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UDP exerts cytostatic and cytotoxic actions in human neuroblastoma SH-SY5Y cells over-expressing P2Y6 receptor

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ABSTRACT

The role of P2 receptors for purines/pyrimidines is not well characterized in neuroblastoma, although a variety of purinergic mRNAs/proteins are expressed in these cells. Among these, the $P2Y_6$ receptor is the only subtype distinguished by UDP-specific activation. In this work, after over-expressing the $P2Y_6$ protein in human neuroblastoma SH-SY5Y cells, we find that UDP arrests cell cycle and induces apoptosis, by counteracting the pathological functioning of neuroblastoma *in vitro*. UDP also causes mitochondrial damage through diffusion of cytochrome c in the cytoplasm, and stimulates caspase-3,7,8 activities, with extensive over-expression of manganese superoxide dismutase. Our data establish the direct toxic role and anti-cancer activity of UDP in a neuroblastoma cell line, and identify the $P2Y_6$ receptor as a novel potential target in anti-tumoural therapies. This constitutes an advancement not only in the knowledge of purinergic signalling, but also in the biological and pathological aspects of neuroblastoma *in vitro*.

Among nervous system tumours, neuroblastoma is the most common extra cranial malignant solid tumour of childhood, with an annual average incidence of about 1.3/100,000 cases (Heck et al., 2009). The tumour is derived from the sympathetic nervous system, in particular from neural-crest-derived neuroectodermal cells, and the main site of origin is within the adrenal medulla (Brodeur, 2003).

Extracellular nucleotides play significant roles in the regulation of cell duplication during cancerogenesis, via cell-surface receptors termed P2 (Ralevic and Burnstock, 1998). These are divided into two subfamilies: seven P2X ligand-gated ion channels ($P2X_{1-7}$) and eight G protein-coupled P2Y subtypes ($P2Y_{1,2,4,6,11-14}$). P2Y receptors are widely distributed in the central and peripheral nervous system and involved in functions ranging from neurotransmission and neuromodulation, to trophic and toxic actions (Burnstock, 2006; Apolloni et al., 2009). Several members belonging to the P2Y family can be defined as pyrimidinergic, and these include subtypes as $P2Y_{2,4}$ stimulated by UTP, $P2Y_6$ activated by UDP, $P2Y_{14}$ stimulated by UDP-glucose. The $P2Y_6$ is thus the only receptor distinguished by UDP-specific activation (Nicholas et al., 1996).

The biological role particularly of P2 receptors in neuroblastoma is still not very well characterized and neither a correlation exists between P2 receptor expression and prognosis in neuroblastoma. However, mRNAs and proteins for various subtypes are expressed and activated in these cells, comprising the $P2Y_6$ protein (Sak et al., 1999, 2001; Lee et al., 2003; Cavaliere et al., 2005). For instance, in both primary neuroblastoma tumours and neuroblastoma cell lines, $P2X_7$ protein is detected and its activation *in vitro* by ATP and 3′-O-(4-benzoyl)benzoyl-ATP (BzATP) induces release of substance P, partially supporting cell proliferation (Raffaghello et al., 2006). Perhaps via $P2Y_{11}$ receptor subtype, extracellular ATP can moreover stimulate neurite outgrowth in mouse neuroblastoma neuro2a cells lacking nerve growth factor receptor (Lakshmi and Joshi, 2006). In addition, 2 methylthio-ATP and UTP inhibit cAMP accumulation in neuroblastoma-glioma hybrid cells (Song and Chueh, 1996). Finally, while a brief activation of the pyrimidinergic $P2Y_4$ subtype by UTP enhances neuritogenesis, a prolonged stimulation instead induces cell death mostly in $P2Y_4$-transfected SH-SY5Y neuroblastoma cells (Cavaliere et al., 2005). The specific aim of this work was thus to investigate the role of the $P2Y_6$ receptor and to compare it to that established for the $P2Y_4$

Abbreviations: MnSOD, manganese superoxide dismutase; PBS, phosphate-buffered saline; PI, propidium iodide.

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subunit. The features that $P2Y_6$ and $P2Y_4$ are both pyrimidinergic receptors, that UTP (preferential agonist for $P2Y_4$ receptor) is constitutively degraded into UDP (preferential agonist for $P2Y_6$ subunit) in the extracellular environment, and that $P2Y_6$ and $P2Y_4$ can form hetero-oligomeric complexes, have represented the rationale for their direct comparison within the same cellular context of human neuroblastoma SH-SY5Y cells. The results *in vitro* that we show in this work will thus contribute to expand the general knowledge about the $P2Y_6$ receptor, and to possibly identify its manipulation as a strategy for the understanding and potential relapse of neuroblastoma.

