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A Purinergic Trail for Metastases

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Abstract:	Nucleotides and nucleosides have emerged as important modulators of tumor biology. Recently acquired evidence shows that when these molecules are released by cancer cells or surrounding tissues, they act as potent pro-metastatic factors favoring tumor cell migration and tissue colonization. Nucleotides and nucleosides should therefore be considered a new class of pro-metastatic factors. In this review, we focus on the pro-metastatic roles of nucleotides and discuss future applications of purinergic signaling modulation in view of anti-metastatic therapies.

To the Editorial Office
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Dear Dr. Schaffhausen,

We are very thankful to you and the reviewers for deep revision and useful advices. We have accepted suggested corrections and modified the text accordingly. Changes are marked in red. We hope the amended version of the review is now suitable for publication in *Trends in Pharmacological Sciences*.

We look forward to hearing from you at your earliest convenience,

Kind Regards

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A Purinergic Trail for Metastases

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Keywords

P1 receptors, P2 receptors, extracellular nucleotides, tumor, chemotaxis.

Abbreviations

AC, adenylyl cyclase; ADA, adenosine deaminase; AMP, adenosine monophosphate; ADO, adenosine; ALP, alkaline phosphatase; Caco-2, human epithelial colorectal adenocarcinoma cell line 2; CAF, cancer-associated fibroblasts; CBK, creatine kinase brain-type; ecto-nucleoside triphosphate diphosphohydrolase CD39; CD73, ecto-5'-nucleotidase; DC, dendritic cells; MMP, metalloproteinase; NF-κB, nuclear factor-kappaB; NPP1, nucleotide pyrophosphatase/phosphodiesterase-1; NTPDases, ectonucleoside triphosphate diphosphohydrolases; RAGE, receptor for advanced glycation end products; VEGFR-2, vascular endothelial growth factor receptor 2.

Summary

Nucleotides and nucleosides have emerged as important modulators of tumor biology. Recently acquired evidence shows that when these molecules are released by cancer cells or surrounding tissues, they act as potent pro-metastatic factors, favoring tumor cell migration and tissue colonization. Nucleotides and nucleosides should therefore be considered a new class of pro-metastatic factors. In this review, we focus on the pro-metastatic roles of nucleotides and discuss future applications of purinergic signaling modulation in view of anti-metastatic therapies.

The Purinergic Extracellular "Apparatus" of the Cell

Purine and pyrimidine nucleotides (ATP, ADP, UTP, UDP) and nucleosides such as adenosine (ADO) are fundamental molecular building blocks, intracellular modulators, and energy providers required by cells to live. A more recently appreciated feature of nucleotides and nucleosides is their ability to play other roles outside the cell, where they can be synthesized, released and transported in different ways [1-3]. For example, formation of ATP on the extracellular side of the cell membrane results from the activity of the plasma membrane enzyme F0F1 ATP synthase, catalyzing the phosphorylation of extracellular ADP to ATP as well as its dephosphorylation [4].

ATP can also be released as a consequence of physiologic or pathologic stimulation of the cell. To the first category, we can ascribe the release of the nucleotide from neurons, secretory cells, astrocytes, muscle cells, fibroblasts, macrophages, erythrocytes, platelets, dendritic cells, neutrophils, hepatocytes, and cholangiocytes [5,6]. Analogously, a physiological release of ADO has been observed from neuronal cells [7,8], kidney [9], cardiomyocytes [10], and immune cells [11].

Under pathological conditions release of ATP, but also UTP and UDP is due to very different cell stressors [12], mainly damaging the plasma membrane such as in the case of endothelial damage, intoxication by bacterial toxins or platelet clotting, or as a consequence of bacterial or viral infection, or again because of mechanical stress or allergen contact. In these cases, ATP functions as an "alarm signal" alerting and activating in an autocrine/paracrine manner the surrounding immune cells to fight microbes and/or initiate tissue reparative responses [13-15] or to localize and engulf apoptotic cells [16]. Many stimuli are endowed with the ability to induce a release of ATP and other nucleotides from normal cells [17-19], but this is true also for tumor cells [20-23].

Once in the extracellular *milieu*, ATP (and ADP) are quickly hydrolyzed to AMP (by the ecto-nucleoside triphosphate diphosphohydrolase, NTPDase/CD39) and subsequently to ADO (by the ecto-5'-nucleotidase also indicated as CD73) [24-27]. Among nucleotide and nucleoside degrading

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enzymes, we also have to mention alkaline phosphatase (ALP), nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1, CD203a) which is abundantly present in glioma and prostate cancer cells, and NTPDase2 expressed for example in human colon HT-29 cells and preferentially hydrolyzing ATP. Extracellular ADO is then eventually transformed into inosine, which is inactive at P1 receptors, by the enzyme adenosine deaminase (ADA, CD26) (Figure 1).

If ATP is not degraded, it binds to a group of purinergic receptors, the P2 receptors which, according to their different intracellular signaling pathways and selectivity toward nucleotides, are currently classified as ionotropic P2X (P2X1–7) or metabotropic P2Y (P2Y1, 2, 4, 6, 11–14) receptors [28,29]. P2X receptors are cationic channels activated by extracellular ATP and selective for monovalent and divalent cations (Na^+, K^+, Ca^{2+} and Mg^{2+}). P2Y receptors are G-protein-coupled plasma membrane receptors with seven spanning motifs, an extracellular amino terminus and an intracellular carboxy terminus [30]. Eight human P2Y subtypes have been identified and characterized. They are: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14. P2Y receptor subtypes show heterogeneity in G-protein coupling, agonist specificity and intracellular signaling. P2Y1, P2Y12 and P2Y13 subtypes are preferentially activated by ADP, while UDP is an agonist at P2Y6. P2Y2, P2Y4 and also P2Y6, are activated by UTP, while P2Y11 (as well as P2Y1 and P2Y2) are activated by ATP. P2Y14 is activated by UDP sugars such as UDP-glucose [30]. The biological actions of ADO are mediated by four G-protein-coupled receptors known as P1 receptors and currently grouped into four subtypes: A_1, A_{2A}, A_{2B} and A_3. They are also known as ADORA1, ADORA2A, ADORA2B or ADORA3, respectively [11, 31-33]. A_1, A_{2A}, and A_3 show a higher affinity for ADO than A_{2B}. Differences among subtypes also concern affinity towards specific G protein families and effects on the enzyme adenylyl cyclase (AC) which is inhibited by A_1, and A_3, while it is activated by A_{2A} and A_{2B} subtypes [31].

Transformed cells show abnormalities both in intracellular purine metabolism and in extracellular purinergic signaling [34]. Similarly to normal cells, cancer cells need ATP as an energy source to live,

replicate and migrate; however, differences concerning ATP production have been described. It has long been known that in transformed cells, glycolytic generation of the nucleotide can assume a predominant role (**Warburg effect**). Recent observation shed light on at least two previously unknown systems for ATP provision of cancer cells. In hypoxic conditions, colon metastatic cells migrating to the liver release creatine kinase brain-type (CBK), an enzyme which catalyzes phosphorylation of extracellular creatine, generating phosphocreatine that is transported back into the cell by the SLC6A8 carrier. Phosphocreatine is then used intracellularly by the tumor cell to generate ATP [35]. Another system utilized by the tumor cell to intercept ATP is internalization of the extracellular nucleotide by **macropynocytosis** [36] (i.e., through formation of macropynosomes which are plasma membrane invaginations that form intracellular compartments mainly used by the cell for the uptake of extracellular fluids). Macropynocytosis confers to cancer cells at least two indubitable advantages: (1) ATP can be endocytosed from the intracellular space and then released intracellularly, thus causing a large increase in the concentration of the cellular energy pool, and more importantly (2) sequestration of extracellular ATP represents a condition that improves survival and resistance of cancer cell to drug treatments [37].

