

Emerging roles of P2X receptors in cancer

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Abstract

Tumor microenvironment composition strongly conditions cancer growth and progression, acting not only at cancer itself but also modifying its interactions with immune, endothelial and nervous cells. Extracellular ATP and its receptors recently gained increasing attention in the oncological field. ATP accumulates in cancer milieu through spontaneous release, tumor necrosis or chemotherapy exerting a trophic activity on cancer cells, modulating the cross talk among tumor, and surrounding tissues. Accordingly, ATP gated P2X receptors emerged as central players in tumor development, invasion, progression and related symptoms. Indeed, P2X receptors are expressed and functional on tumor cells itself-but also in immune-infiltrate and nearby neurons. In this review, we summarize recent findings on P2X receptors role in tumor cell differentiation, bioenergetics, angiogenesis, metastasis and associated pain, giving an outline of the potential anti-neoplastic activity of receptor agonists and antagonists.

Introduction

In recent years, a growing body of literature highlighted the importance of extracellular ATP in cancer. ATP is an abundant biochemical component of cancer microenvironment where it acts, through its own receptors, as growth promoting factor, danger signal and represents the main source of the immunosuppressant adenosine. Receptors for extracellular ATP belong to two subfamilies: seven transmembrane domains, G-coupled P2Y receptors and P2X ion channels. The growth promoting activity of P2Y receptors in cancer is well known and has been recently covered in excellent reviews [1, 2]. Here we give an overview of P2X role in tumor proliferation, progression and related pain and discuss the potential of these receptors as therapeutic targets in cancer.

P2X receptors (P2X1-7) are ATP gated ion channels, mediating the passage through the plasma membrane of Na^+ , Ca^{2+} and K^+ [3]. Two transmembrane domains, an extracellular loop plus N and C terminal intracellular tails form each receptor subunit. P2X subunits assemble into functional homo or hetero-trimers showing diverse pharmacological properties and functional characteristics [4]. Crystal structure resolution of P2X4 revealed a dolphin like receptor subunit shape, extracellular loop being the body and transmembrane domains forming the tail of the animal [5]. P2X7 receptor exposure to millimolar concentrations of ATP causes the opening of a large unselective membrane pore, that has been associated to cytotoxicity [6]. Interestingly, pore dilation is not a P2X7 exclusive as it is also activated by heteromeric P2X2/P2X5 receptors [7]. The different activity and cellular expression of P2X receptors is at the basis of their role in several patho-physiological processes such as pain sensation, inflammation and related diseases [8]. P2X receptors are key modulators of cancer-associated immune responses, pain sensing and are emerging as central players of tumor proliferation, vascularization and spreading.

ATP in the oncogenic milieu

ATP was isolated and identified as the main regulator of endergonic cell reactions in the early ages of 20th century. Subsequently, the seminal work of Burnstock, clearly demonstrated that ATP also exerts an important role in extracellular signaling [9]. Once secreted in the extracellular space, ATP is easily degraded to ADP and adenosine by ubiquitous extracellular ectonucleotidases such as CD39 and CD73 [10]. Ectonucleotidases, adenosine and its receptors are involved both in cancer proliferation and host immune system modulation [10]. Adenosinergic immune suppression mediated by A2 receptors is an

established data [11] and plasma membrane enzymes responsible for its generation (i.e. CD39 and CD73) are well known to favor carcinogenesis [12, 13]. Extracellular generation of adenosine requires ATP, which accumulates in tumor microenvironment during cancer development [14] and is associated to regulation of cancer cell metabolism and tumor immune-cells cross-talk [15, 16]. Interestingly, a good candidate for ATP secretion in the extracellular space is the P2X7 receptor in its large pore conformation [17, 18]. ATP can be released both from tumor or immune infiltrating cells via several mechanisms, such as granule exocytosis and plasma membrane channels (i.e. ABC cassette proteins) [1]. Stimuli causing ATP secretion from tumor cells also include response to mechanical deformation, hypoxia, necrosis and ischemia [19]. However, whatever is the source of extracellular ATP, accumulating evidence confirms its high concentration in tumor milieu [14, 20] and renders of great interest the study of ATP receptors functions in cancer.

Role of ATP and P2X receptors in immunogenic cell death

A peculiar type of cell death, associated to extracellular ATP release, is immunogenic cell death (ICD). ICD is a form of cell death caused by chemotherapeutic cytostatic agents such as oxaliplatin and anthracyclines, or radiotherapy [21]. ICD of cancer cells can induce an antitumor immune response against dead-cell antigens through activation of dendritic cells and a specific T cell response [22]. Immunogenic apoptosis of cancer cells displays the classical hallmarks of apoptosis such as phosphatidylserine exposure, caspase activation and mitochondrial depolarization, but it differs from this type of death in surface exposure or secretion of damage-associated molecular patterns (DAMPs) [23] and in the ability to cause an immune response against tumor cells [21, 24]. DAMPs are intracellular molecules that, when released, are able to activate an inflammatory response [25]. ATP is an example of non-protein DAMP [26], while examples of intracellular proteins that act as DAMPs are heat-shock proteins [27], high-mobility group box 1 [28] and calreticulin [29].

