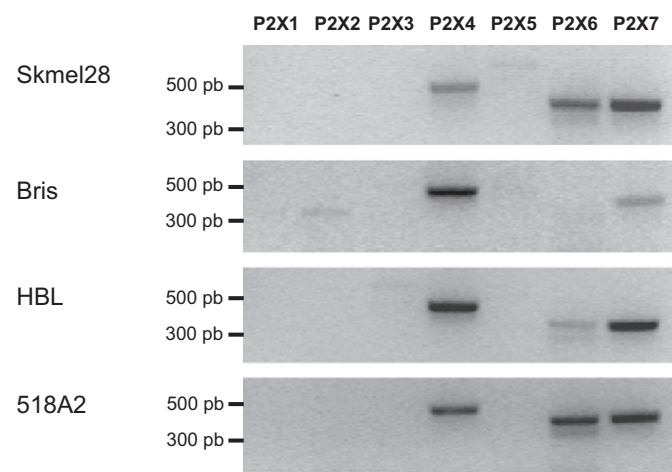

Fig. 2. Expression profile of P2 receptors in human melanoma cancer cell lines. Representative RT-PCR experiments analysing the expression of mRNA for all the P2X receptors, and showing the expression of P2X7 in Skmel28, Bris, HBL and 518A2 melanoma cells. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum. Reverse transcription-PCR experiments were done using standard protocols and PCR primers indicated in our previous publication [119]. Briefly, the temperature profile for PCR was 4 min at 94 °C followed by amplification for 40 cycles, each consisting of 1 min at 94 °C, 30 s at 60 °C, and 1 min at 72 °C and a final extension for 2 min at 72 °C. PCR products were analyzed by electrophoresis in 1.8%-agarose gels containing ethidium bromide, and visualised by UV trans-illumination.

cell lines such as ACN, GI-CA-N, HTLA-230, GI-ME-N, LAN-5, LAN-1, SK-N-BE-2 and SH-SY-5Y, and at the plasma membrane of at least ACN cells [42]. Even though more characterisations are needed, it can be nonetheless postulated that this immunostaining is representative of the expression of a non-C-terminus truncated variant, such as the P2X7A.

4.1.7.2. Glioma. Gliomas are malignant primary tumours of the central nervous system, deeply infiltrating adjacent tissues in the brain, and patients bearing these tumours have a very poor prognosis. The principal therapy actually used for the management of disease is the surgical resection of the tumour, and there are attempts in improving the efficacy of other treatments such as radiotherapy by manipulating the purinergic signalling. In this context there are several in vitro experiments that have been performed in either human or rat glioma cell lines. As such P2X7 is expressed, in mRNA and proteins, in human U-138MG, U-251MG and M059J, in rat C6 and in mouse GL261 glioma cells [194–197]. To date, there is no study comparing the levels of P2X7 in human glioma versus non-glioma samples.

4.1.8. Colorectal cancers

In an IHC study performed with a limited number of human samples, full-length P2X7 immunoreactivity was identified in the basolateral side of the normal epithelium, while in colon adenocarcinoma, the immunoreactivity was distributed throughout the cells. In all the five cases studied, P2X7 levels were similar between cancer cells and normal cells [178]. P2X7 was also identified in human HCT8, Caco-2 and murine MCA38 (P2X7a but not k variant) and CRC MC-26 colorectal cancer cell lines [193,198,199].

4.1.9. Ovarian cancer

In a recent study, P2X7 proteins were identified by IHC to be in similar levels in biopsies from non-cancerous and cancerous human ovarian biopsies [200]. This study also showed functional expression of P2X7 at the plasma membrane of SKOV-3 and CAOV-3 ovarian carcinoma cells [200].

4.1.10. Uterine cancers

Uterine cancers certainly represent the cancer types for which there is the less ambiguity so far. Indeed, multiple publications, coming from the team of G. Gorodeski, demonstrated that mRNA and proteins for the full-length P2X7 were expressed in epithelial cells of normal uteri such as endometrial, endocervical and ectocervical tissues, and was reduced in cervical squamous cell carcinomas as well as in endometrial and endocervical adenocarcinomas. Furthermore, there was a reduced expression of P2X7 in hyperplastic pre-cancerous endometrium compared to normal endometrium. These observations have led the authors to propose that P2X7 expression could be used as a biomarker for uterine epithelial cancers [178,201,202]. The reduction in the P2X7 expression was claimed to result from the over-expression of micro RNA miR-186 and miR-150 in cancer cells [203]. Furthermore, the dominant negative P2X7J variant truncated in the C-terminus, was identified in cervical and normal cancer cells. The expression levels of P2X7J were very similar in normal and cancer cells, but the expression of P2X7A was higher in normal cells. Authors proposed this P2X7J variant to represent a new marker for cervical cancer or of defective apoptosis by imposing a dominant negative inhibitory effect on P2X7A and thereby preventing the cell death due to the stimulation of P2X7A by BzATP [87,89].

