The P2X7 receptor is a key modulator of PI3K/GSK3β/VEGF signaling network: evidence in experimental neuroblastoma

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Abstract

Neuroblastoma (NB) is an aggressive pediatric tumor, responsible for 15% of cancer related deaths in childhood, lacking an effective treatment in its advanced stages. The P2X7 receptor for extracellular ATP was associated to NB cell proliferation and recently emerged as promoter of tumor engraftment, growth and vascularization. In an effort to identify new therapeutic options for neuroblastoma, we studied the role of P2X7 receptor in NB biology. We first analyzed the effect of P2X7 activation or down-modulation of the main biochemical ways involved in NB progression: the PI3K/Akt/GSK3β/*M*YCN and the HIF1α/VEGF pathways. In ACN human NB cells, P2X7 stimulation enhanced PI3K/Akt while decreasing GSK3β activity. In the same model P2X7 silencing or antagonists administration, reduced the activity of PI3K/Akt and increased that of GSK3β, leading to a decrease in cellular glycogen stores. Similarly, P2X7 down-modulation caused a reduction in HIF1α levels and VEGF secretion. Systemic administration of two different P2X7 antagonists (AZ10606120 or A740003) in *nude/nude* mice reduced ACN-derived tumor growth. An even stronger effect of P2X7 blockade was obtained in a syngeneic immune-competent neuroblastoma model: Neuro2A cells injected in *AlbinoJ* mice. Together with tumor regression, treatment with P2X7 antagonists, caused down-modulation of the Akt/HIF1α axis, leading to reduced VEGF content and decreased vessel formation. Interestingly, in both experimental models, P2X7 antagonists strongly reduced the expression of the probably best-accepted oncogene in NB: *MYCN*. Finally, we associated P2X7 overexpression with poor prognosis in advanced stage NB patients. Taken together our data suggest that P2X7 receptor is an upstream regulator of the main signaling pathways involved in NB growth, metabolic activity and angiogenesis, and a promising therapeutic target for neuroblastoma treatment.

Key words: P2X7, neuroblastoma, PI3K, GSK3β, *MYCN,* VEGF

Introduction

One of the new frontiers in oncology is the understanding of microenvironment activity on tumor cells. Extracellular ATP, an abundant constituent of the oncogenic milieu, is emerging as new and potent regulator of cancer growth progression and immune response modulation[1-4]. In tumor microenvironment, ATP acts as trophic factor, danger signal and main source of the immunosuppressant adenosine [5]. Extracellular ATP is the natural ligand of P2Y metabotropic and P2X inotropic receptors, among which P2X7 is the best candidate responsible for cancer-associated ATP effects, as it has shown an intriguing ability to confer a growth advantage to cancer cells in *in vivo* models [1]. The growth promoting activity of P2X7 may be related to the proliferative advantage conferred to tumor cells under limiting growth conditions, such as serum and glucose deprivation [6-8], to the stimulation of vascular endothelial growth factor (VEGF) secretion and to the facilitation of extracellular matrix invasion [1,2,9]. Although, P2X7 expression and activity has been reported in several cancers [10,11] tumor-promoting pathways activated by P2X7 are largely unknown.

Neuroblastoma (NB) is a common neuroendocrine childhood tumor causing 15% of pediatric cancer deaths. This malignancy presents as a highly heterogeneous disease ranging from spontaneously regressing to refractory forms. Therapeutic intervention for advanced stage patients is rarely successful. Advanced stage patients show 42% survival rate at 5 years, despite aggressive multimodality therapy[12]. Due to similarities of neuroblastoma cells to neurons, the activity of P2X7 receptor in NB cell lines has been investigated by different studies [13]. P2X7 has been attributed a role in NB cells exocytosis[14]and in the regulation of neuronal differentiation [15,16]. We and others have previously shown that P2X7 receptor is involved in NB cell proliferation both *in vitro* [16,17] and *in vivo* [1]. However, an in depth investigation of the signaling cascade activated by P2X7 and responsible for stimulation of NB growth was still missing.

The phosphatidylinositol-tris-phosphate kinase 3 (PI3K) signaling network is a recognized oncogenic pathway activated in NB [18]. The PI3K pathway comprises PI3K itself, Akt and glycogen synthase kinase 3β (GSK3β) [19]. PI3K/Akt signaling promotes increased GSK3β phosphorylation, that is associated with reduced GSK3β activity [19]. In its un-phosphorylated form GSK3β mediates proteasome degradation of *MYCN,* the oncogene most frequently overexpressed in NB [20]. Moreover, aggressive NB shows high microvessels density, indicative of intense neo-angiogenesis, due to PI3K-dependent VEGF activity [21]. However, the PI3K/Akt pathway is of pivotal importance also in non-cancerous cell survival and the therapeutic activity of drugs interfering with these biochemical ways was lower than expected [22]. Therefore, novel tumor-specific up-stream pharmacological targets able to modulate the PI3K/Akt axis are eagerly sought.

In this study, we report the identification of P2X7 as an upstream modulator of the PI3K/Akt pathway. We show that P2X7 increases PI3K/Akt activation, HIF1α expression, VEGF secretion, GSK3β inactivation, regulating *MYCN* oncogene and glycogen accumulation. Interestingly, two different P2X7 antagonists were found to be highly effective in reducing NB growth *in vivo,* in both xenogeneic and syngeneic murine models. Finally, high P2X7 levels were associated with poor overall survival in a cohort of NB patients. Taken together, our data point to P2X7 as a new oncogene and a promising therapeutic target in neuroblastoma.