ATP release from dying cells constitutes a “find me” signal for the recruitment of dendritic cells, monocytes and macrophages [30]. ATP also acts as pro-inflammatory molecule interacting with P2X7 receptor expressed by dendritic cells and macrophages and causing the activation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome [31]. In a recent work Martins and colleagues, demonstrated the molecular mechanism of ATP secretion during ICD, a process that involves molecules central in autophagy (ATG5, ATG7 and BCN1), lysosomal exocytosis (LAMP1,

VAMP1), apoptosis (caspases), membrane blebbing (ROCK1, myosin II) and plasma membrane permeabilization (PANX1) [32]. ATP is also released following the treatment with antineoplastic chemotherapeutic drugs, such as anthracyclines and oxaliplatin, that induce ICD triggering anticancer immune response [32]. Moreover, overexpression of the cell surface ATP-degrading enzyme CD39 abolishes ICD, thus rendering cancer cells resistant to chemotherapy [20, 33]. On the same line, accumulating evidence suggest that the ATP-P2X7 pathway which links the stress response and death of cancer cells to T lymphocyte-mediated anticancer immunity, is important also for a better efficiency of chemotherapy *in vivo*. In fact, P2X7 null mice and mice lacking any of the NLRP3 inflammasome components or IL-1 receptor 1 do not respond to chemotherapeutics inducing ICD [16]. Moreover, anthracyclin-based adjuvant chemotherapy is inefficient in patients with breast cancer who express a P2X7 loss of function allele [34]. These data suggest that ATP-dependent P2X7-mediated inflammasome activation is relevant for a better efficacy of anticancer therapies in patients [16, 31].

P2X receptors expression in cancer

P2X receptor expression, investigated by RT-PCR, Western blotting or immunohistochemistry, has been reported in several cancer cell lines or tumor derived specimens [35]. Among different tumor types, haemopoietic lymphoproliferative disorders are showing the higher levels of P2X receptors and were the first models in which an association between P2X expression and oncogenesis was suggested [36-39]. The earlier report on P2X7 expression in B chronic lymphocytic leukemia (B-CLL) dates back to 1989 [40], since then different studies have analyzed P2X7 function and polymorphic expression in this disease. In 2002, we related P2X7 overexpression with aggressive variants of B-CLL [38]. Subsequently, genetic linkage studies associated P2X7 1513 A to C loss of function polymorphism with B-CLL development in an Australian population [41]. However, these data were not confirmed by studies evaluating wider patients' cohorts [42]. On the contrary, a gain of function variant of the receptor was first identified in B-CLL patients [43].

More recently, Chong *et al.* analysed mRNA expression of P2X receptors in Chinese paediatric acute leukemia reporting an over-expression of P2X1, P2X4, P2X5 and P2X7 in patients versus healthy controls [37]. Interestingly, P2X4 and P2X7 tended to be contemporary overexpressed and their increased levels associated with relapse of acute leukaemia [37]. P2X5 mRNA was up modulated in several solid tumours [44] and in almost all hematologic malignancies [45, 46], B-CLL and B-ALL

showing the highest expression of the receptor [44]. Interestingly, increase of P2X5 in cancer was identified due to the properties of a polymorphism of the receptor in activating graft versus leukaemia reaction. Graft versus leukaemia is a mechanisms that occurs post allogeneic, HLA-identical, transplantation of leukemic patients. Donor's cytotoxic T lymphocytes recognize some non-HLA antigens on recipient leukemic cells and kill them. A polymorphic variant of P2X5 was identified as one of these antigens that are known as minor histocompatibility antigens [45]. Although not a lot has been reported on other P2Xs, several solid tumours have been shown to express the P2X7 receptor those include breast [47], prostate [48], colon [49], renal [49], cervical cancers [50], neuroblastoma [51], melanoma [52] and papillary thyroid carcinoma [53]. Interestingly, expression of P2X7 in papillary thyroid carcinoma was associated with poor prognosis [54] and lymph node metastasis formation [55]. A loss of function polymorphism of P2X7 was also associated to reduced aggressiveness and metastatic dissemination in prostate cancer [56]. Conversely to what observed in other cancer types, P2X7 expression was down modulated in adeno and cervical squamous carcinomas [50, 57]. However, in the same type of cancer a loss of function splice variant of P2X7 (P2X7-j) was reported to be over-expressed [58]. Therefore, the presence of other truncated forms of P2X7 associated to increased cell proliferation [18] cannot be excluded.

P2X receptors in cell proliferation and cancer cell metabolisms

One of the characteristics acquired by tumor cells, which is central in the early stages of tumor formation, is the ability to proliferate in unfavorable conditions such as growth factors and nutrients deprivation [59]. Although the P2X7 receptor was originally identified as a pro-apoptotic cytotoxic receptor [6], its involvement in cell proliferation was soon evident [60, 61]. Indeed, P2X7 transfection was shown to confer a growth promoting activity in serum starvation to various cell types including B-lymphocytes, pro-myelocytes and human embryonic kidney cells [61, 62]. In these models, P2X7-mediated proliferation was dependent on spontaneously released ATP, as administration of the nucleotide degrading enzyme apyrase reverted this phenotype [61, 62].

The first cell type in which P2X7 activation has been related to growth is lymphocytes. In a seminal paper, Baricordi *et al.* showed that ATP acting at P2X7 was able to increase T lymphocytes proliferation after T cell receptor activation [60]. Following studies confirmed these data, showing that P2X7 receptor triggers the activation of the main pathways involved in lymphocytes proliferation, including FAK and IL-2 secretion [63], and that P2X7 inhibitor oxidized ATP hampers the expansion

of effector T cells [64]. On the same line, Junger and colleagues reported that T cell receptor stimulation causes release of extracellular ATP, leading to P2X7-dependent nuclear factor of activated T cells (NFAT) activation and IL-2 secretion [65]. Intriguingly, in this model ATP release seemed to be sustained by P2X1 and P2X4, more than P2X7 itself [66]. NFAT is a key player in P2X mediated proliferation also in non-lymphocytes cells [18, 49, 67-69] and this is not surprising given that NFAT is activated by calcium flux, mediated by the opening of P2X channels. Interestingly, NFAT activation seems to be at the basis of P2X7 mediated proliferation also in osteoblasts [69]. The altered bone phenotype of P2X7 null mice [70] prompted a series of investigations to define P2X7 role in osteoblasts-osteoclasts biology (for recent reviews see [71, 72]). While the role of P2X7 in osteoclast activation is still controversial, a clear role of the receptor in osteoblast proliferation and osteodeposition emerged [73, 74]. Moreover, in osteoblast like MC3T3-E1 cell line, P2X7 activation triggered, via PI3K, both lactate release and increased glucose metabolism [74]. Numerous nervous system cell types showed P2X7 dependent proliferation those include different neuroblastoma cell lines [49, 51, 75].