5. Role of P2X7 receptors in determining tumorigenesis

5.1. P2X7 receptors in cancer cells

As indicated in the previous section, P2X7 is often found to be expressed in cancer biopsies and cancer cells, and multiple studies have assessed the cellular properties related to cancer progression or

prevention the receptor could be associated with or even trigger. However, depending on the cell types or the ways by which P2X7 were studied, the results appeared to strikingly different and sometimes apparently opposite indicating that P2X7 could be pro-cancerous or even anti-cancerous. A detailed analysis of the method used could be informative to highlight apparent discrepancies.

5.1.1. Pro-cancerous activities of P2X7 in cancer cells

5.1.1.1. Promoting cell survival, proliferation and tumour growth. In murine melanoma B16 cells, P2X7 was found to be responsible for the extracellular release of ATP in acidic conditions (pH 6.4) mimicking the acidosis found in the core of solid tumour as a result of the hypoxia. This release resulting in extracellular ATP in the 10 nM range, which was prevented by pharmacological antagonism using A438079 or by expressional repression of P2X7 using small interfering RNA (siRNA), was responsible for an increase in cancer cell proliferation in vitro. The increase in cell proliferation was due to another purinoreceptor than the P2X7, which remains to be identified [192]. Therefore P2X7 was indirectly promoting cancer cell proliferation, by releasing extracellular ATP, and its pharmacological inhibition using oxidised-ATP (oATP) significantly inhibited the tumour growth of B16-bearing syngenic mice [192]. γ-radiations (0.5 Gy) were also proposed to induce ATP release by the same melanoma cells partially through P2X7 [204].

A P2X7 variant not truncated in its C-terminus, and possibly the P2X7A, is expressed in human neuroblastoma biopsy samples and in numerous neuroblastoma cell lines. In ACN cells the receptor is expressed at the plasma membrane and is thought to be functional. ATP and/or BzATP stimulations induced increases in the $[Ca^{2+}]_i$, plasma membrane depolarisation and YO-PRO-1 uptake. This latter parameter was prevented by incubation with KN-62 [42]. ATP stimulation also caused cell rounding, membrane blebbing and the release of vesicles into the extracellular space, without activation of caspase-3. P2X7 activation was associated to cancer cell growth through the paracrine release of the trophic substance P from nucleotide-activated neuroblastoma cells. Further evidence supporting the role of P2X7 in controlling cell survival and proliferation of neuroblastoma cells was shown that the treatment of mouse neuroblastoma Neuro-2a cells with the ATP hydrolase apyrase or with P2X7 antagonists, oATP or BBG, decreased cancer cell viability and cell number. Conversely, the pharmacological inhibition of P2X7 in these cells promoted neurite outgrowth and neuronal differentiation of cells [205,206]. One could hypothesise that P2X7 activity in neuroblastoma cells maintains an undifferentiated and proliferative phenotype that could be representative of cancer cell characteristics and cancer stem cells properties.

P2X7 is expressed at the plasma membrane of SKOV-3 and CAOV-3 ovarian carcinoma cells. In these cells, BzATP stimulation increased the $[Ca^{2+}]_i$ and the levels of phosphorylated Akt (pAkt) and ERK (pERK), which were prevented by the use of A438079. BzATP did not induced cell death in these two cell lines. The mechanical stimulation of cells produced by a flow of physiological solution was responsible for an increase in the $[Ca^{2+}]_i$ that was abolished in the presence of apyrase, therefore suggesting release of ATP. Furthermore, the incubation of cells with apyrase, with the P2X7 antagonist AZ10606120, or their transfection with the P2X7 E496A mutant inducing a dominant negative effect on the wild-type receptor, reduced cell viability by about 30%. Taken together these results led the authors to suggest that basal activity of P2X7, induced through the auto-release of ATP, increased pERK and pAkt levels and maintained cell viability [200].