A very recent finding is that extracellular ATP may also contribute to the chemoresistance of cancer cells by increasing the expression of multidrug resistance associated proteins (i.e. proteins endowed with the ability to extrude anticancer drugs from the tumor cell), thus reducing the intracellular drug concentration and consequently its toxicity. An important observation is that in colorectal cancer cells, the multidrug-resistance-associated protein 2 is upregulated by extracellular ATP [37]. In line with this evidence, the expression of multidrug-resistance proteins has been associated to poor prognosis in cancer patients [37].

Another piece of the complicated “ATP puzzle” is the expression by cancer cells of enzymes deputed to control extracellular ATP availability. Already many years ago, F1F0 synthase expression was

detected on the cell membrane of cancer cells, and it is possible that this enzyme may condition availability for P2 receptors of its substrates ATP and ADP [38]. Another important finding concerns the ability of cancer cells to secrete soluble enzymes involved in ATP synthesis. Hence, human breast cancer cells liberate the adenosine 5'-diphosphate transphosphorylase (**sNDPK**), catalyzing the synthesis of ATP in the extracellular *milieu.* The authors suggest that this event stimulates neo-vascularization through P2Y1-dependent endothelial cell migration and VEGFR-2 engagement [39].

The above evidence highlights the extreme complexity of the purinergic system with its synthetic and catabolic pathways, pointing out the high degree of interconnection between extracellular and intracellular enzymes, transporters and receptors. Moreover, one has to consider that in the tumor microenvironment, the purinergic machinery undergoes a marked reorganization whose resulting response is often aimed at sustaining and magnifying pro-tumorigenic mechanisms.

Tumor Cells and Extracellular ATP: a Complex Relationship

A common state shared by the majority of transformed tissues is an aberrant, sometimes referred to as "disordered", purinergic signaling network, implying overexpression or, in some cases, decreased expression of single P1 or P2 receptor subtypes and/or ecto-nucleotidases. Therefore, extracellular nucleotides and nucleosides present in the tumor niche find an abnormal enzymatic and/or receptor arrangement inducing potentially negative responses (such as inhibition of the immune response against tumor cells) able in many cases to favor tumor growth, invasion and metastasis [40]. For example, an aberrant purinergic context plays a pivotal role in subverting the immune context and response against tumors, thus favoring growth and progression of the neoplasia. To this purpose, it is worthy to mention the immunosuppressive effect of adenosine monophosphate (AMP) on lymphocyte activity [41] or that of ADO, by both receptor-dependent and

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receptor-independent mechanisms, on NK cytotoxic activity, T helper 1 contribution, expansion of myeloid-derived suppressor cells (MSDC) and M2 macrophage polarization [42-47].

Studies have shown that the extracellular *milieu* surrounding tumor cells contains elevated ATP concentrations, reaching in some cases the hundred micromolar range [48-49]. This is peculiar given that extracellular ATP concentration is normally so low that is barely detectable in normal tissues, in physiologic conditions. Accumulation of ATP in the extracellular *milieu* of the tumor site is likely due to different and often concurrent situations. It is important to note that although cancer cells express ectonucleotidases, they can show a low extracellular ATP hydrolysis rate and sometimes an increased ecto-5'-nucleotidase activity, as in the case of human glioma cells. Given that ATP acts as a proliferative stimulus for glioma cells, an increase in its concentration may represent an advantage for tumor growth [50]. In other tumors, such as astrocytoma, the release of ATP is paralleled by that of UTP and UDP-glucose, a condition favoring activation of multiple P2 receptors [5,51].

Of note, the ADO system contributes to the regulation of cancer growth and dissemination by interfering with the processes of proliferation, apoptosis and metastasis through the engagement of ADO receptors, which are highly expressed on the neoplastic cells. It has been observed that ADO exerts opposing effects on cancer cell growth, promoting cell proliferation in some cases and curbing this process in others, based on which and in what extent ADO receptor subtype is engaged in selected tumors [40]. The net cellular response to purinergic stimulation depends on the availability of the nucleotide/nucleoside in the extracellular environment. Increasing studies demonstrate that the majority of purinergic processes, modulated by activation of purinergic receptors, are regulated by a few dominant enzymes, which are actively involved in the rapid degradation of extracellular nucleotides, and thus, in shaping the purinergic response, depending on the metabolic conditions of the tissue [50]. In this regard, the CD39/CD73 pathways (see Figure 1) pivotally involved in leading the shift from an ATP-driven pro-inflammatory environment to an immunosuppressive *milieu*

sustained by ADO, undergoes dynamic changes based on the pathophysiological context in which it is embedded. In particular, it has been observed that CD39 and CD73 are highly expressed in several human solid neoplasias, where they actively contribute to cancer cell proliferation and dissemination [53-55]. By contrast, the expression of the catabolic enzyme ecto-adenosine deaminase as well as nucleoside transporters is decreased in cancer tissues, with consequent increases in ADO concentrations within the cancer *milieu* [40].

Another frequent aberration found in cancer cells is the high levels of expression of the pore-forming P2X7 receptor [56-58]. This implies at least two opposite consequences: i) an increased P2X7-mediated signaling that, depending on the tumor type, can stimulate either proliferation [59-60] or cell death [62-63], and (ii) an increased ATP release, favoring cancer cell migration and metastasis [20,38,64-67] (Figure 3). Damage due to noxious side effects on healthy tissues, as a consequence of antitumor chemotherapy or radiotherapy, induces the release of intracellular ATP, alerting and attracting immune and non-immune cells to the lesion sites and stimulating production of cytokines, chemokines, growth factors and bioactive phosphosphingolipids [68-71]. Hence, ATP released by dying apoptotic cancer cells as a consequence of chemotherapeutic treatment with oxaliplatin stimulates P2X7 receptor expressed by dendritic cells (DC). This induces NLRP3/ASC/caspase-1 inflammasome activation and subsequent interleukin-1beta (IL-1beta) secretion [70]. Production of IL-1beta is pivotal for polarization of IFN-gamma-producing $CD8^+$ T lymphocytes, cytotoxic for the tumor [69]. ATP secretion by dying cancer cells upon immunogenic chemotherapy occurs through a pathway that involves nucleotide transfer from lysosomes to autolysosomes and that is **Lysosomal Associated Membrane Protein 1 (LAMP1, CD107a)** and caspase-dependent [72].

A positive effect exerted against tumors has been obtained through the pharmacological treatment of cancer cells with ATP. This maneuver is aimed at increasing the up-take of cytotoxic

drugs by P2X7-overexpressing transformed cells, potentiating for example the cytotoxic effects of 4-hydroperoxycyclophosphamide (4HC) towards leukemia cells, minimally influencing viability of normal hematopoietic stem cells [73]. On the same line, ATP administration magnifies the effect of etoposide (VP16) treatment in lung adenocarcinoma cell lines, allowing elimination of cancer cells, another ATP-linked important application useful for purging leukemic cells before autologous stem cell transplantation [73-74]. Although encouraging results have been obtained, further investigation on this issue is needed to clarify why the cytotoxic activity of anti-tumor drug /ATP co-administration, sometimes shows a biphasic effect on tumor growth inhibition and apoptosis, suggesting that the mechanism involved is likely more complex than previously thought [75].

The relevance of the tumor microenvironment for tumor growth, invasiveness and metastasis has been highlighted in different ways and by many scientific reports. Several lines of experimental studies have shown that extracellular nucleotides and nucleosides are also endowed with the ability to induce and modulate the migratory properties of tumor cells [22, 76-78]. Consistent with these findings, tumor cells attracted by released nucleotides can egress the primary site and migrate to secondary sites, thus invading previously untouched tissues. Since liberation of nucleotides occurs from cells as a consequence of the treatment of patients with antitumor-drugs, metastatic dissemination of the surviving cancer cells would therefore be an extremely dangerous side effect of antitumor therapy toxicity [71].