1. Materials and methods

1.1. Cell culture and pharmacological treatment

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's/F12 medium, with 10% heat inactivated foetal bovine serum, glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Sigma–Aldrich, MI, Italy), at 37 °C in 5% CO_2. UTP, UDP and uridine (Sigma–Aldrich) were added directly to the cells in complete culture media, for the indicated times.

1.2. Human (h)$P2Y_6$ stable clones

Human $P2Y_6$ ($hP2Y_6$) plasmid was constructed as described in D'Ambrosi et al. (2007). SH-SY5Y cells in 35-mm plates were stably transfected with empty pCMVTag2B vector (mock) or $hP2Y_6$ receptor cDNA using Lipofectamine™ 2000 (Invitrogen, Paisley, UK), in serum-free medium (Opti-MEM, Invitrogen) for 24 h. The transfected cells were maintained under selection with 200 μg/ml of antibiotic G 418 (Sigma–Aldrich) and single clones were isolated, expanded and screened, as described below.

1.3. Total RNA isolation and RT-PCR

G 418-resistant SH-SY5Y clones were screened for $hP2Y_6$ expression by RT-PCR. In details, cells were lysed in TRIZOL® and total RNA was extracted. RNA isolated was subjected to reverse transcription (RT) for 50 min at 42 °C using SuperScript™ II (200 units) and First Strand Buffer (Life Technologies, Milan, Italy), in the presence of 25 mM oligo dT, 10 mM DTT and 500 nM dNTP mix. Amplifications of cDNA obtained from RT were then performed in PCR buffer (Promega Corp., Madison, WI) containing dATP, dGTP, dTTP, dCTP (each used at 2.5 mM), in the presence of 1 μM of each $hP2Y_6$ specific primer (sense: 5′-ATGGATTACAAGGATGACGACGAT-3′; antisense: 5′-AGCCATGCCATAGGGCATATAGT-3′), and 2.5 units of GoTaq DNA Polymerase (Promega Corp.). PCR products were obtained after 30 cycles, at annealing temperature of 60 °C. As positive controls, cyclofilin primers (sense: 5′-TTGTCCATGGCAAATGCTGGACCA-3′; antisense: 5′-TGCTCTCCTGAGCTACAGAAG-GAA-3′) were inserted in each PCR for 21 cycles. Amplification products (15 μl out of 50 μl) and 100 base-pair markers (1 μg) were subjected to electrophoresis on 1% agarose gel containing 1 μg/ml ethidium bromide, and then photographed under UV light, using Kodak Image Station (KDS IS440CF 1.1). At least 10 different SH-SY5Y $hP2Y_6$ stable clones were screened and analyzed.

1.4. Immunofluorescence microscopy

Mock and $hP2Y_6$ transfected SH-SY5Y cells were fixed for 15 min in 4% paraformaldehyde, permeabilized for 5 min in PBS containing 0.1% Triton X-100. The cells were then incubated for 1.5 h at 37 °C with rabbit anti-P2Y6 (1:500, Alomone, Jerusalem, Israel), mouse anti-Cyt c (1:400, Promega Corp.), or rabbit anti-MnSOD (1:300, Stressgen, San Diego, CA), in 1% BSA in PBS. The cultures were stained for 1 h with Cy3-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch, West Grove, PA, USA), or Cy2-conjugated donkey anti-mouse IgG (1:200, Jackson Immunoresearch). Nuclei were stained with Höechst 33258 (1 μg/ml) for 5 min, and the cells were finally mounted and cover-slipped with gel/mount antifading (Biomeda, CA, USA). Immunofluorescence was analyzed using a reflected fluorescence microscope (BX51, Olympus) equipped with an F-View digital camera and the Cell-F Digital Imaging Software. Quantification of immunofluorescence was performed by direct counting only of those cells presenting a bright fluorescent signal over background. A range of 20–50 cells per field was counted in at least five different random fields.

1.5. Ca^{2+} imaging

The extracellular solution at pH 7.4 contained in mM: 135 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 Hepes and 10 glucose. The calcium-free extracellular solution was prepared by removing $CaCl_2$ and adding 0.5 mM EGTA. Intracellular calcium measurements were performed by using the fluorescent Ca^{2+} indicator fura-2 acetoxymethyl ester (fura-2 AM). Both mock and $hP2Y_6$ transfected SH-SY5Y cells were loaded for 45 min at 37 °C with 5 μM fura-2 AM dissolved in extracellular solution. The microperfusion chamber containing the coverslip was placed on the stage of an inverted fluorescence microscope Nikon TE200 (Nikon, Tokyo, Japan), equipped with a dual excitation fluorimetric calcium imaging system (Hamamatsu, Sunayama-Cho, Japan). Low-density seeded cells were continuously perfused at a rate of 2.5 ml min^{-1}. Emission florescence of selected cells was passed through a narrow-band filter and recorded with a digital CCD camera Hamamatsu C4742-95-12ER. Monochromator settings, chopper frequency and complete data acquisition were controlled by Aquacosmos Ratio software U7501-01 (Hamamatsu). The sampling rate was 0.3 or 0.5 Hz. Fura-2-loaded cells were excited at 340 and 380 nm, and fluorescence was measured at 510 nm. The fluorescence ratio F340/F380 was used to monitor $[Ca^{2+}]_i$ changes as described in Alloisio et al. (2006).