Several studies have described P2X7-dependent microglial or glial cell proliferation [76-79]. On the contrary, P2X4 receptor seems to have a pro-apoptotic role in activated microglia, as its blockade prevents microglial loss due to neuro-inflammation [80]. Accordingly, P2X7 was reported to be expressed on both glioma cells [81] and infiltrating microglia [82], while P2X4 expression was limited to microglial cells [83]. Proof of concept of the involvement of P2X7 receptor in tumor growth was given in 2012 when in an *in vivo* study we clearly demonstrated a connection between P2X7 expression and tumor growth [49]. Transfection of P2X7 in cells that naturally does not express it, such as HEK293 human embryonic kidney cells or CT26 colon carcinoma cells caused an increase in tumor engraftment and growth. Consequently, silencing or pharmacological blockade of the receptor in P2X7-endogenously-expressing murine models of neuroblastoma and melanoma caused tumor regression [49].

Although the mechanisms underlying P2X7 mediated proliferation are far to be uncovered we know that they include an alteration of cell metabolism. The need for a reorganization of metabolic pathways in cancer cells depends on the increased requirement of biosynthetic intermediates often in the absence of a constant supply of oxygen and metabolites [84]. In 1930s Otto Warburg described a link between mitochondrial dysfunction and tumorigenesis [84]. He observed a significant increase in glycolysis and lactate production in the presence of oxygen without a substantial increase in oxidative phosphorylation

[84]. Since then, aerobic glycolysis (also known as “Warburg effect”) is known to be the preferred metabolic pathway adopted by cancer cells, in presence of oxygen. However, recent studies suggest that aerobic glycolysis and mitochondrial oxidative phosphorylation are both at the basis of energy production in cancer [84]. As described by Warburg, aerobic glycolysis needs a large amount of glucose as substrate for glycolytic enzymes [85]. But also the most abundant free amino acid in human body L-glutamine has long been known to be essential for cancer cell growth [86]. These nutrients are so important to cancer cells that they over-express glucose and glutamine transporters [87]. Glutamine and glucose provides the carbon skeletons, NADPH, and ATP to build new cancer cells. We recently investigated the metabolic effects of the P2X7 receptor showing that its expression favors cells proliferation not only in the absence of serum in the culture medium but also in glucose deprivation [15]. According with Warburg, P2X7-expressing cells showed a higher lactate output, they over-expressed several of the key glycolytic enzymes (i.e. G3PDH, PFK, PKM2, PDH and PDHK-1) and the ubiquitous glucose transporter Glut-1. Moreover, P2X7 positive cells exhibited larger depots of the glucose substitute glycogen [15]. Interestingly, the previously demonstrated P2X7-dependent mitochondrial-efficiency increase [62] was maintained in glucose deprivation [15]. Thus, P2X7 confers to cancer cells adaptability to unfavorable milieu conditions via up-regulation of glycolytic enzymes and a more efficient use of intracellular glycogen stores. A study by Estrella and colleagues further elucidated the role that lactate, ATP and adenosine may have in tumor microenvironment [88]. According with them, the acidic microenvironment established thanks to P2X7-dependent release of lactate, ATP derived adenosine will drive extracellular matrix invasion and tumor metastasis [88].

P2X in angiogenesis

Tumor growth and metastasis formation are angiogenesis-dependent processes [89]. As tumor develops and augments its size, distance between cancer cells and blood vessels progressively increases, compromising exchange of oxygen, nutrients and waste products. To overcome this tough condition, tumor needs to stimulate the formation of new vessels. Moreover, without new vasculature cancer cells cannot metastasize to another organ.

Angiogenesis is a complex multistep phenomenon, which consists of endothelial cell proliferation and migration, degradation of extracellular matrix and morphogenesis/capillary tube formation of endothelial cells [90, 91]. A key angiogenic molecule that promotes all these processes is vascular

endothelial growth factor (VEGF) [92, 93]. VEGF gene transcription is activated by hypoxia-inducible factor 1 (HIF-1), in case of marked reduction in local level of oxygen (hypoxia) [94].

Involvement of P2X receptor in vessel formation is largely unknown. It was reported that P2X2 and P2X4 are up regulated by hypoxia in hippocampal cultures [95]. Moreover, P2X2 is overexpressed by neonatal mouse retina after oxygen-induced retinopathy, a model for retinal neovascularization [96].

Based on actual knowledge, among P2X receptors, P2X7 is the only one that mediates VEGF secretion. P2X7-dependent VEGF release was documented in different cancer cell lines [49, 81, 97] as well as in primary human monocytes [98] and human embryonic kidney fibroblasts (HEK293) transfected with P2X7 [49]. Experimental *in vivo* studies showed that P2X7 expressing tumors had a thick vascular network and stained positive for VEGF [49, 97]. Pharmacological blockade or silencing of P2X7 reduced VEGF release and vessel formation [49], thus confirming receptor participation in tumor associated angiogenesis.

Furthermore, silencing of HIF-1 α , a subunit of the nuclear transcription factor for VEGF gene, down-modulates P2X7 expression in cancer cells [99] and P2X7 feeds back on HIF-1 α as overexpressing the receptor up-modulates HIF-1 α [15, 100].

We can conclude that P2X7 is the main P2X receptor involved in tumor angiogenesis. P2X7 sustains vessel formation mediating VEGF secretion by cell types that are closely related to generation of tumor vasculature, which is endothelial cells, cancer cells itself and probably tumor associated macrophages (Fig.1).