With a series of excellent studies, Adinolfi and Di Virgilio and collaborators demonstrated that P2X7 displays a trophic activity in the absence of pharmacological exogenous activation [207]. They have proposed the idea that P2X7 could support different cellular aspects that are critical to the oncogenic transformation, to cancer cell proliferation and tumour growth through a basal activity of the receptor. They initially demonstrated that the transfection of Human Embryonic Kidney

(HEK293) cells with the full-length human P2X7, having the capacity to induce membrane permeabilisation, increased the resting mitochondrial potential, the basal mitochondrial Ca^{2+} concentration and the intracellular ATP content, and thus enhanced to whole mitochondrial activity which increased cell proliferation and conferred to the cells the ability to grow in the absence of serum. These effects were dependent on an autocrine/paracrine tonic stimulation by released ATP, as they were abolished in the presence of apyrase. On the other hand, the sustained stimulation of P2X7 by exogenous ATP had opposite effects and resulted in cell death [208]. Later on, using the same cellular model, they demonstrated that P2X7 transfection increased the ability of the endoplasmic reticulum to accumulate, store, and release Ca^{2+}. Furthermore, P2X7 expressing cells survived and proliferated in serum-free conditions and were resistant to apoptosis triggered by ceramide, staurosporin, or intracellular Zn^{2+} chelation. The nuclear factor of activated T cells complex 1 (NFATc1) was strongly activated in the P2X7 transfectants in the absence of exogenous agonist. All these effects were abrogated in presence of oATP or apyrase, further supporting the initial finding that P2X7, under tonic conditions in response to basal ATP release, had an anti-apoptotic activity or even promoted cell growth [209]. The P2X7B variant retaining its ion channel activity but unable to induce membrane permeabilisation, had similar effects, and cells over-expressing this variant showed the ability to infiltrate into soft agar, like tumour cells would do. The co-expression of the full-length P2X7A with the P2X7B induced the formation of heterotrimers that potentiated all the cellular effects observed in the presence of P2X7B alone (i.e. increased intracellular Ca^{2+} and Ca^{2+} release from the ER, potentiation of NFATc1 activation, increased cell survival and infiltrating colonies) and increased the intracellular contents of ATP as well as the release of ATP [146]. They further demonstrated that the expression of P2X7 promoted tumour growth in vivo. HEK293 cells expressing P2X7 exhibited a more tumorigenic and anaplastic phenotype than control cells in vivo when inoculated subcutaneously into nude mice. The growth of these tumours was reduced by intratumoral injection of oATP. P2X7-expressing tumours secreted high amounts of VEGF and showed a more developed vascular network than control tumours. This tumoral growth and neoangiogenesis of P2X7-expressing tumours were blocked by intratumoral injection of the VEGF-blocking antibody Avastin (bevacizumab), as well as the pharmacologic or molecular inhibition of P2X7. Similar results were obtained using mouse CT26 colon carcinoma cells transfected with mouse P2X7 receptor inoculated into syngenic immunocompetent BALB/c mice. Tumour growth was also inhibited by the blockade of P2X7 endogenously expressed in B16 mouse melanoma and in ACN human neuroblastoma cells and inoculated to syngenic C57Bl/6 or nude mice, respectively [210].

5.1.1.2. Induction of pro-invasive properties. In the rat C6 glioma cell, mRNA and proteins of P2X7 are expressed, and the stimulation with BzATP induced an increase in the $[Ca^{2+}]_i$ and ethidium uptake, that were prevented by the incubation with oATP, suggesting that the receptor might be functional. Furthermore, the stimulation of C6 cells with BzATP was responsible for the up-regulation of P2X7 mRNA and protein expression. Stimulation with BzATP also induced a four-fold increase in cell migration [195]. Another study performed in same C6 cells as well as human U-138MG cells, indicated that glioma cells were more resistant to cell death and to the induction of caspase-3/7 activity upon stimulation with high extracellular concentrations of ATP (5 mM) and BzATP (100 μM) in comparison with non-cancerous organotypic hippocampal slice cultures [211].