Results

P2X7 increases PI3K/Akt activity in ACN human NB cells. PI3K, that plays a critical role in cancer cell growth, has been recently associated to NB progression [18]. Thus, we investigated whether P2X7 could modulate PI3K activity. To this aim, we utilized the ACN human NB cell line, which proliferates both *in vitro* and *in vivo* in a P2X7-dependent fashion [1,17]. Treatment of ACN cells with either P2X7 synthetic agonist Benzoyl-ATP (Bz-ATP), or its natural ligand ATP, caused a 30% increase of PI3K

activity, as assessed by measuring phosphatidylinositol-tris-phosphate (PIP_3) production (Figure 1a). PI3K activation was followed by enhanced phosphorylation of Akt (PhAkt(Ser473)) (Figure 1b). PhAkt(Ser473) was dose dependently up modulated upon Bz-ATP application, reaching a 100% increase at the maximal dose applied (300 μM). Akt activity was increased even more by millimolar concentrations of ATP (Figure 1b, c). In a similar fashion, P2X7 activation augmented GSK3β phosphorylation (PhGSK3β(Ser9)) (Figure 1b, d), thus causing a decrease in its activity.

P2X7 silencing or blockade reduces extracellular ATP levels. In an effort to determine whether P2X7 receptor could affect the concentration of ATP in NB microenvironment, we evaluated the effect of P2X7 loss or blockade on ATP secretion. P2X7 silencing was obtained by stable transfection of two shRNA constructs (refereed as shRNA1 and shRNA2; see Supplementary Figure 1a, b); while P2X7 activity was antagonized by administration of AZ10606120 and A740003 at the lowest effective concentrations in blocking P2X7-dependent intracellular calcium flux (300 nM AZ10696120; 5 μM A740003, Supplementary Figure 1c). P2X7 down-modulation by either silencing or drug administration resulted effective in reducing ATP release form ACN cells (Figure 2a), suggesting that the receptor would activate an autocrine/paracrine loop positively influencing NB growth.

P2X7 down-modulation decreases PI3K/Act activity and cell growth while increasing GSK3β function. Once ascertained that P2X7 can influence the levels of its natural agonist ATP in NB , we evaluated if receptor down-modulation could also negatively affect the PI3K/AKT axis. In accordance with results obtained with P2X7 agonists production of PIP_3 was almost halved by P2X7 silencing while antagonism with AZ10606120 or A740003 caused a reduction in PIP_3 amount of 33 and 75 %, respectively (Figure 2b). P2X7 blockade by shRNA2 or both antagonists tested, reduced slightly but significantly PhAkt(Ser473) (Figure 2c, d, f, g). On the contrary, GSK3β activity was strikingly increased by P2X7 down-modulation as demonstrated by decrease in its phosphorylation at serine 9

(Figure 2c, e, f, h). Increased activity of GSK3β ranged from 15%, obtained with AZ10606120 to 60% achieved by both shRNA1 and 2 silencing (Figure2 e, h). In an effort to evaluate whether P2X7 antagonism was effective in substantially reducing PI3K/Akt mediated NB cell proliferation, we measured ACN cell growth following administration of previously tested P2X7 antagonist and PI3K inhibitors LY294002 and NVP-BEZ2235. All compounds caused a significant and comparable reduction of ACN cell growth (Figure 2 i). Interestingly, if compared to single drug administration, jointed treatment with P2X7 and PI3K blocking drugs did not caused an additional reduction of cell growth, suggesting that, in our model, P2X7 impacts on proliferation acting as upstream-regulator of the PI3K pathway.

P2X7 influences glycogen accumulation in NB cells. Active GSK3β blocks glycogen synthase activity, resulting in depletion of glycogen stores [23]. To confirm the link between P2X7 and GSK3β activity, we evaluated whether P2X7 could influence glycogen accumulation. ACN cells silenced for P2X7 by shRNA1 and shRNA2 showed an evident reduction of glycogen stores over control scramble shRNA, as assessed by PAS staining (Figure 3a-c). P2X7 antagonists determined a similar reduction in glycogen staining intensity in ACN cells (Figure. 3f-h). Notably, both P2X7 silencing and antagonists induced a significant decrease in the number of PAS positive cells (Figure 3 d, i) and in their total glycogen content, assessed by measuring glucose generated from glycogen hydrolysis (Figure 3e, j).

P2X7 silencing or blockade decreases HIF1α/VEGF levels. To further extend our understanding of P2X7 role in NB we investigated whether this receptor could be involved in angiogenesis, which is critical in NB progression and has been associated to PI3K/Akt axis [24]. Therefore, we tested whether P2X7 could affect the most relevant pathway causing vessels sprouting i.e. HIF1α-VEGF [25]. Both

P2X7 partial loss and antagonism induced a striking reduction (≈50%) of HIF1α protein content (Figure 4a). As expected, secretion of VEGF was consequently reduced (Figure 4b).