P2X in cell migration and cancer metastasis

Cell migration is an important process involved in different events, from embryonic development to wound healing and immune responses. Ability to move from one site to another is also a propriety that cancer cells use to colonize different tissues and organs, giving rise to metastatic foci. Understanding the mechanisms implicated in cell migration is thus relevant to improve pharmacological treatment of metastatic tumors and so clinical outcome of patients with high invasive cancers.

For tumor migration to occur, cancer cells must be able to digest the extracellular matrix and to bypass tight junctions among non-cancerous cells. Proteolytical disruption of tumor surrounding tissue is made by extracellular proteases like matrix metalloproteinases and cysteine cathepsins [101, 102]. P2X7 receptor activation has been involved in the spreading of tumor cells from primary tumor site [18, 103]. In fact, P2X7 promotes cell release of different proteolytic enzymes such as cathepsins and matrix

metalloproteinases [103-106] (Fig.1). In glial cells, P2X7 dependent activation of extracellular proteases proceeds from lysosomal release of cathepsins to extracellular degradation of tissue inhibitor of metalloproteinases, leading to matrix metalloproteinase 9-dependent migration of these cells [107].

Recently, it was reported that P2X7 activation enhanced breast and lung cancer cells invasiveness, through not only protease release but also acting at cytoskeletal remodeling [103, 108, 109]. In human lung cancer cells, P2X7 mediates TGF beta-dependent actin reorganization and migration [108]. Moreover, P2X7 pharmacological blockade impairs breast cancer cell dissemination in a zebra-fish model of metastasis [103, 109]. These findings imply that P2X7 receptor not only promotes tumor growth but is also involved in metastases development. Thus, P2X7 blocking drugs could be also employed as anti-metastatic agents, as suggested by good results of *in vivo* experimental models [109].

Expression and function of P2X receptors in stem cells

Recently, the cancer stem cell (CSC) theory has emerged as an attractive hypothesis for tumor development and progression. The theory suggests that tumors consist of subsets of cells with functional heterogeneity and that only a small subset of these cells (CSC) within tumor bulk exhibits the capacity to initiate and sustain tumor growth, invasion, metastasis and recurrence [110]. CSCs have been identified in both hematological malignancies and solid tumors, including multiple myeloma [111], liver [112], brain [113], colorectal [114], lung [115], and pancreatic cancers [116]. CSCs have the characteristics of stem cells as they are capable of both self-renewal and differentiation into diverse cancer cells [110].

Thus, investigating the molecular signaling involved in cell plasticity and differentiation in stem cell would be of help not only in the regenerative medicine field but also in cancer as deregulation in the balance between proliferation/differentiation often triggers tumor transformation.

Purinergic signaling is already present at the early stages of embryogenesis, being involved in cell proliferation, migration and the differentiation of a wide variety of structures [117, 118]. Accumulating evidence suggest an influence of ATP, through P2X purinergic receptors, on embryonic stem cells (ES) [119, 120]. Mouse ES cells (E14TG2a) expressed almost all members of the ionotropic family P2X, which are P2X2, P2X3, P2X4, P2X5 and P2X7 [119-122]. In these cells, ATP promotes cell proliferation, acting at P2X3 and P2X4 [119]. Moreover, a P2X7 antagonist reduced colony forming ability of ES cells *in vitro*, suggesting that P2X7 receptor is required to increase their staminal potential and survival [120]. P2X receptor activity is involved in hippocampal neurogenesis by inducing

proliferation of hippocampal progenitor cells [123] and participating in the formation of neuronal networks [124]. Accordingly, in cultured hippocampal neurons P2X7 receptor promotes axonal growth and branching [125]. On the contrary, P2X7 functional decrease or silencing was associated to differentiation of neuroblastoma cells [126, 127].

Also in the olfactory epithelium (OE) purinergic signaling acts as a paracrine signal regulating neurogenesis [128]. OE is an epithelium unusual for its remarkable regenerative capacity and that sustains neurogenesis of olfactory receptor neurons [129]. Basal cells of adult mouse OE express functional P2X receptors (P2X1, P2X2, and P2X3) that are responsible for injury-induced proliferation of these cells [128].

The role of P2X receptors in proliferation and differentiation has been investigated also in hematopoietic stem cells (HSCs). Human HSCs are identified by the expression of CD34 antigen, a cell membrane phosphoglycoprotein present on human bone marrow, peripheral blood and cord blood progenitors [130]. Lemoli and colleagues showed that P2Xs (P2X1-7) are expressed and functionally active on CD34⁺ hematopoietic progenitors, in lineage-negative CD34⁻ progenitors, as well as in CD34⁺ -derived long-term culture-initiating cells [131]. Moreover, HSCs release ATP from intracellular compartments that positively influences proliferation and differentiation of these hematopoietic progenitors via activation of P2X1 and P2X4 [132].

As we have reported here, P2Xs are expressed by several progenitor cells, like MSCs, neural precursor cells and HSCs and their expressions vary with differentiation toward different lineages.

P2X receptors are thus involved in stem cell plasticity, commitment and might be implicated in the early phases of carcinogenesis, when deregulation in proliferation/differentiation balance gives rise to malignant cells and CSCs (Fig.1).

The pivotal role of ATP and P2X receptors in cancer pain

Pain is an unpleasant sensation common to cancer patients. During course of cancer, pain sensation is present at any time, with different frequency and intensity, and it is progressively increasing in the advanced stages [133]. Pain sensations experienced by patients depend upon cancer type [134], site of primary and metastatic cancer development and finally on treatment, i.e. chemotherapy, radiotherapy [135-137] or surgery [138]. The three classical types of pain, i.e. neuropathic [139, 140], somatic/nociceptive [137] and inflammatory, can be identified in all cancer phases [136, 141]. In fact,

cancer expansion generate nerve pain caused by pressure on nerves [142] or the spinal cord [143], as well as soft tissue pain [144].