In highly invasive MDA-MB-435 s human breast cancer cells was, P2X7 was found to be fully functional as activation of the receptor by ATP gave rise to inward currents, increases in the $[Ca^{2+}]_i$ and, to less degree, ethidium uptake. The activation of the receptor by ATP also led to the release of active cysteine cathepsins responsible for the proteolytic degradation of the extracellular matrix and promoting cancer cell invasiveness, with very mild effect on cell viability [132,163]. To notice, in these studies, the pharmacological inhibition of P2X7 by both

competitive and non-competitive antagonists, as well as its reduced expression by the use of small interfering RNA significantly inhibited basal invasion in the absence of stimulation with an exogenous agonist. This would favour the hypothesis of a basal activity of the receptor that could be due to local and autocrine / paracrine release of ATP by cancer cells. P2X7 was also expressed in A549, PC-9 and H292 lung cancer cells and its exogenous activation by ATP or BzATP, as well as the autocrine release of ATP by cancer cells mediated by TGF-β1, also led to the increase in cell invasiveness that was prevented by synthetic (A438079) or natural (emodin) antagonists of the receptor [132,183,184]. Interestingly, the migration of PC-9 cells was under the dependence of EGFR receptors harbouring constitutively active mutations, and inhibition of their activity with AG1478 and gefinitib inhibited both cell migration and the expression of P2X7 proteins [184].

A study indicated that PC-3 human prostate cancer cells express mRNA for P2X7 receptor, and while application of ATP was responsible for the inhibition of cancer cell growth and viability, it was not prevented by using the P2X7 antagonist KN-62 and was more likely mediated by P2X5 [29]. In this study, it was not clear whether P2X7 was functional, since ATP failed to induce membrane permeabilisation, assessed by the Lucifer yellow uptake assay. However, the ability of the receptor to induce a characteristic ATP-mediated inward current was not assessed and the involvement of P2X7 in other cancer properties was not specifically studied. In a recent study performed with PC-3 cells, P2X7 was found to enhance the invasiveness of DU-145 in PC-3 human prostate cancer cells [109]. These facts question on the use of ATP-induced membrane permeabilisation as an indicator of P2X7 functional expression.

5.1.1.3. Metabolic adaptation to tumour microenvironment. Hypoxic episodes occurring in some areas of tumours would stabilise and activate the hypoxia-inducible factor-1α (HIF-1α) that would increase the expression of P2X7. In both MCF-7 and MDA-MB-231 breast cancer cells, hypoxia induced a HIF-1α-dependent increase of P2X7 expression which in turn was responsible for sustaining cancer cell survival and invasiveness through the activation of the ERK1/2 and Akt pathways and the extracellular activity of MMP-2 and MMP-9 gelatinases [177]. In PC-3 prostate cancer cells hypoxic episodes were associated with HIF-1α stabilisation and translocation into the nucleus and to the increased expression of P2X7 [212] which would also increase cancer cell invasiveness [109].

These results are in line with those obtained in the lab of Di Virgilio showing that the overexpression of the full-length P2X7 was responsible for a metabolic adaptation of cells towards a glycolytic phenotype, even in aerobic conditions [213]. This phenotype is well known as being the Warburg effect and is associated with neoplastic development and cancer aggressiveness [214]. This metabolic shift, was characterised by the upregulation of HIF1α, the glucose transporter Glut-1, the glycolytic enzymes glyceraldehydes 3-phosphate deshydrogenase G3PDH, phosphofructokinase PFK pyruvate kinase PKM2 and pyruvate dehydrogenase kinase 1 PDHK1, along with the reduced expression of the pyruvate deshydrogenase PDH, and allowed the cells to proliferate in the absence of serum and in the very stringent environmental conditions that could be found in developing tumours [213].

5.1.2. Anti-cancerous activities of P2X7 in cancer cells

5.1.2.1. Induction of apoptosis. In MCF-7 and MDA-MB-231 breast cancer cells, P2X7 is proposed to negatively regulate cell proliferation and tumour growth by inducing apoptosis and that the reduction of its expression by miR-150 which is upregulated in cancer tissues would reduce cancer cell death and promote cancer cell and colony growth [179].

In the human A431 squamous cell carcinoma cell line P2X7 is found to be expressed and, while its functionality was not clearly demonstrated, its stimulation with high doses of ATP, ATPγS and BzATP reduced cell numbers in vitro. These results, together with the finding that P2X7

seemed to be expressed in apoptotic/necrotic foci of basal cell and squamous cell biopsies, the authors of the study claimed that P2X7 was involved in the ATP-mediated apoptosis in cancer cells [190]. Similarly, P2X7 mRNA and protein are expressed in human HCT8 and Caco-2 colon adenocarcinoma cell lines and both ATP (1 mM) and BzATP (100 μM) increased the $[Ca^{2+}]_i$. This was reduced in the presence of oATP or KN-62. Stimulation of cancer cells with high concentrations of ATP (2 mM) for 48 h reduced the cell viability as shown by measuring the proportion of hypodiploid cells using flow cytometry. This was attributed to the induction of apoptosis in cancer cells. Pre-treatment of cells with oATP inhibited this proapoptotic effect of ATP by about 50%, which led the authors to conclude that P2X7 was involved in apoptosis of intestinal epithelial cells but another P2 receptor might also contribute [198]. Yaguchi and collaborators also identified that high concentrations of ATP reduced Caco-2 cancer cell growth through the inhibition of PKC, but since they were unable to identify P2X7 mRNA expression, they proposed that another unknown receptor was involved [215].