It has recently been proven that bone marrow cells flushed from mice subjected to irradiation as well as treatment with **vincristine**, release ATP, UTP and ADO. This event is likely due to cells dying upon treatment and the subsequent liberation of factors increasing tissue receptiveness towards tumor cell metastases [79]. Of note, tumor growth can itself cause the release of nucleotides from neighboring tissues. For example, the growth of gliomas induces glutamate liberation with consequent neuronal intoxication and death followed by ATP liberation that act as chemoattractant

and thus prompting cancer cell migration (Figure 3). *Clostridium difficile* toxins induce the release of UDP (a P2Y receptor agonist) from human epithelial colorectal adenocarcinoma (Caco-2) cells, and activation of the P2Y6 subtype causes an increase of CXCL8 expression [12]. This represents a paradigm for purinergic-mediated chemokine induction, where cell death or tissue damage cause the release of intracellular nucleotides that act as danger signals to induce chemokine expression and secretion. These events attract immune cells but potentially also cancer cells expressing receptors for the secreted chemokines. Cell chemotaxis also plays a crucial role in tumor biology, and there is evidence that in cancer microenvironment, chemotaxis pathways can be reprogrammed in favor of tumor cell dissemination [80]. Accordingly, chemotaxis of carcinoma and tumor-associated inflammatory and stromal cells is mediated by chemokines, chemokine receptors, growth factors and growth factor receptors [80]. As recently acquired, chemokine secretion is also stimulated by nucleotides in transformed cells, thus increasing their possibility of dissemination and colonization of healthy tissues.

Nucleotides Released from Tumor Cells Stimulate Chemokine Secretion

Cancer metastatization is driven by a plethora of factors, among which chemokines and their receptors play a central role [80]. For example, the chemokine CCL2/MCP-1 is involved in metastatization of myeloma, breast cancer, prostate cancer, bladder cancer, and renal cancer [81]. Similarly, an increase in IL-8/CXCL8 concentration has been detected within the microenvironment of different tumors, where it stimulates proliferation, angiogenesis, migration, and metastatic invasion [82]. Expression of CXCR6 by non-small cell lung carcinoma (NSCLC) promotes its metastatic diffusion by modulating **metalloproteinases (MIPs)** [83], and CXCR6/CXCL16 signaling has also been shown to promote breast cancer progression [84]. This cytokine and its receptor are considered prognostic factors in prostate cancer [85].

A relationship between tumor secreted chemokines and extracellular nucleotides has emerged some years ago. In this regard, P2X7 and P2Y6 receptor stimulation mediates the secretion of the chemokines IL-8/CXCL8 and MCP-1/CCL2 as well as proliferation in human glioma cells [86], and P2X7 stimulation induces MCP-1, IL-8 and VEGF secretion in rat C6 glioma cells [87,88]. Interestingly, CXCL16 is also linked to the purinergic signaling network, given that P2X7 receptor stimulation causes shedding of CXCL16 from human B myeloma, and the process is dependent on the metallopeptidase ADAM10 [89].

Several data points to a different role for ADO in modulating chemokine production in the tumor microenvironment. Hence, ADO inhibits expression of chemokines in tissues invaded by metastatic-like melanoma cells, hindering for example CXCR3-cognate chemokine pathways and consequent T-cell infiltration [90]. On the same line, it has been observed that under hypoxic conditions ADO, via interaction with A_{2A} and A_{2B} receptors, inhibits CCR7 expression on T cells. Since CCR7 is involved not only in lymphocyte homing but also in T-cell protection from apoptosis, it is conceivable that ADO may weaken this function holding a critical role in hypoxia-induced apoptosis of T cells [91]. On the other hand, it has been reported that ADO, via activation of A_{2A} and A_{2B} receptors, upregulates CXCR4 expression and its protein level at the surface of HT29 colonic cancer cells. This event, enabling the neoplastic cells to respond to CXCL12, increases their rate of migration and proliferation [92]. Of note, secretion of the pro-inflammatory chemokine IL-8/CXCL8 in HT29 cancer cells is linked to purinergic receptors activation, which in turns depends on expression and activity of the ectoenzymes NTPDase2, adenylate kinase (ADK) and CD73 that condition nucleotide and nucleoside concentration of the extracellular *milieu*. In particular, IL-8/CXCL8 secretion is sustained by the presence of an "ATP halo" maintained by ADK and NTPDase2 activity, while in this experimental model (human colon HT-29 cells), the presence of ADO deriving from CD73 activity does not affect IL-8/CXCL8 release [93]. Increasing attention has been giving to a previously

neglected cell type contributing to cancer growth an invasiveness, i.e. cancer-associated fibroblasts (CAF), whose impact on promotion of cancer development occurs via secretion of multiple cytokines and chemokines. Recent data point for a role of the CAF SDF-1/CXCR4 pathway in endometrial cancer progression [94] or for CCL5 and IL-6 secretion in the invasiveness of prostate cancer [95]. Similarly, activation of the CAF CXCL12/CXCR4-mediated pathway promotes invasiveness of gastric cancer [96]. Therefore, a deep investigation of the purinergic signaling of CAF and its participation in the modulation of chemokine and cytokine secretion during cancer development would be highly needed.

These findings shed new light on important aspects of cancer biology and progression, linking purinergic receptor activation with chemokine secretion by tumor cells. This axis could likely be used as a potential prognostic marker and perhaps by a new therapeutic target.

Metastatic Cells Use Extracellular ATP and ADP as a Trail

Cell chemotaxis is a complex coordinated series of events that allows cells to move in a particular direction within a chemical gradient [80]. Purinergic receptors play an important role in nucleotide- and nucleoside-induced chemotaxis in normal [97] as well as in cancer cells. As an example, the ATP and UTP activated P2Y2 receptor is endowed with the ability to induce cell motility and oriented migration in human lung cancer cells [79], while rat schwannoma cells incubated *in vitro* with UTP migrate and secrete MMP-2 via a MAPKs phosphorylation-dependent mechanism [98].

Single purinergic receptors interact physically with plasma membrane molecules, such as **integrins**, that are involved in cell migration. The Gcoupled P2Y2 receptor is involved in chemotaxis induced by UTP. This receptor subtype contains an integrin-binding domain in its first extracellular loop that is made up of the three arginine-glycine-aspartic aminoacids (RGD). This evidence supports

the hypothesis that this receptor can interact directly with integrins. The event was confirmed by the ability of an anti-αVβ3/β5 integrin antibody to inhibit P2Y2 signal transduction in K562 erythroleukemia cells [99]. This important finding was then elegantly demonstrated also in human 1321N1 astrocytoma cells, which require a P2Y2-RGD-dependent interaction with α(v) integrins to activate G(o) and initiate G(o)-mediated signaling events leading to UTP-induced cell migration [100].

The mRNA and protein levels of P2Y2 are markedly higher in human hepatocellular carcinoma as well as in the human hepatocellular carcinoma cell lines HepG2 and BEL-7404, in comparison with normal human hepatocytes [101]. Extracellular ATP, which is also an agonist at the P2Y2 receptor, promotes proliferation and migration of cancer cells in nude mice [101-102].

The highly metastatic breast cancer cell line MDA-MB-231 releases ATP levels higher than the low-metastatic breast cancer cell line MCF-7 [77] and shows higher P2Y2 activity than the same low-metastatic cell line [77]. Activation of the P2Y2 subtype by ATP or UTP in MDA-MB-231 cells increases matrix metalloproteinase-9 (MMP-9) activity, vascular endothelial growth factor (VEGF) production and phosphorylation of vascular endothelial (VE)-cadherin of the endothelium, with MDA-MB-231 cells crossing of the endothelial barrier [76]. These authors elegantly demonstrated in a mouse model that tumor growth and metastatization are dramatically reduced in P2Y2-shRNA-transfected MDA-MB-231 cells, confirming the crucial role of this subtype in breast cancer growth and metastatization [76].