Normally the onset of cancer related pain is characterized by intermittent episodes, rapidly transformed into continuous chronic sensations [145, 146]. Furthermore, anti-cancer treatments or surgical cancer removal cause mainly nociceptive and neuropathic pain [136]. Indeed, patients undergoing surgery could be affected by phantom pain [147]: a pain arise in a part of the body that has been removed. When classifying cancer pain, not only the source or anatomical localization of cancer has to be considered, but also the severity. The pain patients' experience can be divided in three classes: mild, moderate and severe [148, 149]. Indeed, during cancer progression, there is also a developing of pain from acute to chronic [150] persisting even in case of complete eradication of cancer. The necessity to eradicate not only cancer but also to manage cancer pain is an open clinical challenge [151-153].

The role of ATP and its receptors in pain sensations, postulated by Burnstock in 1996 [154], has been extensively proved [155, 156]. The main P2X receptors involved in pain signaling are P2X3, P2X2/3, P2X4 and P2X7 [155, 156]; which are involved in cancer pain too [2]. P2X3 and P2X2/3, receptors abundant in dorsal root (DRG) and trigeminal ganglion, localized on small-to-medium diameter C-fiber and A δ sensory neurons, have a crucial role in nociceptive transmission and mechano-sensory transduction [157]. The well-known contribution of DRG neurons in cancer bone hyperalgesia [158] was also characterized by P2X3 receptor up-regulation and increased pain sensation in murine models of bone pain [159, 160]. Increased P2X3-P2X2/3 expression in DRG neurons was also confirmed in melanoma-bearing mice [161], where spontaneous pain behavior induced by the tumor was alleviated by P2X receptor antagonists [161] (Fig.2).

The involvement of P2X4 and P2X7 receptor in cancer pain have to be considered more "indirect" in comparison to that of P2X2/3 receptors [160]. In fact, P2X4 and P2X7 are expressed mainly on non-neuronal cells surrounding the neurons involved in pain perception [162, 163]. However, P2X4 and P2X7 play a critical role in inflammatory and neuropathic pain, participating and supporting the function of P2X2 and P2X3 on DRG neurons [163-165] (Fig.2). P2X4 receptor is up-regulated in tumor associated macrophages and microglia [83], probably playing a function also in the development of cancer pain. P2X7 has been speculated responsible for hypersensitivity in neuropathic and inflammatory pain states [166]. In fact, recently Huang *et al.* [167] demonstrated a function for P2X7 receptor in the induction and maintenance of bone cancer pain *in vivo*, using antagonist and siRNA

strategies. Nevertheless, different reports underlined contrasting information using different P2X7 null mice strains. Chessel and colleagues showed an absence of responsiveness to noxious thermal or mechanical stimuli in C57BL/6 of $p2x7^{-/-}$ mice [168], confirmed by anti-nociceptive role of P2X7 receptor antagonists [168-171]. *Vice versa*, BALBcJ P2X7-deficient mice demonstrated a susceptibility to cancer-induced bone pain [172]. Discrepant results obtained in different mice strains are possibly ascribable to different P2X7 polymorphism expressed by C57BL/6 and BalbcJ mice that would affect pain related phenotypes of WT counterparts of P2X7 less mice [160, 166, 171, 173].

P2X pharmacological strategy against cancer

In the 80s Burnstock and Kennedy proposed the division of P2 receptors in P2X and P2Y subgroups on the basis of their pharmacological behavior [9]; furthermore, specific agonists and antagonists are characterized for the different P2X1-7 receptors [8]. Specificity and efficacy of the different P2X receptors blockers has been covered by different reviews [8, 174, 175] and other dedicated manuscripts in this special issue. Here, we focus our attention on the possible application of P2X agonists, antagonists and inhibitors to eradicate or reduce cancer progression and cancer related symptoms (Fig.2).

P2X antagonist/inhibitor strategy

Various P2X antagonists have been tested in animal models with different purposes: reducing cancer growth and cancer pain. The main class of P2X antagonist tested for their ability to ease cancer pain acts at P2X2/3 receptors. The P2X2/3 specific antagonist A-317491 [176], was tested in both rat and mice models of bone cancer pain, showing respectively a continuous [159, 177] or transient analgesic action [177]. Differences reported seem to be ascribable to poor central nervous system (CNS) penetration of this antagonist thus limiting its clinical use [178]. AF-353, another P2X2/3 and P2X3 antagonist characterized by oral bioavailability and CNS penetration, attenuates bone cancer pain [178]. Interestingly, Suramin, an unspecific P2X antagonist administered in phase III clinical trials to patients of prostate cancer significantly improved their quality of life by reducing pain sensation [179]. Similarly, different P2X7 antagonists are in pre-clinical or clinical trial phases for the treatment of neuropathic and inflammatory pain, with promising results [174, 180]. Among these compounds, A-740003, a specific P2X7 antagonist, showed anti-nociceptive effects in neuropathic pain models [181] but it was not tested in cancer models. The *in vivo* and *in vitro* anti-nociception effects of another