In A375 human melanoma cells, mRNA for P2X7 was detected, and proteins were immunostained by using an antibody recognizing an epitope in the C-terminus of the receptor. Even though, it remains unknown if these cells express or not other splice variants, the one detected was not truncated in the C-terminus and could correspond to the full-length P2X7A. The stimulation of cells with hundreds of micromolar BzATP induced membrane permeabilisation to YO-PRO-1, the activation of caspase-3/7 and resulted in a decrease in cell survival. While the co-application of KN-62 only mildly reversed cell death, it was suggested that P2X7 activation was pro-apoptotic and that its pharmacological targeting (activation) could provide a novel treatment of the disease [30]. Following this hypothesis, they pursued with an in vivo model in which athymic mice were subcutaneously inoculated with A375 human melanoma cells. Mice were then treated with daily intraperitoneal injections of 1 mL D-PBS containing 50 mM ATP for 39 days. The tumour volume and animal weight were measured over the course of the experiment. The treatment with ATP was shown to decrease the tumour volume by 50% in 7 weeks in treated mice, and prevented the weight loss observed in untreated animals [216]. These results supported the idea that ATP could be used as a treatment for melanoma, but the exact mechanism and the receptors involved in the effects observed in this experimental in vivo model were not demonstrated.

In a model of skin neoplasia induced by the administration of the carcinogenetic and promoting agents, DMBA (7,12-dimethyl-benz(a) anthracene) and TPA (12-O-tetradecanoylphorbol-13-acetate) respectively, on the skin of FVB female mice, it was shown that the direct application of BzATP reduced the proportion of living animals bearing tumours, and the size of lesions [217]. This effect was attributed to the induction of apoptosis in cancer cells stimulated by 100 μM BzATP.

No information was given on the expression of the different splice variants and particularly of those being truncated in the C-terminus that would be ineffective in inducing membrane permeabilisation. In in vitro experiments, authors of this study showed that the application of 100 μM BzATP increased the $[Ca^{2+}]_i$, induced membrane permeabilisation to ethidium and induced apoptosis in normal keratinocytes from wild-type mice, while it failed to induce ATP in normal keratinocytes coming from P2X7−/− mice. In chimio-induced cancer tissues, it appeared that the expression of the full-length P2X7, monitored by both western blotting and immunohistochemistry, was decreased compared to the normal skin. These results prompted the authors to conclude the diminished pro-apoptotic effect of BzATP in cancer keratinocyte was due to a lower expression of P2X7 as compared to normal keratinocyte. They also proposed that the activation of apoptosis in cancer cells through the activation of P2X7 with BzATP could represent a novel chemotherapeutic growth-preventing modality for skin cancers in vivo [217]. While this study is of sure interest it did not consider the participation of other P2 receptors in vivo, and the risk and side effects that could be triggered by such treatments. Furthermore, the P2X7 receptor is depicted as a pro-apoptotic receptor being

down-regulated in cancer cells. Indeed, apoptosis is induced when stimulating the cells with high doses of BzATP. However, such pharmacological and prolonged stimulations of P2X7 can trigger cellular responses that are not the same than those activated by the receptor under basal conditions. As such it is possible that such experiments do not allow the determination of the real role played by P2X7 in cancer biology.

While the release of low concentrations of ATP (in the 10 nM range), mediated by basal activity of P2X7 in B16 mouse melanoma cells, was indirectly inducing an increase in cancer cell proliferation, high concentrations of ATP (in the 3 mM range) thus possibly directly activating the receptor and inducing membrane permeabilisation had opposite effects [192]. Therefore the level of ATP released, as well as the mode and time-scale of the activation of P2X7, might result in striking different effects. Further supporting this idea, it has been proposed that high concentrations of ATP, which could be released by dying cancer cells in necrotic areas of tumours, could directly kill adjacent tumour cells via P2X7. Therefore, maintaining high concentrations of ATP by inhibiting the ectonucleotidase CD39 would have an anti-tumour effect [218]. While such a hypothesis is tempting, it is hard to understand how very aggressive tumours, showing multiple necrotic foci, still continue to naturally grow and to metastasise.