In addition, activation of the P2Y2 receptor induces hypoxia-inducible factor-1α expression, lysyl oxidase secretion and collagen crosslinking, thus contributing to the creation of global pro-metastatic conditions [77]. Highly metastatic cells show higher ERK and PKC phosphorylation levels that can be downregulated with the ATP hydrolyzing enzyme apyrase or by knocking down the P2Y2 subtype [77]. These events are not ancillary, as ERK and PKC inhibitors effectively reduce the

metastatic ability and expression of mesenchymal markers [77]. In prostate cancer, metastasis is driven by cooperation between the P2Y2 and EGF receptors, again through the activation of ERK1/2 proteins [68]. The effects mediated by P2Y2 activation in cancer also lead to the modification of the tumor niche. Indeed, nucleotides secreted from the highly metastatic breast cancer cell line MDA-MB-231 induce the liberation of P2Y2-dependent lysyl oxidase with the consequent crosslinking of extracellular matrix collagen, facilitating tumor cell colonization and metastasis [103]. Unfortunately, the P2Y2 subtype is not the only P2 actor participating in metastatic progression of cancer. Indeed, the P2X7 subtype has also been described as a crucial player in ATP-driven metastasis of prostate cancer. Pharmacological, genetic and molecular approaches have clearly shown that down-regulation of P2X7 expression in this and other cancers corresponds to a considerable attenuation of the migratory properties of cancer cells *in vitro*, and abrogation of tumor invasiveness and metastatic capacity in nude mice. For ATP-mediated prostate cancer invasiveness, the involvement of EMT/invasion-related genes and of PI3K/AKT and ERK1/2 signaling pathways have been hypothesized [21]. As confirmation of the recurrence of this P2X7-mediated activity, it has recently been shown that ATP also stimulates invasion and migration of human T47D breast cancer cells.The process is mainly mediated by the P2X7 subtype, since it is efficiently reduced by knocking-down of this subtype [78]. However, the scenario is likely more complex and might be dependent on P2X7-expressing cell type, on other co-expressed P2 receptors and extracellular ATP concentration. Indeed, it has recently been shown that ATP concentrations higher than 20 µM inhibit migration of breast tumor-derived endothelial cells and that the process is mediated by P2X7 and P2Y11 receptors [104]. Similarly, another study pointed out a different role for ATP and ADO released by bone osteocytes on breast cancer spread; in this case, the migratory ability of tumor cells was inhibited by ATP via P2X7 activation, while ADO had the opposite effect since it promoted cancer cell migration [22].

A commonly found condition in the tumor niche is hypoxia, particularly before cancer-induced neo-angiogenesis. Acidosis and hypoxia in the solid tumor microenvironment are considered factors favoring the chemoresistance and aggressiveness of tumor cells. An important observation obtained from *in vitro* experiments performed in conditions mimicking acidosis and hypoxia shows that these conditions lead to ATP release from B16 melanoma cells through a process mediated by the P2X7 receptor [23]. Another intriguing observation is related to the finding that hypoxia augments expression of P2X7 and the receptor for advanced glycation end products (RAGE), thereby modulating cancer cell invasiveness. The process occurs through Erk1/2 and Akt phosphorylation and nuclear translocation of nuclear factor-kappaB (NF-κB) [105]. Moreover, the main nuclear factor involved in gene regulation during hypoxia (i.e. the **hypoxia-inducible factor (HIF)-1α**)whose silencing in tumor cells causes downregulation of RAGE, P2X7R and NF-κB. On the contrary, BzATP, a very potent P2X7 agonist, stimulates tumor invasiveness through activation of MMP-2 and -9 [105]. In line with these findings, it has been shown that P2X7 receptor activation increases the invasiveness of the highly aggressive human breast cancer cell line MDA-MB-435s, through a mechanism involving Ca^{2+}-activated SK3 potassium channels and cysteine cathepsins [67].

Genetic deletion of the ectoenzyme CD39 precludes formation of new vessels, thus preventing cancer growth in mice implanted with tumors. Moreover, the absence of this enzyme hinders tumor diffusion and metastatization to the lungs, resulting in diminished activation of focal adhesion kinase [54]. Another important finding is that the release of ATP by tumor cells can modulate cell motility through P2X7 receptor activation. It has been nicely observed that the existence in cancer cells of a relationship between TGF-β1 induced cell migration and P2X7-mediated purinergic signaling. Hence, the stimulation of human lung cancer H292 cells with TGF-β1 induces ATP release and autocrine P2X7 activation. The pharmacological antagonization of P2X7 receptor with A438079, or AZ10606120 as well as the degradation of extracellular ATP via incubation with apyrase abrogates

cell migration, which in turn is enhanced by P2X7 stimulation [20]. Investigation on novel and potent P2X7 antagonists able to block P2X7-mediated tumor dissemination is ongoing [106]. The role of ecto-nucleotidases in modulating tumor growth and autophagy has been elegantly demonstrated by two recent studies [107-108]. When considering the involvement of CD39 in the metastatic process, it has been revealed an involvement of this ectoenzyme in a model of colorectal cancer dissemination [53]. In particular, in the mouse model of colorectal metastases, high levels of CD39 expression paralleled an increase of metastasis in the liver. Of note, in human tissues, low CD39 mRNA expression levels within the tumors appeared to correlate with less or delayed colorectal spread and better long-term survival. Recent studies indicate that the expression of CD39 is higher in cells from the bronchoalveolar lavage of patients with non-small cell lung cancer (NSCLC) than in those from chronic obstructive pulmonary disease (COPD) [109]. This was paralleled by significantly lower concentrations of ADP and ATP in BALF, suggesting that CD39 is active in the lung cancer microenvironment. Concerning metastasis, CD39 expression is higher in metastasized than in non-metastasized tumors, and the expression of specific P2 receptors (P2X4, P2X7 and P2Y1) is higher in tumors with distant metastases [109]. This suggests that purinergic signaling may contribute to the metastatic diffusion of the neoplasia. Expression of CD73 is tightly controlled by HIF-1-α and can be up-regulated during hypoxia [110]; accumulating evidence reports that CD73 is heavily involved in the regulation of metastatic processes, and indicates a tight correlation between the expression and activity of this enzyme in cancer cells and their ability to spread [111].

CD73 deficient mice display an increased antitumor immunity and are resistant to experimental metastasis [55]. It has been recently reported that expression of CD73 on tumor cells enhanced tumor metastasis in mice [112]. The tumor metastasis was counteracted by the treatment with the selective A_{2A} receptor antagonist SCH58261 or with the A_{2B} receptor antagonist PSB-1115, indicating a role for both the A_{2A} and A_{2B} receptors in enhancing $CD73^{+}$ tumor cell metastasis.

Moreover, simultaneous inhibition of CD73 and A2A, or blocking of CD73 by the recently introduced human monoclonal antibody MEDI9447 improves the immune-mediated anti-cancer response [41,113].

In vitro and *in vivo* studies confirmed the pro-metastatic effect of CD73-derived ADO, via A_{2B} receptor stimulation [114]. The critical involvement of A_{2B} receptor in driving metastatic process has been corroborated by additional studies showing that its blockade counteracts the survival and metastatic potential of breast cancer cells *in vitro* and suppresses breast cancer colonization in mouse lungs [115]. The molecular mechanisms underlying the pro-metastatic effect of A_{2B} receptors were recently demonstrated. Activation of this receptor subtype is followed by prenylation of the small GTPase RAP1, with a consequent reduction in cell adherent junctions, promoting the subsequent increase in cell diffusion [116]. Pharmacological blockade of A_{2B} receptors inhibits both experimental and spontaneous metastasis. In this context, inhibition of the metastatic process is independent of host A_{2B} as well as of lymphocytes and myeloid cells [117].