P2X7 antagonists cause NB regression in two different *in vivo* models. To investigate whether P2X7 receptor could be a valuable pharmacological target for NB, we studied the effects of P2X7 antagonists on NB cell growth *in vivo*. As a first model, we tested tumors derived by subcutaneous injection of human ACN cells in immune-compromised *nude/nude* mice. AZ10606120 (300 nM), A740003 (5 μM) or placebo (PBS + 0,005% DMSO) were administered intra peritoneum (ip) every two days after the appearance of the tumor mass (day five from cells injection) for a total of 28 days. Treatment with either AZ10606120 or A740003 caused an evident (≈40%) reduction of excised tumor size (Figure 5a, b). Western blot analysis of tumor homogenates (Figure 5c) confirmed the data obtained *in vitro*. Hence, P2X7 antagonists administration decreased both Akt and GSK3β phosphorylation (Figure 5c-e), suggesting a reduction and an increase, respectively, in the activity of these kinases. Considering the two different antagonists tested, A740003 had the most relevant effect on the Akt/GSK3β axis causing 30% reduction in the phosphorylation of both kinases (Figure 5c-e). AZ10606120 *in vivo* administration caused a slight reduction (≈15%) of Akt/GSK3β phosphorylation. These results were consistent with the *in vitro* data, showing a stronger activity of A740003 versus AZ10606120 (Figure 2 e-g). Treatment with both P2X7 antagonists obliterated *MYCN* content in ACN derived tumors (Figure 5c, f). Similarly to the *in vitro* data, P2X7 blockade by A740003 caused a significant reduction of VEGF levels measured in tumor homogenates (Figure 5g).

We next tested the efficacy of P2X7 antagonists against experimental NB obtained by injection of murine Neuro2A NB cells in syngeneic *AlbinoJ* mice. This fully immune-competent strain allowed us to assess whether P2X7 antagonists interfered with the mouse immune system, and how such interference influenced tumor growth, as P2X7 activation has been associated to anti-tumor immune response [26]. In Neuro2A cells, the lower concentration of A740003 effective in blocking P2X7-evoked

calcium rise was of 10 μM (Supplementary Figure 1d), the drug was consequently *in vivo* administered at this concentration. Placebo or inhibitors were administered every two days from the appearance of the first tumor mass (day 8 from the inoculum) until day 15. The effect of P2X7 antagonism in Neuro2A bearing mice was even stronger than that seen in ACN bearing animals. In fact, receptor blockade by both A740003 and AZ10606120 caused an almost 50% reduction of tumor growth rate in live animals (Figure 6a), accompanied by a comparably strong reduction of excised tumors volume (Figure 6b, c). The biochemical pathways affected by anti P2X7 drugs included PhGSK3β(Ser9), whose levels were halved by A740003 (Figure 6d, e), and HIF1α that was strongly decremented by both drugs (Figure 6d, f). Interestingly, P2X7 antagonists strikingly reduced *MYCN* levels (Figure 6d, g) similarly to what observed in the ACN model (Figure 5c, f). Finally, HIF1α amount reduced by P2X7 antagonists (Figure 6d, f) was paralleled by a decreased level of VEGF in tumor homogenates, blood vessels numbers and staining with the endothelial marker von Willebrand factor (Figure 6h-l)

P2X7 expression correlates with poor prognosis of stage IV NB patients. To further investigate P2X7 relevance as a therapeutic target, we correlated patient survival with P2X7 levels in a NB patients' cohort. We performed the gene expression profile of 131 NB patients for which we collected information on INSS stage and overall survival. Patients were divided into two groups according to P2X7 expression levels (high-expressing and low-expressing). Kaplan-Meier survival analysis was performed independently on 56 highly aggressive (stage 4) and 75 not aggressive (stages 1, 2, 3, 4s) tumors using P2X7 expression levels as a variable. We found that higher P2X7 levels significantly correlated with an unfavorable prognosis in stage 4 (Figure 7a, $p>0,05$) but not in other stages (1, 2, 3, 4s) NB patients (Figure 7b). In the examined cohort we did not find any correlation between P2X7 and PI3K, GSK3β or *MYCN* gene expression levels (data not shown), thus suggesting that P2X7 acts post-transcriptionally in modulating both PI3K and GSK3β axis.

Discussion

An increasing body of literature strongly supports P2X7 involvement in cancer cell proliferation, energy production and migration [1,2,7,8,27]. P2X7 expression has been demonstrated in a wide spectrum of tumor types [11,28] including colon carcinoma, melanoma and NB, all of which also showed P2X7-dependent growth *in vivo* [1]. With the present study, we further extended our findings, unveiling PI3K/GSK3β/*MYCN*/HIF1α axis as a new pathway activated by P2X7 in NB. The PI3K/Akt signaling cascade is a central player of tumor cell growth and bioenergetics, that regulates aerobic glycolysis, cell cycle progression and autophagy . In the specific context of NB, PI3K/Akt has been also associated to progression and resistance to chemotherapy [18,29]. In this study, we demonstrated a P2X7-dependent positive regulation of the PI3K/Akt path in NB cell lines and derived tumors. Indeed, P2X7 agonist Bz-ATP increased both PI3K and Akt activity while P2X7 antagonism caused a substantial inhibition of the PI3K/Akt pathway. Interestingly, P2X7 antagonist reduced the levels of extracellular ATP released by NB cells confirming a role for the receptor in the secretion of its own natural ligand in cancer [30].We also provided the first evidence that P2X7 not only modulated the PI3K/Akt pathway but also downstream effectors such as GSK3β and HIF1α [31,32]. In response to agonists binding, P2X7 caused inactivation of GSK3β (Figure 1), while P2X7 antagonists or receptor silencing had an opposite effect on kinase activity (Figures 2, 3, 5 and 6). GSK3β is well known as negative regulator of glycogen synthase, and drives consumption of cellular glycogen. Accordingly, wild type ACN showed increased levels of glycogen in comparison to ACN cells either P2X7 silenced or treated with P2X7 antagonists (Figure 3). Clear glycogen-rich cells are present in many aggressive tumors and glycogen is emerging as energy supply for cancer cells, promoting their survival in metabolic stressing conditions and hypoxia [23,33,34]. Our data, showing a reduction of tumor cell glycogen following P2X7 down-modulation, suggest that P2X7 might favor cell survival acting at this energy source. By activating