In a recent study performed in mouse MCA38 colon adenocarcinoma cells and in mouse B16 melanoma cells, the P2X7a variant (but not the P2X7k variant) is expressed (mRNA) and the stimulation of cells with high doses of ATP (up to 5 mM) and to BzATP (up to 2 mM) was responsible for ethidium uptake and for a reduction in cell viability and in clonogenicity. The proposed mechanism is a decrease in the intracellular content of ATP and a deregulation of the PI3K/Akt and AMPK-PRAS40-mTOR signalling pathways that perturb the balance between cell growth and autophagy [193].

5.1.2.2. Inhibition of cancer cell proliferation and tumour growth. In C6 rat glioma cells, both the pharmacological (BBG) and the molecular extinction (siRNA) were found to have growth promoting effect in vitro and in vivo. These effects were not due to a significant reduction in caspase-3 induction in cancer cells, but rather as a consequence of the over-expression of EGFR, HIF1-α, VEGF and P2Y2 [196]. In GL261 mouse glioma cells, high concentrations of ATP (5 mM) and BzATP (up to 3 mM) induced the uptake of propidium iodide and a rapid increase in extracellular LDH activity, external exposure of phosphatidylserine and a reduction of cell survival measured by MTT cell viability assay, which were claimed to be mediated by P2X7. These effects were not observed in P2X7-expressing rat C6 or human U138 and U87 glioma cells [197].

The effect of P2X7 agonists, ATP and BzATP, in combination or not with irradiation (2Gy) on cell death was studied in human U-138MG, U-251MG and M059J glioma cells. All the three cell lines expressed P2X7 proteins, but the radiosensitive M059J glioma cells significantly expressed higher levels of proteins, and showed a higher ethidium uptake upon ATP (5 mM) stimulation, both in the absence or presence of irradiations, than in the two other cell lines. P2X7 agonists produced cell death in the radiosensitive M059J cell line, but the radioresistant glioma cell lines U-138MG and U-251MG presented resistance to death when treated with either ATP or BzATP [194]. While authors claimed a relevance of P2X7 activation for increasing glioma radiosensitivity, more experiments are necessary to really understand the roles of P2X7 in the acquisition of resistances to therapeutic strategies.

5.1.2.3. Inhibition of cancer cell migration. In a recent study, it is shown that P2X7 is expressed in human MDA-MB-231 breast cancer cells, however its stimulation by the non-hydrolysable analogue ATPγS reduces cancer cell growth, migration, and inhibits the growth of orthotopic xenograft tumours due to the injection of same human cells in the mammary fat pad of nude mice [219]. In this same study, while the activity of P2X7 was not clearly demonstrated, it was proposed that its persistent stimulation with ATPγS reduced mouse

Py8119 breast cancer cell migration in vitro and tumour growth in a model of intratibial injection of cells in C57bl/6 mice [219].

It is widely accepted that the anti-proliferative or pro-apoptotic effect resulting from exogenous stimulation of P2X7 in some cell types are associated with the membrane permeabilisation property. It can be postulated that, in some cancer cells P2X7 is fully functional as an ion channel but not inducing membrane permeabilisation, because of the expression of a C-terminus truncated variant or due to the molecular uncoupling to pore forming channels such as pannexin-1. In such cases, P2X7 would control other cellular properties such as cancer cell invasiveness. If this hypothesis can be verified, it can be proposed that, selective induction of membrane permeabilisation in cancer cells might represent a new strategy to treat cancers, probably more specific than the pharmacological targeting of P2X7.