Concluding Remarks

The aim of the present review was to examine the role of nucleotides and nucleosides as chemotactic factors for tumor cells. Indeed, an important acquisition is that transformed cells exhibit an abnormal purinergic network compared to normal cells. Moreover, differences are present between metastatic and non-metastatic tumors, concerning expression and activity of single molecular components of the purinergic network. It is relevant for clinical purposes that inhibition of specific purinergic receptors inhibits metastatic competence of tumor cells.

An increasing number of reports have convincingly shown that tumor cells and surrounding normal tissues release nucleotides as a response to multiple different stimuli. Among those identified, anticancer drugs play an important role. Hence, leakage and/or release of nucleotides from untouched cancer cells or from therapeutically damaged ones, simultaneously favors besides tumor cell survival and its migration and dissemination in the context of healthy tissues. This effect can be hampered by the parallel decreased degradation rate of extracellular nucleotides due to down-modulation of ecto-nucleotidases. Overexpression of single P1 and P2 receptors is an additive advantage for tumor cells as specific P1 and P2 subtypes support cancer growth and invasiveness, while decreasing efficacy of the immune *surveillance*. Therefore, a frequently observed global effect of these changes is the modification of the tumor microenvironment, with increased ATP and ADO concentrations. By exploiting the great potential of purinergic signaling it has recently been understood that nucleotides and nucleosides play a relevant role in tumor biology as metastatic factors both *per se* and through induction of secretion of multiple chemokines from tumor cells of different origin. In this regard, increasing evidences displayed a critical role of ADO in modulating chemokine secretion (i.e. CCR7, CXCR3 and CXCR4) leading to hypothesize the employment of A_{2A} or A_{2B} receptor antagonist as novel cancer immunotherapy strategy able to counteract the immunosuppressive effects of ADO. This objective needs more addressed investigations in order

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to characterize the involvement of ADO in the several steps leading to cancer cell spread. In particular, it would be interesting to evaluate the role of ADO on other chemokines, such as CCR 2, CCR 9 or CXCR 16 known as deeply involved in the metastatic process. Further studies are directed to define the role of ADO in cancer exosomes, small vesicular structures endowed with a CD39/CD73 machinery. These might be able to "re-educate" myeloid-derived suppressor cells as well as to alter the extracellular matrix into the pre-metastatic microenvironment, paving the way toward metastasis.

Therefore, a priority now is to investigate how nucleotide and nucleotide-mediated signaling acts at the tumor microenvironment to modulate chemokine secretion. It is possible that an inhibition of the purinergic signaling at different levels lead to decrease or abrogate chemokine production during tumorigenesis and therefore limit cancer diffusion. Inhibition of single purinergic receptors would in theory reduce or abolish chemokine secretion, thus hindering metastatic dissemination of cancer cells. Potential pharmacologically relevant receptors to target for therapeutic intervention may be the P2X7, P2Y2 and P2Y1 subtypes which have been linked at least to breast and prostate cancer metastasis [77,78,118].

On the contrary, adoptive immunotherapy would benefit from an increased chemotaxis and domiciliation of tumor-specific T cells [119] or NK to solid tumors and metastases. As a consequence, further investigations on modulation of the migratory properties of these cells by the purinergic signaling network are needed.

Since formation of a nucleotide/nucleoside-rich extracellular *milieu* favors tumor growth and metastasis and high motile activity is stimulated by ATP release and autocrine P2X7-mediated signaling, another challenging question would be how to decrease ATP concentration in the tumor niche. Nonetheless, it is reasonable to assume that recent acquisitions on modulation of tumor

metastatic properties by the purinergic signaling network may allow integration and potentiation of the efficacy of current anti-cancer treatments, especially to counteract cancer metastatic diffusion.

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Conflict of Interest

The authors state no conflict of interest.

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Legend to Figures

Figure 1. Complexity of nucleotide and nucleoside signaling and exchanges between inner and outer plasma membrane. Nucleotides (ATP, ADP, UTP, UDP) and nucleosides (ADO) are produced intracellularly and released extracellularly, where they act as signaling molecules by binding specific purinergic receptors (P2 and P1 receptors). The fate of ATP and ADP depends on expression of plasma membrane ecto-nucleotidases (CD39 and CD73), that sequentially metabolize ATP/ADP to AMP and ADO, which is an agonist for P1 receptors. Extracellular ADO can also be transported into the cytoplasm by specialized plasma membrane transporters (ENT1 and ENT2) or extracellularly degraded to inosine (Ino) by adenosine deaminase (CD26). Extracellular ATP can be sequestered by the neoplastic cell by micropinocytosis, thus implementing the intracellular ATP pool. F1F0-ATP synthase, alkaline phosphatase (ALP), nucleoside pyrophosphatase/phosphodiesterase-1 and 2 (NPP1, NTPDase2) are not depicted.

Figure 2. Tumor growth [1] is responsible for compression of healthy neurons and release of toxic compounds, among which glutamate [2] and subsequent neuronal death [3]. Dying cells release ATP [4] which is chemotactic for tumor cells [5] and thus favoring their migration and colonization of normal tissue.

Figure 3. Multiple effects of ATP on neoplasia. Extracellular ATP elicits different responses in tumor cells. They range from cell proliferation, cell death and stimulation of metastatization, depending on tumor type, purinergic receptor subtype expressed and activated intracellular signaling pathways.

Glossary

Hypoxia-inducible factor 1: (HIF-1) it is a heteromeric transcription factor composed of alpha and beta subunits. Its main role is the modulation of cell transcription in response to hypoxic conditions. It is therefore involved in processes such as revascularization, neoangiogenesis, tumor cell invasion and metastatization.

Integrins: they are transmembrane cell adhesion proteins interacting with extracellular matrix molecules or with cell receptors to favor cell-cell communication. Integrins activate intracellular signaling pathways through interaction with cytoskeletal proteins and are involved in cell survival, proliferation, embryogenesis, blood coagulation, immune defense, tumor cell invasion and metastatization.

Lysosomal Associated Membrane Protein 1: (LAMP1, CD107a) it is a glycoprotein localized across lysosomal and plasma membranes. Cell surface expression of LAMP1 is involved in cell adhesion, migration and invasiveness of tumor cells.

Macropinocytosis: it is an actin-mediated uptake of large molecules (antigens, solutes, nutrients or viruses) by cell membrane ruffling that forms large endocytic vacuoles (with a diameter >0.2 µm), known as macropinosomes. The process plays a fundamental role in cell motility, antigen capture and presentation, metastatic diffusion of tumors or parasite entry.

Metalloproteinases: (MMPs) they are secreted proteases degrading extracellular proteins such as structural matrix components. They also metabolize other proteases, protease inhibitors, chemokines and cell-surface receptors. MMPs are inhibited by proteins known as tissue inhibitors of metalloproteinases (TIMPs) and involved in tumor invasion, metastatic diffusion, inflammation, autoimmune and vascular diseases.

Nucleoside diphosphate kinase: (NDPK) it is an enzyme catalyzing the exchange of terminal phosphate between nucleoside diphosphates (NDP) and triphosphates (NTP) in a reversible manner. Four NDPK isoforms are present in humans (from A to D). They combine to generate functional NDPK hexamers. NDPKs are involved in cell proliferation, development and differentiation.

Vincristine: it is a plant alkaloid with anti-cancer properties pharmacologically used against different neoplasias. It interferes with the microtubular apparatus of the mitotic spindle, thus blocking cell division and favoring apoptosis of cancer cell.

Warburg effect: it is a condition characterized by predominance of aerobic glycolysis on mitochondrial oxidative phosphorylation to generate intracellular ATP. Warburg observed that in contrast to normal tissues, cancers transform glucose into lactate, even when oxygen is sufficient to support mitochondrial oxidative phosphorylation.

Trends Box

- Release of nucleotides and nucleosides in the extracellular *milieu* has been demonstrated in many tissues. Once released extracellularly they play multiple functions by binding to specific cell membrane purinergic receptors.