selective P2X7 receptor antagonist, A-438079, were characterized [169] but this compound fails to alleviate bone cancer pain-related behaviors [172]. This incongruity could be ascribed to the cancer animal models used in different reports [173, 182]. Finally, a role of P2X7 receptor in cancer pain was demonstrated in rat bone cancer models where P2X7 is up regulated in the rostral ventromedial medullar activated glial cells [167]. In this cancer bone model, administration of P2X7 antagonist brilliant blue G (BBG) induced a down-modulation of P2X7, accompanied by a down-regulation of pain perceptions [167]. P2X7 antagonist not only reduce cancer pain, but, even more important, can inhibit tumor growth and dissemination. Different compounds are effective against distinct cancer types. P2X7 receptor antagonism promotes host survival in metastatic models [183] as well as reducing tumor growth [49]. P2X7 receptor inhibitors have the same effect on primary tumors: oxidized ATP (oATP) administration reduce B16 melanoma [184] and CT26 colon carcinoma [49] tumor growth. On the other hand, contrasting reports showed both facilitation [185] and a reduction [186] of experimental glioma growth, after the administration of BBG. Moreover, the different antagonist/inhibitor effects on cell metabolism and differentiation have to be taken in account [126, 187]. Interestingly, ATP and P2X receptors contribution in cancer progression, is indirectly proved also by the efficacy of not-P2X-specific anti-cancer compounds such as the traditional Chinese medicine compound Emodin that inhibits P2X7-mediated cancer cell migration [109]. Other drugs that exert an anti-tumor action through P2X7 are statins. Statins are commonly used cholesterol-lowering drugs [188], that exhibit antitumor effects both experimental models and treated subjects [189]. Interestingly, Mistafa *et al.* [190] proposed that statins would induce tumor growth blockade through P2X7 activation [190]. The authors demonstrated that, in pancreatic cancer cell lines, statins, acting at P2X7 receptor, inhibits cell growth increasing the effect of chemotherapeutic drugs. Interestingly, prolonged exposure of lung cancer cells A549 to the statin atrovastin caused an increase in P2X7 expression accompanied by a reduction in P2X4 protein levels [191].

The P2X agonist strategy

As previously described, tumor cells show high levels of P2X7 receptor thus due to receptor's cytotoxic properties; also application of P2X agonist, such as ATP can be considered a strategy to inhibit tumor growth. In order to reach the millimolar concentrations of ATP required for nucleotide-dependent cytotoxicity and to maintain these concentrations, following ecto-nucleotidases action, very high doses of ATP have to be injected. Administration of ATP or synthetic analogs at high dosage

caused an arrest in tumor growth in case of hormone-refractory prostate cancer [192] and in colon cancer models [193]. Clinical trials with intravenous injection of ATP were performed in patients with advanced non-small-cell lung cancer, showing an improvement in the quality of life and reducing cachexia effects [194]. However, in a following study, the administration of reduced ATP doses through shorter infusions failed to improve the quality of life of pre-terminal patients of different tumor types [195]. Furthermore, oral administration of ATP, in enteric-coated pellets, was ineffective in increasing patients' quality of life, and did not augmented plasma levels of ATP itself or other bioactive metabolites (i.e. ADP, AMP, adenosine, adenine), with the exception of uric acid.

Interestingly, ATP release and P2X7 receptor expression can be enhanced by irradiation [196]. This phenomenon facilitates a positive feedback loop in ATP release and P2X7 receptor expression [197]. Gehring *et al.* [82] demonstrated that this phenomenon happens also in radioresistant glioma, an additive ATP administration enhance pro-apoptotic function, inducing tumor cell death [82]. The beneficial effects of ATP could be also enhanced, as suggested by Ghiringhelli *et al.* [198], blocking ectonucleotidases or adenosine receptors, in order to keep the extracellular levels of ATP at a cytotoxic concentration.

Even chemotherapeutic compounds induce the production of ATP by tumor cells [199], and the pore conformation assumed by P2X7 receptor when exposed to high ATP concentration, facilitate the passage of hydrophilic chemotherapeutic agents [174, 200, 201]. The synergic effects of chemotherapy drugs and ATP seems another promising strategy [28].

Summarizing, accumulating evidence strongly suggest the clinical relevance of ATP and P2X receptors in anti-cancer therapeutic approaches. The adequate anti-cancer strategy, P2X agonists or antagonists, depends on multiple factors, including: genetic of the patients [202], different cancer types and their site of onset, P2X7 receptor tumor expression [203], and cancer-related pain sensations.

The final goal of ATP as therapeutic approach is to induce cellular death in tumor, and act in synergy with chemotherapy or radiotherapy. However, in order to obtain good results, the treated tumor has to be characterized by an endogenous high P2X7 receptor expression. Moreover, the delicate balance between beneficial and detrimental ATP (and adenosine) effect for cancer and for innate immune responses have to be taken in account, as recently characterized in neuroblastoma model [204]. Furthermore, the effect of high ATP dose administrations in patients affected by cancer pain remains unclear.

Based on all these different factors, it is tempting to speculate that a therapy based on P2X agonists administration will expose patients to more risks (i.e. pain development, immune system deregulation) than another one based on P2X antagonist/inhibitors. Moreover, joint administration of different P2X blocking drugs will allow the simultaneous blockage of tumor progression and cancer related pain.

Figure legends

Figure 1. P2X receptors role in cancer growth and progression. P2X receptors pattern expression varies along cell differentiation influencing stem cell fate. Altered imbalance between proliferation/differentiation give rise to cancer cells. Cancer cell P2X receptors sustain tumor proliferation and progression enhancing metabolic pathways and consequently cell growth. P2X7 receptor mediates itself ATP secretion. P2X7 promotes metastasis by favoring new blood vessel formation (via VEGF), extracellular matrix degradation (via protease secretion) and tumor cell migration spreading.

Figure 2. Effect of pharmacological modulation of P2X receptors in cancer.

P2X receptors involvement in pharmacological treatment of tumors occurs at different levels. Some classes of chemotherapeutics, such as anthracyclines, are able to elicit immunogenic cell death of cancer cells. ATP released during this event, acting at P2X receptors, stimulates immune cells to eradicate tumor. P2X receptors themselves are good therapeutic targets for cancer treatment. P2X2, P2X3, P2X4 and P2X7 antagonists reduce cancer pain, while P2X7 antagonist inhibits cell proliferation. High concentrations of ATP, acting as P2X7 agonist cause cancer cells death through receptor's cytotoxic function.