5.2. P2X7 receptors in tumour-associated immune cells

Tumours are infiltrated with different subsets of immune cells and some tumours originate from a chronic inflammatory environment, and thus there is a well-orchestrated functional interaction among inflammatory infiltrates and the tumour [220,221]. P2X7 is highly expressed and controls the function of different immune cells such as monocytes, macrophages, dendritic cells and lymphocytes [222–224]. As detailed in the previous section, tumour microenvironment present high levels of extracellular ATP and therefore activation of the P2X7 is likely to occur in tumours infiltrated by immune cells. However, P2X7 function in tumour-associated immune cells will elicit a pro-tumorigenic response contributing to tumour growth and progression by inducing the production of different pro-tumorigenic factors such as extracellular matrix-degrading enzymes and other bioactive molecules such as VEGF or prostaglandins [225–228]. This is mainly due to the fact that tumour-associated macrophages present an M2 alternative activated phenotype associated with tissue remodeling and the resolution of the inflammation, where P2X7 function uncouples from pro-inflammatory cytokines production [175,229,230]. This event plays a pivotal role in the lack of immunity against tumours and the failure to elicit a robust immune response to remove cancer cells. Tumour-associated macrophages would be immunosuppressive and therefore would prevent the attack of tumour cells by natural killer and T-cells [230]. In this context, immunotherapy treatments against tumours aim to elicit a robust pro-inflammatory response that will prime innate dendritic cells and macrophages towards M1 inflammatory phenotypes for efficient tumour-associated antigen presentation to lymphocytes [231,232]. In this regard, anticancer treatments, such as radiotherapy or some chemotherapeutic agents, besides to induce direct cytotoxic effects on cancer cells, they also mediate immunogenic cell death and therefore elicit an IFNγ-mediated immune response required for the efficacy of these treatments [233,234]. In the tumour environment dying tumour cells succumbing to chemotherapy further increased the activation of P2X7 in macrophages and dendritic cells via the release of ATP. This release of ATP in conjunction with Toll-like receptor (TLR) engagement with other DAMP signals, such as the HMGB1, are essential to elicit a proper anti-cancer immunity [235,236]. In macrophages and dendritic cells, the activation of these pathways converges in the assembly of the inflammasome, a central innate immune sensor for pathogens-associated molecular patterns (PAMPs) and/or DAMPs [76, 237,238]. The NLRP3 inflammasome activated via P2X7, has been described as being responsible for eliciting CD8 + T-cell mediated cancer eradication following anticancer treatments [51,236]. However, NLRP3 and other inflammasomes, including NLRC4 and NLRP6, have been also implicated in tumorigenesis [239]. Therefore in the context of cancer, P2X7 activation during tumorigenesis will play different roles in different types of cancers, being beneficial in certain circumstances and injurious in others. The use of specific drug-like P2X7 antagonists targeting immune cells could be detrimental in the first steps of cancer development by inducing an immune tolerogenic response. In contrast,

when the primary tumour is established, pharmacological blockage of P2X7 could decrease the tumour-promoting function of tumour-associated macrophages [44].

6. Conclusions and perspective on future research direction

From the 1980s, the importance of extracellular adenine nucleotides was demonstrated in carcinogenesis. At that time it was demonstrated that the application of exogenous ATP or ADP had anti-cancerous properties through the inhibition of cancer cell growth, and ATP or BzATP were even proposed as treatment modalities in vivo in animal models and in humans [45–49]. This hypothesis was further supported until recently [50]. However, while treatments with ATP had very strong cytotoxic effects on cancer cells from several tumours, it also appeared that in some other occasions it could have pro-cancerous activities [25]. Besides, treatments with ATP or analogs could initiate nociceptive pathways through the activation of P2X3 receptors on sensory nerves. These intriguing results prompted researchers to study in details the activity and participation of the purinergic signalling, and purinergic receptors, in carcinogenesis and in the progression of cancers, as well as the release mode and concentrations of nucleotides in the tumour site [26]. Results obtained are of primary importance as they could lead to the formulation of novel therapeutic strategies and to the repurposing of purinergic receptor antagonists developed for the treatment of other pathologies in the cancer treatment. Among all P2 receptors studied in the context of cancer initiation and progression, the P2X7 receptor seemed to attract the most of interest and also probably generated the most of controversy on its expression, activity and postulated role. With the exception of some experiments, among which those performed on uterine cancers, most of the studies performed in cancer biopsies and cancer cells indicated the over-expression of P2X7. Multiple studies appeared contradictory in their results when assessing the possible involvement of P2X7 in oncogenic processes. However, a careful analysis of the experiments conducted should help in determining such discrepancies, in proposing new hypotheses and important aspects of P2X7 properties that will have to be examined in the future studies. A critical point will be to clearly analyse, in cancer cells, the presence and intensity of all the functional properties classically attributed to P2X7 (ATP-induced current, intracellular Ca^{2+} increase, membrane permeabilisation, etc.) as they might be associated with different biological functions. It is also obvious that the cells expressing P2X7 in a tumour (cancer cells versus non-cancer cells) are important to consider as they might be associated with either pro-cancerous or anti-cancerous immune properties. Not only the cells are important, but the step of the carcinogenesis or grade of the tumour should be considered. P2X7 activation during tumorigenesis could play different roles in different types of cancers, being beneficial in certain circumstances, and injurious in others. The use of specific drug-like P2X7 antagonists targeting immune cells could be detrimental in the first steps of cancer development by inducing an immune tolerogenic response. In contrast, when the primary tumour is established, pharmacological blockage of P2X7 could result at least in the decrease of the tumour-promoting function of tumour-associated macrophages. It could also reduce the pro-cancerous effect of P2X7 function in cancer cells. Future studies will have to determine the pattern of expression of the different P2X7 splice variants, not only the expression of the full-length P2X7A, in both non-cancerous and cancerous cells, as they could be responsible for various or even opposite regulations of biological functions. Such an analysis was recently initiated with a study performed in osteosarcoma samples and cells [240]. Authors showed that both P2X7A and P2X7B variants were expressed in most osteosarcoma samples analysed and that they were responsible for differential effects on cell growth and matrix mineralisation.