- Ecto-nucleotidases CD39 and CD73 are plasma membrane enzymes converting extracellular ATP/ADP to AMP, and AMP to adenosine, respectively. Therefore, their enzymatic activity is fundamental in determining availability of P2 and P1 receptor agonists and the global outcome of purinergic response.

- Overexpression of P1 or P2 receptor subtypes by cancer cells can support cancer growth and invasiveness while decreasing efficacy of the immune *surveillance*.

Box 1. Outstanding Questions

- Is it possible to pharmacologically target specific purinergic receptors to block metastatic diffusion?
- Is it possible to inhibit local P2X7-mediated ATP release by cancer cells, thus reducing concentration of the nucleotide and its byproducts in the tumor niche?
- Would it be possible to hinder metastatic cancer diffusion by interrupting the sNDPK/P2Y1 axis supporting neo-angiogenesis?

Ferrara, October 21st, 2016

Ms. No. TIPS-D-16-00203

Authors reply to Referees' comments

Referee Comments

Reviewer #1: This is a thorough review about the involvement of purinergic signalling in cancer, with particular emphasis on metastases. They present evidence that ATP released by cancer cells acts as a pro-metastatic factor promoting tumour cell migration. The important role of ecto-nucleotidase in this process is emphasised. The potential use of purinoceptor antagonists for anti-metastatic therapy is discussed.

The background literature is comprehensively covered and the Introduction provides an informed background for the later sections. The arguments are convincingly presented and the figures appropriate. The questions posed are useful.

We are very grateful to the reviewer for appreciating our efforts in making a comprehensive and hopefully intelligible review directed to a wide and presumably heterogeneous audience.

Reviewer #2: Research over the last decade highlighted the important role for extracellular ATP, adenosine and other purines in controlling tumor growth and metastasis.

The authors are well-known experts in the field of purinergic signalling, inflammation and cancer and have already published a number of excellent original research and review articles on this emerging topic (e.g. Antonioli et al., Nature Rev Canc, 2013; Quarona et al., Ann NY Ac Sci, 2014; Ferrari et al., Trends Immunol, 2016). In the present review, the authors are trying to embrace very complex and often controversial area of research. Admittedly, this is not an easy task. In my opinion, the structure and topic of this manuscript should be more focused and straightforward in order to avoid repetition and overlapping with multitude of other similar review articles, and at the same time, to exclude incompletion and gaps in describing particular important pathways and mechanisms.

The referee is perfectly right as we recently wrote some reviews on involvement of the purinergic signaling network in the modulation of physiological and pathological functions in humans. As noticed by the referee it was not at all simple to include into a logical frame. However, we thought it was worthy to try. We thank the reviewer for acknowledging our efforts and for the very helpful comments and advices.

Here are several examples clarifying my statement:

1. The aim of the present review was "to examine the role of nucleotides and nucleosides as chemotactic factors for tumor cells". In fact, substantial part of the manuscript is devoted to the relationships between chemokines and extracellular nucleotides (pages 10-11). However, another recent review article published by the corresponding author (Ferrari et al., Trends Immunol, 2016, Ref #88) already extensively described the link between purinergic signaling, secreted chemokines and their receptors (including IL-8, CXCL8, MCP-1, etc.). The authors should highlight more clearly the novelty of this particular paper, maybe by describing certain characteristic features distinguishing tumor-secreted chemokines and nucleotides from other non-tumor-related inflammatory responses and cell trafficking (if any?). What is known about the role of

extracellular purines in stromal-epithelial interactions in neoplastic cells and the ability of carcinoma-associated fibroblasts to promote tumor progression through production of various growth factors, cytokines and MMPs?

We added new information on this important issue. See pg. 12, line 33 and pg. 13 lines 1-8 or below.

"Increasing attention has been giving to a previously neglected cell type contributing to cancer growth an invasiveness, i.e. cancer-associated fibroblasts (CAF), whose impact on promotion of cancer development occurs via secretion of multiple cytokines and chemokines. Recent data point for a role of the CAF SDF-1/CXCR4 pathway in endometrial cancer progression [94] or for CCL5 and IL-6 secretion in the invasiveness of prostate cancer [95]. Similarly, activation of the CAF CXCL12/CXCR4-mediated pathway promotes invasiveness of gastric cancer [96]. Therefore, a deep investigation of the purinergic signaling of CAF and its participation in the modulation of chemokine and cytokine secretion during cancer development would be highly needed."

New references:

Teng F. *et al*. (2016) Cancer-associated fibroblasts promote the progression of endometrial cancer via the SDF-1/CXCR4 axis. *J Hematol Oncol*. 9:8

Yeh C.R. *et al*. (2016) Estrogen receptor α in cancer associated fibroblasts suppresses prostate cancer invasion via reducing CCL5, IL6 and macrophage infiltration in the tumor microenvironment. *Mol Cancer.* 15:7.

Izumi D. *et al*. (2016) CXCL12/CXCR4 activation by cancer-associated fibroblasts promotes integrin β1 clustering and invasiveness in gastric cancer. *Int J Cancer*. 138:1207-1219

2. By considering the tumorigenic role of nucleotide-releasing and -degrading pathways, it may be relevant to mention recent seminal papers on chemotherapy-induced release of ATP from dying cells and its anticancer immune responses in autophagy-deficient tumors, which can be selectively modulated by local over-expression or inhibition of ecto-nucleotidases (Michaud et al., Science, 2011; Ma et al., Immunity, 2013).

We thank the reviewer for reminding us these important papers on the role of ecto-nucleotidases in cancer. We have now included them, see pg. 17, lines 2-4 and below.

"The role of ecto-nucleotidases in modulating tumor growth and autophagy has been elegantly demonstrated by two recent studies [107-108]."

New references:

Michaud M. *et al*. (2011) Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 334, 1573-1577

Ma Y. *et al*. (2013) Autophagy and cellular immune responses. *Immunity* 39, 211-227

3. The authors also briefly touched another important aspect of purinergic signaling in cancer: the involvement of adenosine in dampening of the antitumor immune responses (e.g. page 7 and 16). In fact, targeting CD39-CD73-adenosine axis (using specific mAbs, small-molecule enzyme inhibitors or A2AR-antagonists), combined with other established immune checkpoint inhibitors (CTLA-4 and PD-1) represents another exciting area of research, which somehow lies beyond the scope of this particular review. Nonetheless, the authors may like to update their review by mentioning several recent reports: Hay CM. et al., Oncoimmunology, 2016; Young A. et al., Cancer Cell, 2016. Furthermore, subsequent to the activation of

A2A and other receptors, extracellular adenosine is known to be further transported and interconverted inside the cells, thereby dampening immune responses and preventing tumor invasion via both receptor-dependent and receptor-independent pathways (Ohta & Sitkovsky, Front Immunol, 2014; Virtanen et al., Mol Canc Res, 2014).

We have now included them in the revised text and Reference section. See pg. 7, lines 22-23 and pg. 8 lines 1-2, or below.

"To this purpose, it is worthy to mention the immunosuppressive effect of adenosine monophosphate (AMP) on lymphocyte activity [41] or that of ADO, by both receptor-dependent and receptor-independent mechanisms, on NK cytotoxic activity, T helper 1 contribution, expansion of myeloid-derived suppressor cells (MSDC) and M2 macrophage polarization [42-47]."

New references:

Hay C.M. *et al*. (2016). Targeting CD73 in the tumor microenvironment with MEDI9447. *Oncoimmunology* 5, e1208875

Ohta A. and Sitkovsky M. (2014) Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol*. 5, 304

Virtanen S.S. *et al*. (2014) Adenosine inhibits tumor cell invasion via receptor-independent mechanisms. *Mol Cancer Res*. 12, 1863-1874

See also pg. 18, lines 1-3.