GSK3β, P2X7 antagonists also destabilize another target of this kinase[35], i.e. *MYCN* (Figure 5b, e, Figure 6 d, g). *MYCN* is a well characterized oncogene in NB and, due to its frequent amplification in poor prognosis patients, it represents a reliable prognostic marker [20]. Remarkably, *in vivo* administration of P2X7 antagonists strongly reduced tumoral levels of *MYCN* in two different experimental NB models (Figure 5 and 6). As far as we know, this is the first time that an association between P2X7 and *MYCN* was reported, further supporting an oncogenic role for P2X7. Moreover, in the patients cohort that we analyzed, the levels of P2X7 mRNA did not correlate with those of *MYCN* (not shown), suggesting that destabilization of *MYCN*, detected in experimental tumor samples (Figure 5c, f), occurred at a post-translational level, likely due to GSK3β phosphorylation [35,36]. *MYCN,* and its positive regulator PI3K, have been postulated to increase NB vascularization, via up-regulation of VEGF secretion [21]. Blood vessel formation is an absolute requisite for tumor progression and accounts for the oncogenic activity of angiogenic proteins such as HIF1α and VEGF [37]. In accordance with its effect on PI3K and *MYCN*, P2X7 also modulated HIF1α and VEGF expression both *in vitro* and *in vivo* models. In ACN human NB cells, P2X7 silencing or its pharmacological blockade caused a remarkable reduction of both HIF1α and VEGF levels (Figure 4). A similar effect was observed in *in vivo* NB models, where P2X7 antagonists down-modulated HIF1α and VEGF secretion (Figures 5, 6) and consequent blood vessels formation (Figure 6 i-e). Despite its recognized oncogenic role in neuroblastoma *MYCN*, is a problematic pharmacological target[38]. Similarly, molecules developed to target the PI3K/Akt path in cancer patients, once tested in clinical trials, showed a limited efficacy, due to toxicity on normal cells [22]. Our data point to P2X7 antagonists as valuable therapeutic alternative to *MYCN* and PI3K/Akt inhibitors in NB. In fact, in our experimental setting, treatment with either AZ10606120 or A740003, significantly downsized tumor dimensions (Figures 5, 6). Reduction of tumor growth was also accompanied by *MYCN*, HIF1α and VEGF down-modulation and up-regulation of the anti-oncogenic kinase GSK3β. These data provide a strong pre-clinical evidence of the efficacy

of P2X7 inhibitors for NB therapy. Interestingly, when co-administered with P2X7 antagonists PI3K inhibitors did not show any significant additional effect on cell proliferation (Figure 2 i) suggesting that, in NB, P2X7 blockers could effectively substitute PI3K inhibitors as therapeutic strategy. Different P2X7 antagonists are under clinical trial for inflammatory pathologies and appear to be safely tolerated by humans [39-41]. Moreover, a clinical trial to cure cancer with a P2X7 blocking antibody was recently launched (Biosceptre International, North Ryde, Australia). Our results may pave the way to the investigation of the efficacy of such molecules in controlled studies enrolling patients affected by NB. One of the main arguments against the use of P2X7 antagonists in tumor therapy is P2X7-dependent activation of anti-tumoral immune response, which could be lost upon receptor blockade, thus favoring tumor progression [4]. However, in our experimental setting, systemic administration of P2X7 blockers caused tumor regression. Moreover, the anti-tumor effect of P2X7 antagonists, was higher in the fully immune-competent Neuro2A bearing mice than in the immune-compromised ACN bearing mice. This difference could be partially ascribed to blockade of P2X7 expressed by suppressor immune cells such as myeloid-derived suppressor cells (MDSC). In fact, MDSCs have been shown to facilitate neuroblastoma growth negatively regulating tumor immune cells infiltration in a P2X7 dependent fashion [42] One of the open challenges in neuroblastoma therapy is the discovery of new and efficacious pharmacologic approaches for highly aggressive patients that show a really limited survival rate. The analysis of P2X7 expression levels in a patients' cohort allowed to identify an association between the receptor and clinical outcome. In fact, P2X7 expression associated with reduced overall survival of stage 4 NB patients, while did not stratified patients in stages 1, 2, 3, 4s. Taken together these data suggest that pharmacological treatment with anti-P2X7 agents could be tailored to aggressive NB patients. Notably, previous studies related P2X7 over-expression to poor outcome and metastatic dissemination in other malignancies such as chronic lymphocytic and acute leukemia,

papillary thyroid and prostate carcinoma [43-46], which, in light of our data, could be considered as eligible pathologies for P2X7 blocking therapy.