1.6. Cell count

Mock and $hP2Y_6$ transfected SH-SY5Y cells were grown either in 96-well opaque-walled tissue culture plates at a density of 10^4 cells/well, or in 24-well flat bottom plates at a density of 3×10^5 cells/well. The cell number was respectively evaluated by CellTiter-Blue® Cell Viability Assay (Promega Corp), or by direct count of intact nuclei. CellTiter-Blue® Reagent (20 μl/well) was added to 100 μl of culture medium, and the cells were then incubated at 37 °C for 3–4 h before recording the $560_{Ex}/590_{Em}$ fluorescence in a multi-label counter (Victor3-V, PerkinElmer Life Sciences). Fluorescence values were normalized with respect to non-treated control values, and expressed as percent viability. For direct count of intact nuclei, the cells were incubated for 5 min at room temperature with a mild detergent, as described (Volonté et al., 1994). In both cases, cells were scored in triplicate and counts represent means ± SEM.

1.7. PI staining

Mock and $hP2Y_6$ transfected SH-SY5Y cells were exposed to 500 μM UDP for the indicated times. They were collected, washed twice with ice-cold PBS, and fixed in 70% ethanol at −20 °C, for at least 1 h. After fixation, the cells were washed once and incubated with 75 kU/ml RNase A (Sigma–Aldrich) and 50 μg/ml propidium iodide (PI, Sigma–Aldrich) in PBS, for 1 h at room temperature in the dark. Fluorescence emitted from the PI–DNA complex was quantified by using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, USA). The percentages of the cells in the different cell cycle compartments were then estimated by applying the Modfit software to each DNA histogram.

1.8. Annexin V/PI staining

In the absence of permeabilization, PI stains necrotic cells. The simultaneous staining of cells with FITC-Annexin V and PI allowed the resolution of viable cells (double negative), early apoptotic cells (Annexin V positive and PI negative), necrotic cells (PI positive), late apoptotic cells (Annexin V positive and PI positive).

To determine the extent of early versus late apoptosis/necrosis, after incubation with 500 μM UDP for 24 h, $hP2Y_6$-SHSY5Y cells were harvested. Adherent and detached cells were pooled, washed and incubated for 10 min at room temperature in the dark, in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) with Annexin V-FITC (5 μl) (BD-Pharmigen) and PI (50 μg/ml). Annexin V-FITC and PI fluorescence were analyzed by using a FACScan cytofluorimeter. In each analysis, 10,000 events were recorded, and percentage of apoptotic cells estimated by means of the CellQuest software packaging (BD).

1.9. Caspase 3/7 activities

$hP2Y_6$-SHSY5Y cells were grown in 96-well white-walled tissue culture plates at a density of 10^4 cells/well. After UDP treatment, MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega Corp.) was first performed to normalize the cell number. Briefly, fluorescence was recorded in the blank, and both control and treated cells, in a multi-label counter (Victor3-V, PerkinElmer Life Sciences) (live-cell fluorescence $400_{Ex}/505_{Em}$; dead-cell fluorescence $485_{Ex}/520_{Em}$). Within the same samples and with the same counter, luminescence was recorded immediately after to evaluate the activities of caspase 3/7, by Caspase-Glo® 3/7 Assay (Promega Corp.), all according to the manufacturer's instructions.

1.10. Protein extraction, SDS-PAGE, and Western blotting

To isolate total protein extracts, cells were harvested with ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), added with protease inhibitor mixture (Sigma–Aldrich). Lysates were kept for 30 min on ice and centrifuged for 10 min at 14,000 × *g*, at 4 °C. Supernatants were collected and protein quantified by the Bradford method (Bradford, 1976). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). After saturation, blots were probed overnight at 4 °C, with mouse anti-Caspase 8 (1:1000, BD Biosciences,) or rabbit anti-MnSOD (1:2500), and finally incubated for 1 h with horse radish peroxidase-conjugated secondary antibodies and visualized using ECL Advance Western blotting detection kit (Amersham Biosciences). Analysis and quantifications were performed by Kodak Image Station.