List of abbreviations:

ALL: Acute lymphoblastic leukemia; ATP: Adenosine triphosphate; BBG: brilliant blue G; CLL: chronic lymphocytic leukemia; CNS: central nervous system; CSC: cancer stem cell; DAMP: damage-associated molecular patterns; DRG: Dorsal root ganglion; ES: embryonic stem cells; ICD: immunogenic cell death; HIF-1: hypoxia-inducible factor 1; HSC: hematopoietic stem cells; IL: interleukin; NFAT: nuclear factor of activated T cells; NLRP3: NACHT, LRR and PYD domains-containing protein 3; OE: olfactory epithelium; p38-MAPK: p38 mitogen-activated protein kinases; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; VEGF: Vascular endothelial growth factor;

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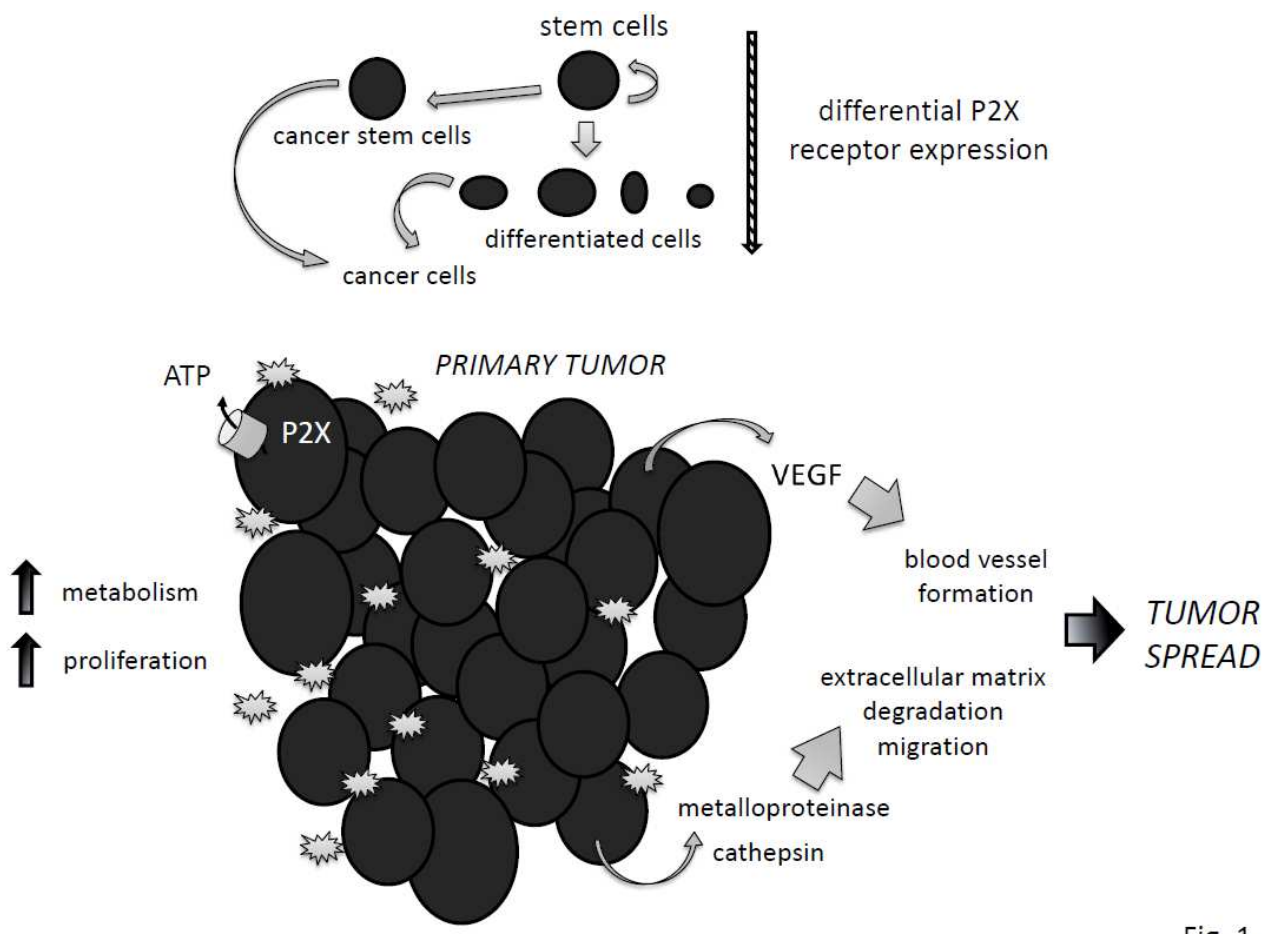