"Moreover, simultaneous inhibition of CD73 and A2A, or blocking of CD73 by the recently introduced human monoclonal antibody MEDI9447 improves the immune-mediated anti-cancer response [41,113]."

New reference:

Young A. *et al*. (2016). Co-inhibition of CD73 and A2AR Adenosine Signaling Improves Anti-tumor Immune Responses. *Cancer Cell* 30, 391-403

4. By describing the role of acidosis and hypoxia in the solid tumor microenvironment (p.14-15), it would be relevant to mention that the expression levels of CD73 and also nucleoside transporters are tightly controlled by HIF-1-alpha and can be up-regulated during hypoxia (as shown by Prof. Holger Eltzschig and colleagues).

We thank the reviewer for giving us the chance to include this important point. We have now included the indicated reference and the following statement. See pg. 17, lines 16-17 or below:

"Expression of CD73 is tightly controlled by HIF-1-alpha and can be up-regulated during hypoxia [110];"

New reference:

Hart M.L. *et al*. (2011) Hypoxia-inducible factor-1α-dependent protection from intestinal ischemia/reperfusion injury involves ecto-5'-nucleotidase (CD73) and the A2B adenosine receptor. *J Immunol*. 186, 4367-4374

5. The author's statement that F0F1 ATP synthase catalyzes the phosphorylation of extracellular ADP to ATP (page 3 and 5) remains questionable and highly controversial. While certain components of ATP synthase indeed are expressed on the cell surface and may serve as receptor for angiostatin and other multiple ligands, no compelling evidence confirming the contribution of this enzyme to extracellular ATP synthesis by using

proton-motive force had been provided so far (for more details, see Yegutkin GG, Crit Rev Biochem Mol Biol, 2014; also Ref #24).

We are grateful to the reviewer for giving us the chance to better clarify this point. It has been demonstrated that at least in HUVEC (Moser T.L. *et al*. 2001, *Endothelial cell surface F1-F0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin*. Proc Natl Acad Sci USA 98, 6656–6661) and in 3T3-L1 adipocytes (Kita T. and Arakaki N. 2015. *Contribution of extracellular ATP on the cell-surface F1F0-ATP synthase-mediated intracellular triacylglycerol accumulation*. Biomed Res. 36, 115-120) cell-surface F1F0-ATP synthase is essential for extracellular ATP production. Authors showed that inhibition of F0F1-ATP synthase by angiostatin or by antibodies against α and β subunits of the enzyme inhibited extracellular ATP production.

6. The authors consider CD39-CD73 axis as predominant nucleotide-inactivating pathways, whereas the information on other enzymes is either missing or remains scattered. For instance, data on selective expression of NTPDase2 on HT29 cancer cells (page 11) should be supplemented by note that this enzyme preferentially hydrolyzes ATP as substrate. No information was provided about other important purinergic ectoenzymes, nucleoside pyrophosphatase/phosphodiesterase-1 (NPP1, known to be abundantly expressed in glioma and prostate cancer cells), alkaline phosphatase (ALP), as well as adenosine deaminase (ADA).

We have now included these important enzymes involved in the extracellular nucleotide catabolism in the abbreviation list, cited them in the revised text and mentioned in the revised Fig. 1. See pg. 2; pg. 4, line 24 and pg. 25, lines 1-5.

"Among nucleotide and nucleoside degrading enzymes, we also have to mention alkaline phosphatase (ALP), nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1, CD203a) which is abundantly present in glioma and prostate cancer cells, and NTPDase2 expressed for example in human colon HT-29 cells and preferentially hydrolyzing ATP. Extracellular ADO is then eventually transformed into inosine, which is inactive at P1 receptors, by the enzyme adenosine deaminase (ADA, CD26) (Figure 1)."

7. Following up my previous concern, I would not consider Figure 1 as a proper illustration for "Complexity of nucleotide and nucleoside signaling and exchanges...". This rather simplified cartoon is missing important info regarding the alternative homeostatic pathways through extracellular NPP1, ALP and ADA, as well counteracting AdK and NDPK reactions. Further metabolism of the uptaken nucleosides through intracellular adenosine kinase or ADA enzymes is also not depicted here. Probably, the title for this Figure should be something like "Schematic view of major nucleotide and nucleoside signaling and exchanges..." The authors can mention then in the figure legend or main text that in practice this turnover cascade is more complex and involves many other extra- and intracellular players.

We have implemented Fig. 1 and changed figure caption as suggested. See revised Fig. 1.

In summary, I wish to emphasize again that this is a timely and thorough review written by the experts in this field. I would not request to take into account all the above extensive concerns and references. But hopefully taking into account some of these constructive comments might help the authors to strengthen and further improve the reviewed manuscript.

We thank the reviewer for appreciation. We took into account each single comment and performed a thorough revision of our MS.

Other comments:

* Page 2, lines 19-20: Common abbreviation for ecto-nucleoside triphosphate diphosphohydrolase is NTPDase (rather than CD39), while CD39 is often employed as another name for NTPDase1. Likewise, while CD73 has been widely used as another name for ecto-5'-nucleotidase, strictly speaking it cannot be considered as a "real abbreviation" for ecto-5'-nucleotidase.

We have amended the sentence as follows: "(by the ecto-nucleoside triphosphate diphosphohydrolase, NTPDase/CD39) and subsequently to ADO (by the ecto-5'-nucleotidase also indicated as CD73) [24-27]." See pg. 4, lines 22-24.

* Page 4, line 10-12: Probably, this sentence should be modified in order not to mislead the readers that only P2Y11R is activated by ATP. In fact, ATP may also serve as a ligand for P2Y1 (along with ADP) and P2Y2 (equipotent to UTP) receptors.

We have now modified the sentence as follows: "while P2Y11 (as well as P2Y1 and P2Y2) are activated by ATP." See pg. 5, line 16.

* Page 1, Trend Box, line 7: CD73 is not a transmembrane protein. This ectoenzyme is attached to the plasma membrane via GPI-anchor.

We apologize for inattention. The statement has been amended, see revised Trends Box or below:

"Ecto-nucleotidases CD39 and CD73 are plasma membrane enzymes converting……"

* In general, the manuscript is well written. However, there are several typos and misprints throughout the text, which should be re-checked and corrected accordingly. For instance:

We apologize for imprecisions. We have now thoroughly revised the text and hopefully amended all misspellings and grammar errors.

"Another frequent (rather that frequently) aberration"... (p.8, line 1).

Corrected. See pg. 9, line 7.

"encouraging (rather than incouraging) results"... (p.8, line 24).

Corrected. See pg. 10 line 6.

"CD39 is active... (rather than "was is active", p.15, line 23).

Corrected, see pg. 17, line 12.

"Breast cancer cell line" (not "ell line", Fig.3). We have amended it, see revised Fig. 3.