Evidence reported in this study indicates P2X7 as a clear oncogenic driver in neuroblastoma, affecting the best known pathways involved in tumor growth and progression and patients prognosis. We also gave preclinical demonstration of P2X7 antagonists efficacy in neuroblastoma treatment, prompting the use of these drugs in clinical trials.

Materials and Methods

Reagents and antibodies. P2X7 antagonists AZ10606120 and A740003 were purchased from Tocris Bioscience (Ellisville, MS, USA). The two compounds were dissolved in DMSO, to reach a 100 mM stock concentration and subsequently diluted in PBS. PI3K antagonists LY294002 and NVP-BEZ235 were from Selleckchem (distributed by Aurogene, Rome, Italy). Anti P2X7 receptor polyclonal antibody was from Sigma Aldrich. Human monoclonal anti-Akt, PhAkt(Ser473) and anti-myosin IIa Abs were acquired from Cell Signaling (Milan, Italy). Human monoclonal GSK3β and PhGSK3β (Ser9), Abs were from Epitomics, (Histo-Line Laboratories, Milan, Italy) whereas anti-*MYCN* antibody was from Abcam (Cambridge, UK). For murine samples, monoclonal anti-PhGSK3β(Ser9) (Thermo Scientific, Tema Ricera, Bologna, Italy) and HIF1α (Abcam, UK) were used together with polyclonal anti-GSK3β (Thermo Scientific, Tema Ricerca, Bologna, Italy) anti-MYCN and Von Willebrand Factor Abs (Abcam, UK). Secondary anti rabbit or mouse HRP-conjugated antibodies and Liquid DAB Substrate Chromogen System were acquired from DAKO (Dako, Milan, Italy). Bouin's , Mayer's Hematoxylin and Eosin Y solutions were purchased by Sigma.

Cell cultures and proliferation assay. ACN and Neuro2A cells were grown in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 1-x non-essential aminoacids. ACNshRNA1, ACNshRNA2 silenced, or ACN scrambled cell clones were previously

obtained in our laboratory [1]. To evaluate ACN proliferation rate, $1x10^4$ cells were plated overnight in complete medium, and then maintained in serum free medium containing, were required, anti-P2X7 and/or anti-PI3K drugs for 48 h. Cells were counted with the help of ImageJ software (developed by Wayne Rasband, NIH, USA).

PI3K activity assay. PI3K activity was evaluated measuring PIP_3 with the competitive *in vitro* PI3-Kinase activity ELISA Pico (Echelon Biosciences Inc., Salt Lake City, UT, USA), as per manufacturer's indication.

Extracellular ATP measure. Extracellular ATP levels were determined by luminometric assay using the Enliten ATP assay system (Promega, Italia, Milan, Italy). A total of 2×10^4 cells per sample were incubated with P2X7 antagonist or left untreated for 48 h. Immediately before the measure ATP was stabilized by replacement of cell supernatant with 100 µl of diluent buffer (FireZyme). ATP concentration was meausured with a Victor 3 multilabel counter (Perkin Elmer) equipped with a Wallac liquid injector (Perkin Elmer) that allowed rapid injection of the luciferin-luciferase solution (100 µl). The total amount of ATP revealed was then normalized on cell numbers.

Western blot analysis. Cell lysates and tumors homogenates were obtained in lysis buffer (300 µM sucrose, 1mM K_2HPO_4, 5,5 mM D-glucose, 20 mM Hepes, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) supplemented, when required, with 0,1% Triton or 0,5% IGEPAL. Samples were loaded in 8% Bolt-SDS pre cast gels (Life Technologies, USA). Membranes were incubated with the primary antibodies overnight at 4 °C. Anti P2X7 receptor Ab was used at a dilution of 1:200 in TBS (50 mM Tris, 150 mM NaCl, pH 7,6) plus 3% non-fat milk and 0,1% BSA. For human samples, anti-*MYCN*, GSK3β and PhGSK3β(Ser9) were diluted 1:1000 in TBS plus 5% BSA. For murine samples, anti-*MYCN* was diluted 1:160 in TBS plus 5% BSA. Anti- HIF1α was diluted 1:1000 in TBS plus 2% non-fat milk. An anti-Myosin IIa Ab, diluted 1:1000 was used as loading control. Subsequently, membranes were incubated with a secondary goat anti- mouse or rabbit, HRP-conjugated antibody at a

1:2000 dilution in TBS-t (TBS, 0.1% Tween 20). Protein bands were visualized by enhanced chemiluminescence detection kit (GE Healthcare, Milan, Italy). Densitometric analysis was carried out with ImageJ software and data were normalized on Myiosin IIa.

Measure of intracellular calcium concentration. Changes in the cytosolic free calcium concentration were measured in a thermostat controlled (37°C) and magnetically stirred Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Milan, Italy) with the fluorescent indicator fura-2/acetoxymethyl ester (fura-2/AM) as previously described[47]. This assay was used to assess concentrations of the antagonists effective in blocking P2X7 activity in ACN or Neuro2A cells (see Supplementary Figure 1).

Glycogen detection and measure. $2x10^4$ ACN cells were let adhere overnight in complete medium, and then maintained in serum free medium for 4 h (scramble or ShRNAs) or 48 h (P2X7 antagonists). Glycogen was revealed with PAS Kit (Sigma). Cells were fixed with 96% methanol and analyzed with a Leica DMIL LED phase contrast microscope equipped with a 20 x objective and a color camera (Leica Microsystems, Germany). Total and PAS positive cells were counted with the help of ImageJ software. Total glycogen content at 48 hours was also evaluated with Glycogen Colorimetric Assay Kit II (BioVision, San Francisco, USA) as per manufacturer instructions.