Fig. 1

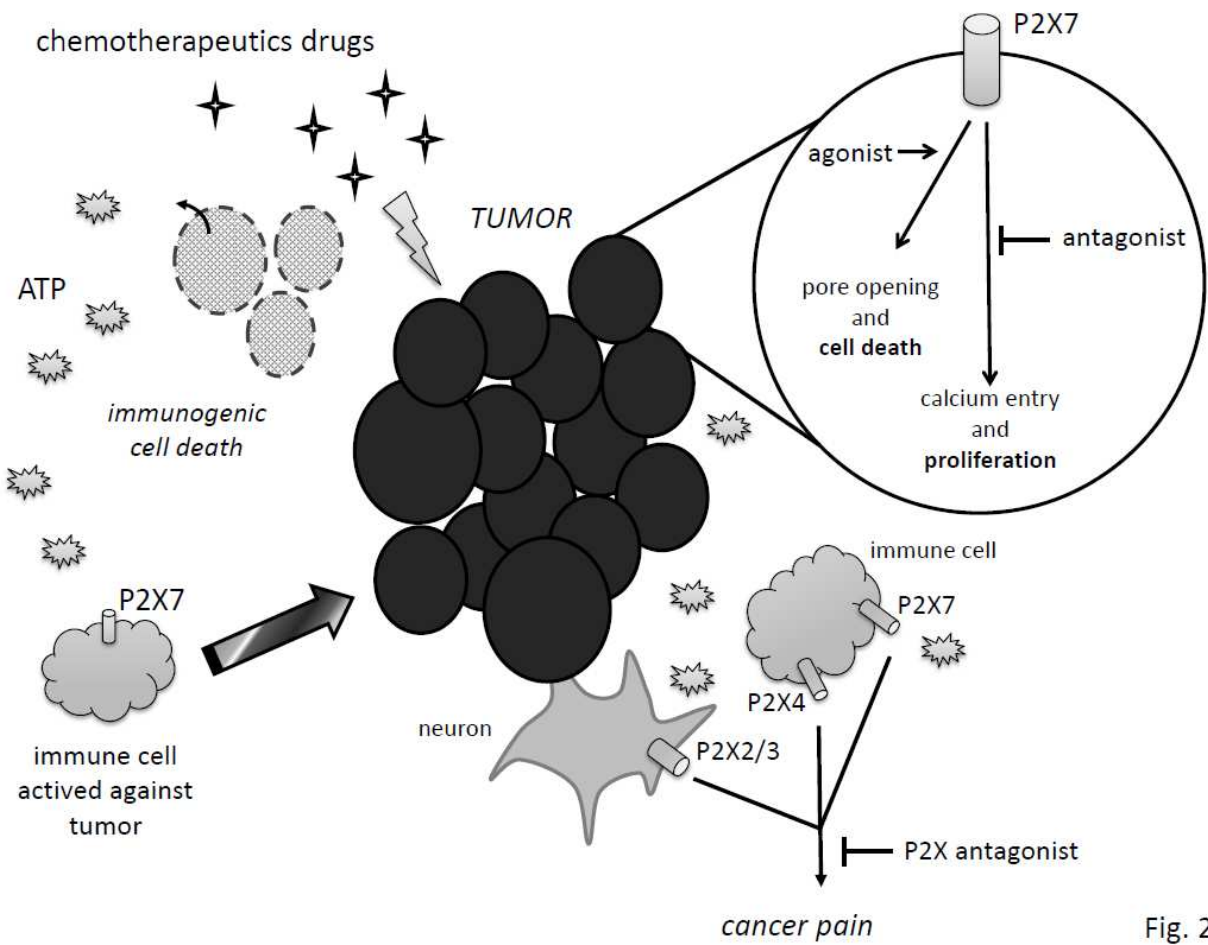
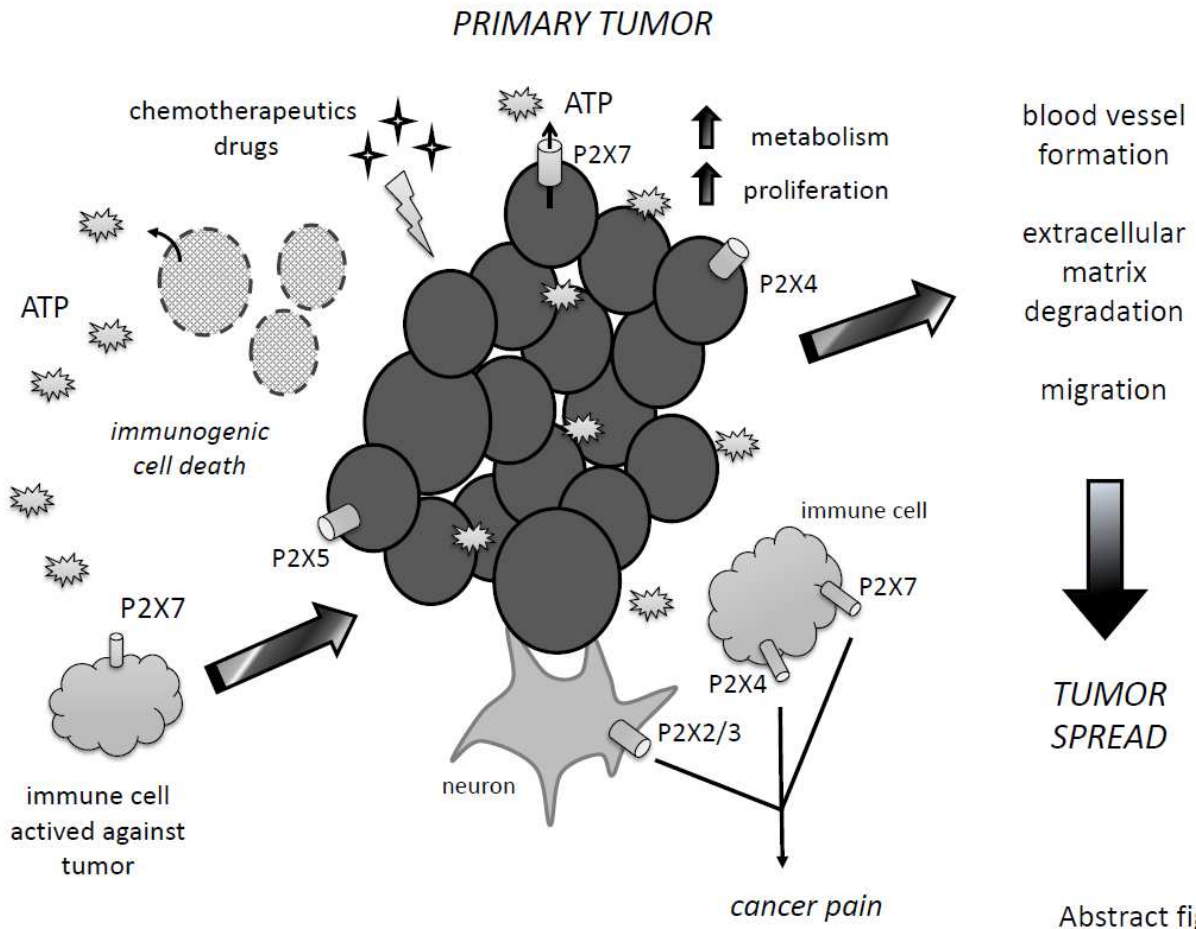


Fig. 2



Abstract figure