Figure 1 Revised

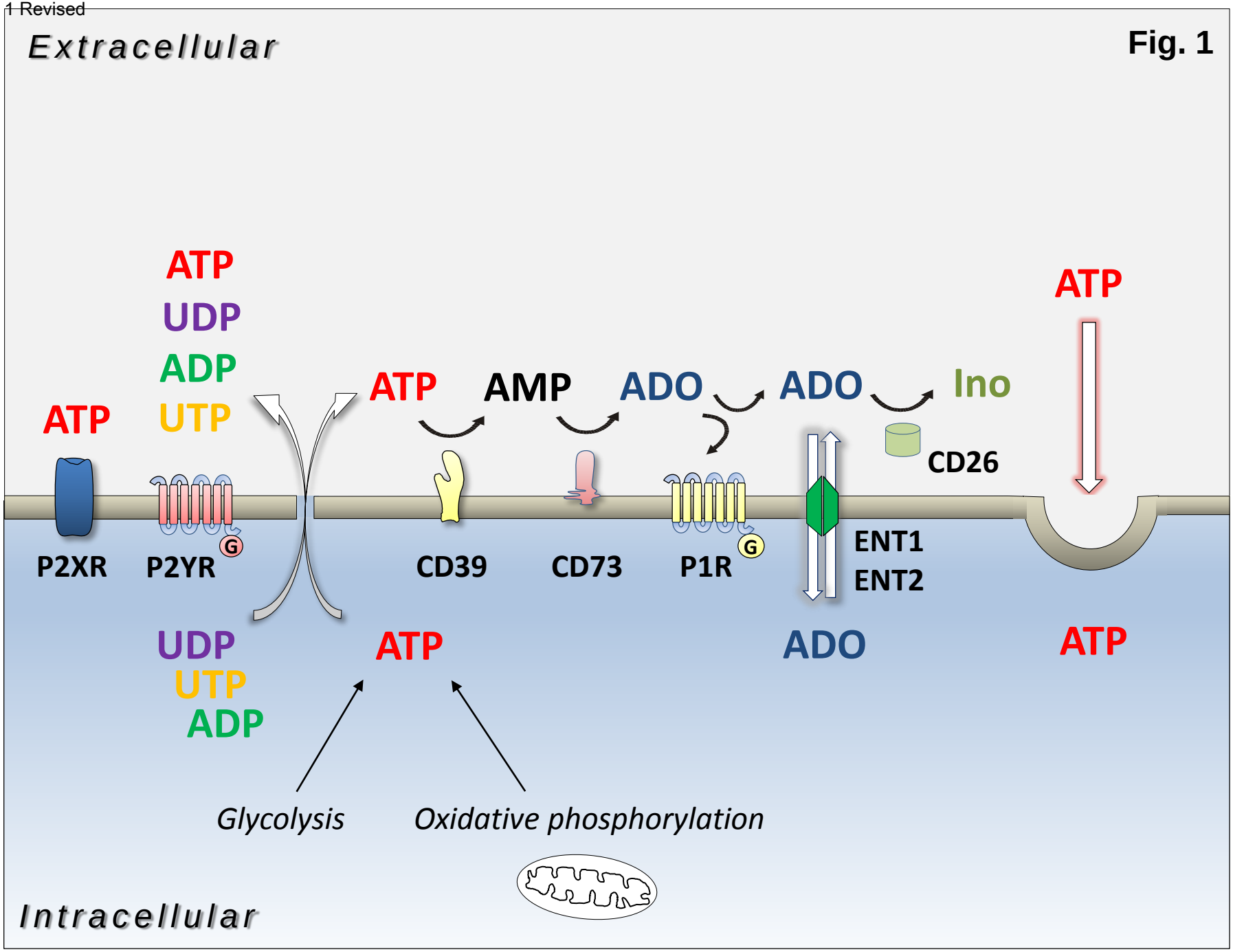


Fig. 2

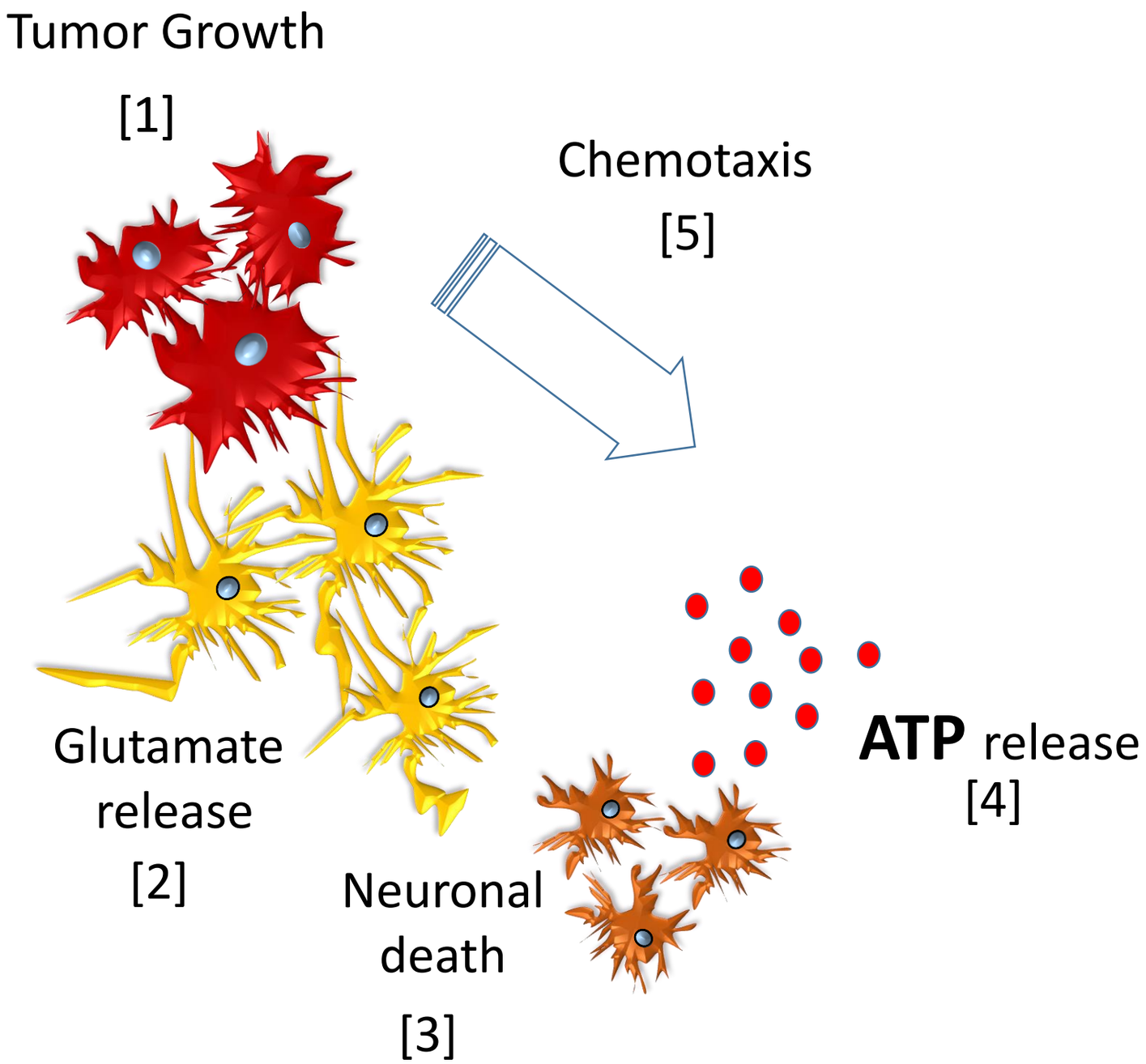
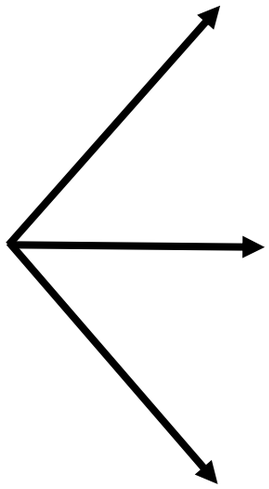

Tumor Growth
[1]
Chemotaxis
[5]
Glutamate release
[2]
Neuronal death
[3]
ATP release
[4]

Figure 3 Revised

Fig. 3

ATP



Cell Death

Epidermoid cervical carcinoma [56]

Melanoma [57]

High grade bladder cancer [59]

Colorectal carcinoma [106]

Cell Proliferation

Breast cancer [53]

Prostate carcinoma [54]

Lung epithelial tumor [55]

Neuroblastoma [58]

Motility
Metastatization

Lung cancer cell lines [20]

Breast cancer cell lines [38]

HEK293-P2X7B [60]

Breast cancer cell line MDA-MB-435s [61]

PC-3M 1E8 prostate cancer cell line [62]

Breast cancer cell line MDA-MB-231 [71]

Hepatocellular carcinoma [92]