HIF1α and VEGF ELISA assays. Nuclear HIF1α content was evaluated with the HIF1α Human ELISA Kit (Abcam, Cambridge, UK), as per manufacturer's indication. VEGF was measured in cell supernatants or tumor homogenates with VEGF ELISA Kit (R&D Systems, Minneapolis, MN USA), according to manufacturer's indication.

Tumor generation and *in vivo* drug administration. Experiments were performed using 4-5 weeks old, female *nude/nude* or *AlbinoJ mice* (Harlan Laboratories, Italy). All animal procedures were in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/109, OJL 358, Dec. 1, 1987, and NIH Guide

for the Care and Use of Laboratory Animals). To induce tumor formation $3\ x10^6$ ACN [1] or $1x\ 10^6$ Neuro2A[48] cells were inoculated into subcutaneous fat of the right limb of *nude/nude* or *AlbinoJ* mice, respectively. Total mice injected were 36 (six for condition). To assess general health conditions and monitor tumor volume, animals were examined every two days. Five-eight days after tumor cell inoculum, tumor masses became detectable reaching a volume range from 10 to 100 mm^3. Animals were divided in 3 groups for each strain and treated with placebo (PBS + 0,005% DMSO, N = 6), AZ10606120 (300 nM, N = 6) or A740003 (5-10 µM, N = 6). Treatments were administrated as a 100 µl intraperitoneal injection every two days after first tumor mass detection. All drugs were dissolved in sterile placebo solution. Tumor size was measured with a caliper, and volume was calculated, approximating the tumor mass to an ellipsoid, according to the following equation: volume = $\pi/6$ [w1 x $(w2)^2$], were w1 = major diameter and w2 = minor diameter. *AlbinoJ* and *nude/nude* mice were euthanized before the appearance of clear signs of discomfort, respectively 15 and 33 days after cell inoculum. Tumor specimens were post-mortem excised and either kept at -80°C or fixed in Bouin's solution to be embedded in paraffin.

Histology and immunohistochemistry. Vascular network was evaluated by counting the number of blood vessels per microscopic field with a × 10 objective in tumor sections stained with haematoxylin/eosin .For the immunohistochemical detection of von Willebrand factor, sections were rehydrated, washed in TBS, blocked for 1 hour at room temperature (RT) in this same medium supplemented with 10% BSA, incubated for 1 hour at RT with TBS containing 1% BSA and anti-von Willebrand factor antibody (1:2000). Slides were then washed twice in TBS containing 0.025% Triton X-100 and endogenous peroxidase activity was blocked by 20 min incubation at RT in 0.3% H_2O_2-containing TBS. After several rinses in TBS, sections were incubated for 1 hour RT with 1% BSA-containing TBS, and HRP-conjugated goat anti-rabbit IgG antibody (1:200). Tissue sections were then washed twice in TBS, and peroxidase activity detected with Liquid DAB Substrate Chromogen System

(Dako). Nuclear counterstaining was performed with Mayer's haematoxylin. Sections were dehydrated, mounted with EUKITT (Kindler GmbH, Freiburg, Germany), and images acquired and analysed with a Nikon eclipse 90i digital microscope equipped with a NIS-elements software (Nikon Instruments Europe, Anstelveen, The Netherlands).

Patients and gene expression analysis. The correlation of P2X7 expression with survival was investigated in a 131 neuroblastoma patient's cohort. Eighty-eight patients were enrolled by the Academic Medical Center (AMC; Amsterdam, Netherlands) [49,50]; 21 patients were collected by the University Children's Hospital, Essen, Germany and were treated according to the German Neuroblastoma trials, either NB97 or NB2004; 22 patients were collected at Gaslini Institute (Genoa, Italy) and were treated according to Italian AIEOP or SIOPEN protocols. Informed consent was obtained from all patients in accordance with institutional policies in use in each country. In every dataset, median follow-up was longer than 5 years and tumor stage was defined as stages 1, 2, 3, 4, or 4s according to the International Neuroblastoma Staging System (INSS[51]). The gene expression profiles for the 131 tumors were obtained by microarray using Affymetrix Gene Chip HG-U133 plus2.0. Raw data were processed by MAS5.0 normalization according to Affymetrix guideline. The probe set associated to P2X7 gene was 207991_at. Expression data are available to registered users in the "R2: microarray analysis and visualization platform" (http://r2.amc.nl) (AMC and Essen patients) or in the BIT-neuroblastoma Biobank of the Gaslini Institute (http://www.gaslini.org).

Statistical analysis. Data shown in diagrams are means ± standard error of the mean. Significance was calculated with the Student's t test tanks to GraphPad Instat software (GraphPad Software, Inc.). Probability of patients' overall survival was calculated with Kaplan Meir method, and significance of the difference between Kaplan Meier curves was calculated by log-rank test using Prism 4.03 (GraphPad Software, Inc.).