From this overview of the literature, it can be postulated that basal activity of P2X7 in cancer cells from an established tumour could be pro-cancerous [146,208,210], while the exogenous stimulation of P2X7 [50], or the endogenous stimulation following anticancer treatment and cell death [51], with high concentrations of extracellular ATP could be anti-cancerous (Fig. 3). However, it is now understood that the exogenous stimulation of cells with (high) concentrations of hydrolysable or non-hydrolysable agonists, for a long period of time, even if giving interesting results in vitro, might not reflect what is really happening in tumours in vivo. As such, the results obtained might be considered
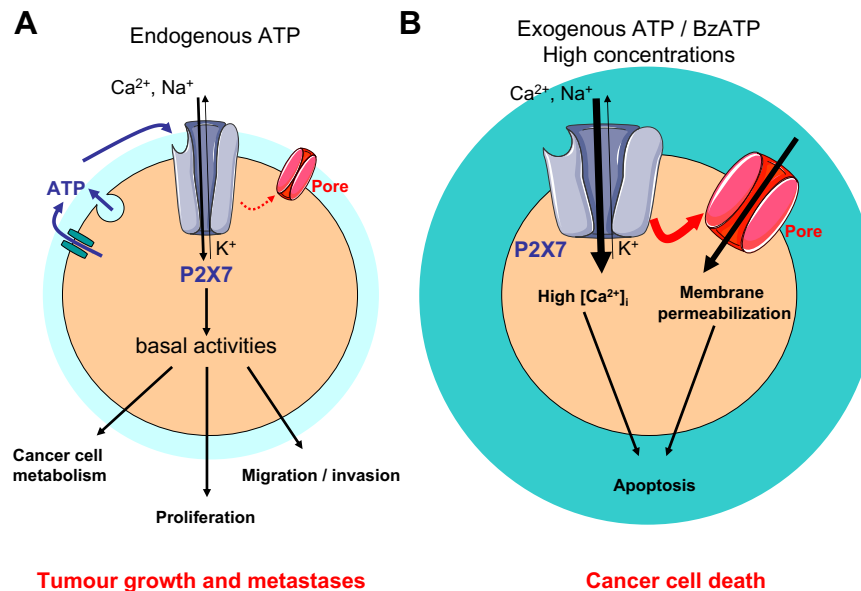

Fig. 3. Schematic representation of the participation of P2X7 receptor in epithelial cancer cell biology. (A) In epithelial cancer cells, the autocrine/paracrine release of ATP, through transmembrane diffusion or vesicular release is responsible for a pericellular halo that activates P2X7 into a basal mode activity, with no or weak membrane permeabilization, thus increasing cell metabolism and promoting cell proliferation, migration and invasiveness. These properties would promote tumour growth and progression towards metastatic steps. (B) The exogenous stimulation with high concentrations of ATP or BzATP would tend to overactivate P2X7 receptors into a cytolytic mode, due to the high increase of intracellular Ca^{2+} concentration and to the membrane permeabilization. This would induce cancer cell apoptosis.

respectfully with these limitations. In line with this aspect, it appears that P2X7 could have a basal activity, due the autocrine / paracrine release of ATP in concentrations that are supposed to be much lower than those used in vitro to stimulate the receptor.

Furthermore, it can also be postulated that P2X7 expression and functionality in cancer cells, together with the endogenous ATP in the tumour, represent key parameters for the selection of cancer cells with an aggressive phenotype. This was formulated as being the "Run or die hypothesis": the increase of the extracellular ATP concentration in the tumour could represent a stressful selective pressure for from which many cancer cells would die while some other, that do not undergo apoptosis, that are the most invasive, would be selected [44].

Acknowledgements

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