Figure legends

Figure 1. P2X7 receptor stimulation increases PI3K/Akt while decreasing GSK3β activity in ACN cells. (**a-d**) ACN cells were treated with P2X7 agonist Bz-ATP and ATP for 48 hours. (**a**) PI3K activity was evaluated measuring PIP_3 levels. Red: untreated, dark green: 100 μM Bz-ATP, light green: 200 μM Bz-ATP, blue: 1 mM ATP, cyan: 2 mM ATP; N=9, ***p<0,001 ; **p<0,01. (**b**) Representative Western blot showing the effect of P2X7 stimulation on PhAkt(Ser473), total Akt, PhGSK3β(Ser9), total GSK3β, Myosin II. (**c**) Densitometry of PhAkt(Ser473) normalized on Myosin II. N=6; ***p<0,001 ; **p<0,01, *p<0,05. (**d**) Densitometry of PhGSK3β(Ser9) normalized on Myosin II. N=6; ***p<0,001; *p<0,05.

Figure 2. Silencing or inhibition of P2X7 receptor reduces ATP secretion, PI3K activity, PhAkt(Ser473) and PhGSK3β(Ser9) levels in ACN neuroblastoma cells affecting their proliferation. (**a**) Extracellular ATP levels measured in supernatants of ACN cells. Red: ACN scrambled control, cyan: ACN transfected with anti-P2X7 shRNA1, blue: ACN transfected with anti-P2X7 shRNA2; ocher: untreated ACN control, light green: 300 nM AZ10606120, dark green: 5 μM A740003. N=10; ***p<0,001, **p<0,01 ;(**b**) PI3K activity was evaluated measuring PIP_3 levels. Red: ACN scrambled control, cyan: ACN transfected with anti-P2X7 shRNA1, blue: ACN transfected with anti-P2X7 shRNA2; ocher: untreated ACN control, light green: 300 nM AZ10606120, dark green: 5 μM A740003. N=9; **p<0,01; *p<0,05. (**c-e**) Western blot analysis of ACN cells silenced for P2X7 receptor (ShRNA1, ShRNA2) or scramble control. (**c**) Representative immunoblot of PhAkt(Ser473), total Akt, PhGSK3β(Ser9), total GSK3β, Myosin II. (**d**) Densitometry of PhAkt(Ser473) normalized on Myosin II. N=6, *p<0,05. (**e**) Densitometry of PhGSK3β(Ser9) normalized on Myosin II. N=6, ***p<0,001. (**f-h**) Western blot analysis of ACN cells treated for 48 hours with either 300 nM AZ10606120 or 5 μM A740003. (**f**) Representative immunoblot of PhAkt(Ser473), total Akt, PhGSK3β(Ser9), total

GSK3β, Myosin II. (**g**) Densitometry of PhAkt(Ser473) normalized on Myosin II. N=6, *p<0,05. (**h**) Densitometry of PhGSK3β(Ser9) normalized on Myosin II. N=6, **p<0,01; *p<0,05.(**i**) Proliferation at 48 hours of ACN cells treated with P2X7 antagonists AZ10606120 (300 nM), A740003(5 μM), PI3K inhibitors NVP-BEZ235 (1μM), LY294002 (1μM) administered singularly or in combination. Data shown represents fold increase in cell numbers on time 0 after 48 hours in serum starvation. Reported significance was calculated versus vehicle. N=15 ***p<0,001.

Figure 3. P2X7 receptor down-modulation decreases ACN cells glycogen stores.(**a-d**, **f-i**) ACN cells were stained with PAS to reveal glycogen. (**a**) ACN transfected with scramble shRNA control, (**b**) ACN silenced for P2X7 with shRNA1. (**c**) ACN silenced for P2X7 with shRNA2. (**d**) Percentage of PAS positive cells. Red: ACN scramble, cyan: ACN shRNA1, blue: ACN shRNA2. N=9,***p<0,001.(**e**) Intracellular glycogen evaluated enzymatically following cell lysis (see materials and methods), red: ACN scramble, cyan: ACN shRNA1, blue: ACN shRNA2. N=15,*p<0,05 (**f**) ACN untreated control. **(g)** ACN cells treated for 48 hours with 300 nM AZ10606120. (**h**) ACN cells treated for 48 hours with 5 μM A740003. (**i**) Percentage of PAS positive cells. Ocher: untreated control, light green: AZ10606120, dark green: A740003. N=9, ***p<0,001. (**j**) Intracellular glycogen evaluated enzymatically following cell lysis (see materials and methods) Ocher: untreated control, light green: AZ10606120, dark green: A740003. N=15, ***p<0,001,*p<0,05

Figure 4. Silencing or pharmacological inhibition of P2X7 reduces HIF1α protein content and VEGF release. (**a**) HIF1α amount revealed by ELISA assay in ACN scramble (red), ACN shRNA1 (cyan), ACN shRNA2 (blue), ACN untreated (ocher) or treated with 300nM AZ10606120 (light green), 5μM A740003 (dark green). N=6; ***p<0,001; **p<0,01. (**b**) VEGF levels measured by ELISA assay in ACN scramble (red), ACN shRNA1 (cyan), ACN shRNA2 (blue), ACN untreated (ocher) or treated

with 300 nM AZ10606120 (light green) or 5 μM A740003 (dark green). Treatment with P2X7 inhibitors was performed for 48 hours. N=4; ***p<0,001; **p<0,01.

Figure 5. P2X7 antagonist reduces growth of ACN cells injected in *nude/nude* mice. ACN derived tumors were generated in *nude/nude* mice as described in materials and methods. 300 nM AZ10606120, 5 μM A740003 or placebo (PBS + DMSO 0,005%) were ip administered every two days from first tumor mass appearance (day 5) for a total of 28 days. (**a-b**) P2X7 inhibitors reduce excised tumor dimensions. (**a**) Representative explants obtained from mouse treated with placebo, 300 nM AZ10606120 or 5 μM A740003. (**b**) Volume of excised tumor masses was calculated as described in materials and methods. Ocher: placebo; light green: AZ10606120; dark green: A740003. N=6; **p<0,01; *p<0,05. (**c-f**) Western blot analysis of tumor homogenates. (**c**) Representative immunoblot of PhAkt(Ser473), PhGSK3β(Ser9), total GSK3β, total Akt, *MYCN*, Myosin II. (**d**) Densitometry of PhAkt(Ser473) normalized on Myosin II. N=9, *p<0,05. (**e**) Densitometry of PhGSK3β(Ser9) normalized on Myosin II. N=9, * p<0,05. (**f**) Densitometry of *MYCN* normalized on Myosin II. N=9, ***p<0,001. (**g**) VEGF amount in tumor homogenates revealed by ELISA, ocher: placebo; light green: AZ10606120; dark green: A740003. N= 9, ***p<0,001.

Figure 6. P2X7 antagonists cause growth arrest of Neuro2A derived experimental neuroblastoma**.** Neuro2A cells derived tumors were generated in syngeneic *AlbinoJ mice* as described in materials and methods. AZ10606120 300 nM, A740003 10 μM or placebo (PBS + DMSO 0,005%) were ip administered every two days from first tumor mass appearance (day 8) for four doses. (**a**) P2X7 blockade slows down tumor growth. Tumors were measured in live animals with a manual caliper. Ocher: placebo; light green: AZ10606120; dark green: A740003. N= 6, ***p<0,001. (**b-c**) P2X7 antagonism reduces tumor dimensions. (**b**) Representative explants obtained from mouse treated with placebo, 300 nM AZ10606120 or 10 μM A740003. (**c**) Volume of excised tumors, ocher: placebo; light

green: AZ10606120; dark green: A740003. N = 6, **p<0,01, *p<0,05. (**d-g**) Western blot analysis of tumor homogenates.(**d**) Representative immunoblot of PhGSK3β(Ser9), total GSK3β, *MYCN*, HIF1α, Myosin II. (**e**) Densitometry of PhGSK3β(Ser9) normalized on Myosin II. N=6, *p<0,05. (**f**) Densitometry of HIF1α normalized on Myosin II. N=6, *p<0,05. (**g**) Densitometry of *MYCN* normalized on Myosin II. N=6, *p<0,05, *** p<0,001. (**h**) VEGF levels measured in tumor homogenates. Ocher: placebo; light green: AZ10606120; dark green: A740003. N=6, **p<0,01, *p<0,05.(**i-k**) Neuro2A derived tumors stained with anti-von Willebrand Factor antibody to identify vascular endothelia. (**i**) Placebo, (**j**) AZ10606120, (**k**) A740003. (**l**) Number of blood vessels per microscopic field obtained with a 10-x objective. Ocher: placebo; light green: AZ10606120; dark green: A740003. N=10, ***p<0,001.

Figure 7. Correlation of P2X7 expression with neuroblastoma patients survival. (**a**) Overall survival analysis performed on 56 stage 4 neuroblastoma patients. Black and red lines indicate patients whose P2X7 mRNA expression was respectively lower or higher than the cutoff set at 8,5. p<0,05. (**b**) Overall survival analysis performed on 75 patients with 1, 2, 3, 4S stages neuroblastoma. Black and red lines indicate patients whose P2X7 mRNA expression was respectively lower or higher than the cutoff set at 22. The p-value is not significant.

Supplementary Figure 1. P2X7 characterization in ACN and Neuro2A neuroblastoma cell lines. (**a**) Bz-ATP (200 μM) dependent changes in cytosolic free calcium concentration ($[Ca^{2+}]_i$) of scramble (red) or P2X7 silenced (ShRNA1, cyan; ShRNA2, blue) ACN cells. (**b**) Representative immunoblot showing reduced P2X7 levels in ShRNA1 or ShRNA2 ACN cells as compared to WT or scramble control. (**c**) Bz-ATP (200 μM) dependent changes in ($[Ca^{2+}]_i$) of ACN cells following 5 minutes administration of either AZ10606120 (300 nM) or A740003 (5 μM). (**d**) Bz-ATP (200 μM) dependent

changes in ($[Ca^{2+}]_i$) in Neuro2A cells following 5 minutes administration of either AZ10606120 (300 nM) or A740003 (10 μM). **(c-d)** Ocher: untreated; light green: AZ10606120; dark green: A740003.

Supplementary Figure 2. Visual Abstract.

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Abbreviations list: **NB**: neuroblastoma; **VEGF**: vascular endothelial growth factor; **PI3K**: phosphatidylinositol-tris-phosphate kinase 3; **Bz-ATP**: benzoyl ATP; **GSK3β**: glycogen synthase kinase 3 β; **PIP_3**: phosphatidylinositol-tris-phosphate; **PhAkt(Ser473)**: Akt phosphorylated at Ser473; **PhGSK3β(Ser9)**: GSK3β phosphorylated at Ser9 , **PAS**: periodic acid Shiff, **ip**: intra peritoneum

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