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urine receptor ligands able to blastoma stem cells

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First of all, we would like to thank both reviewers for their suggestions that prompted us to revise the manuscript. We do hope to have fulfilled their requests and that the manuscript is now acceptable for publication.

Reviewer #1:

1) In Methods (Lactate dehydrogenase assay) is described that the analysis of this enzyme was performed after 24 hours of treatment with P1 and P2 receptor agonists on the GSC. However, in the experimental protocol is described that analyzes were performed on the fourth day after seeding. Please correct this information or better explain when the LDH assay was performed.

Reply: We corrected the wrong information. The assay was performed following the usual protocol, that is on the fourth days after cell seeding (see at pag. 11, the last line).

2) Check the Statistical Analysis. Why did the authors use Student's t test?

Reply: we checked the statistical analysis that was completely re-done using the ANOVA one way and the Dunnett's post hoc test. In this way, the most part of the results were more significant than those reported in the previous version of the manuscript.

3) Three samples were analyzed in this study. The sample obtained from patient 1 and 2 showed very similar molecular characteristics, but the sample obtained from patient 3 showed very different molecular characteristics compared to samples 1 and 2 (table 1). Furthermore, the results obtained with the sample 3 were often different from sample 1 and 2. The authors should increase the sample size to have more statistical power.

Reply: we agree with the reviewer that we should have increased the sample size to have more statistical power, but to accomplish this aspect we would have to repeat a lot of experiments. Thus, we think that the results we have presented are a suggestion needing further investigation.

4) The authors should explore the results from patient 3:

- How are the differences observed in Table 1 could be related to the results obtained with agonists and antagonists of the purinergic receptors?

Reply: As shown in the Fig. 1, the presence of the receptors is nearly the same in the three cell samples. So, we hypothesized that the difference observed in the cell responsiveness to the purinergic agonists and antagonists might be due to intrinsic and different cellular mechanisms against induced damages that confer a greater malignancy to some tumor cells (see the sentences at the end of the pag. 18, last two lines, and at the beginning of the pag. 19, the first four lines, in the Discussion section).

- Why does the sample 3 have a lower sensitivity to ATP and BzATP in comparison to sample 1 and 2? Could the expression or functionality of P2X7R be involved in the response to agonist in this patient?

Reply: The expression of P2X7 seems to be the same in all examined samples but we cannot rule out a different function of these receptor in the sample 3 or a different capability of the cells to repair cell damages, as reported above.

5) The authors should quantify the bands obtained by Western Blot (Figure 1).

Reply: we added the densitometric analysis of the bands of Western Blots in Fig. 1

6) What are the possible mechanisms involved in the reduction of proliferation when apyrase was added with BzATP (Figure 2A)?

Reply: we tried to explain this aspect suggesting that apyrase, reducing the amount of ATP in the extracellular medium, reduced its trophic contribution thus enhancing the BzATP cytotoxic effect, as reported in the Discussion at page 20, line 10-14.

7) Which other ectonucleotidases, such as NTPDase 1, 2 or 3, do these cells express? This data would be interesting to justify the results obtained with the treatment of ATP 500uM + MRS1220 (table 3).

Reply: we performed further experiments to show the presence of NTDase, in particular of CD39, that is also considered a marker for stem cells.

8) Please, change the Figure 3. The pattern of the graphics is very similar, leading difficult understanding of the Figure.

Reply: we revised the Figure 3 and we hope that in the new version it is more understandable.

9) The authors should add "data not shown" to the results obtained with other P1R antagonists (pg. 15 line 34; "inhibitory effects of adenosine on the growth of GSCs").

Reply: we followed the reviewer's advice.

Reviewer #2:

…. the paper is too long and sometimes difficult to be followed. I would thus suggest to shorten/simplify the text, especially as far as the Results section is concerned, in order to facilitate the extrapolation of the main message(s) of the study. The references are too many as well.

Reply: we tried to accomplish this request reducing either the length of the manuscript (especially that of the Results section, reduced by about a page) or the references' number (from 90 to 75).

A second comment concerns the fact that, since the Authors affirm that their cells "mirror the phenotype and genotype of primary tumors more closely than serum-cultured cell lines do" (and they are probably right), their results should be compared with (and discussed on the light on) the findings previously obtained under different experimental conditions. For instance, the apparent discrepancy between the present data and those of Ledur et al. (2012) , who reported that 100 μM of ATP did not influence the number of tumor spheres when grown in serum free medium supplemented with growth factors, should be discussed.

Reply: we included the requested comment in the discussion (see at pag. 19 from line 11 until the end and at pag. 20 lines 1-2.)

Furthermore, the Authors should cite, and take into account in the discussion, a recent study showing a prominent antiproliferative effect of A1 and A2B receptor agonists on CSCs isolated from human GBM cells (Daniele et al., Cell Death and Disease 2014).

Reply: we added the suggested reference (see at pag. 19,last line, in the Discussion section).

The observation that Bz-ATP + TMZ significantly decreased the amount of GSCs arrested in the G2/M phase and enhanced GSC accumulation in the S phase in comparison to TMZ alone does not seem correct, since according to Table 4 the effects of Bz-ATP + TMZ look the same of those exerted by Bz-ATP alone.

Reply: we agree with the reviewer and we corrected this aspect in the text (see at pag. 17 of the Results section, lines 12-14).

As a final suggestion, the Authors should rewrite the sentence concerning the "notable progress" in the therapy of GMB, since-unfortunately- the progress cannot actually be defined as notable.

Reply: also this was done (see the third line of Introduction).

NOTE: we changed one affiliation as at the beginning of the new year some authors passed to another Department, being the previous one no longer active (see the sixth line of the first page)

Potentiation of temozolomide antitumor effect by purine receptor ligands able to restrain the *in vitro* growth of human glioblastoma stem cells

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ABSTRACT

Glioblastoma multiforme (GBM), the most common and aggressive brain tumor in humans, comprises a population of stem-like cells (GSCs) that are currently investigated as potential target for GBM therapy. Here, we used GSCs isolated from three different GBM surgical specimens to examine the antitumor activity of purines. Cultured GSCs expressed either metabotropic adenosine P1 and ATP P2Y receptors or ionotropic $P2X_7$ receptors. GSC exposure for 48h to 10-150μM ATP, P2R ligand, or to ADPβS or MRS2365, $P2Y_1R$ agonists, enhanced cell expansion. This effect was counteracted by the PY_1R antagonist MRS2500. In contrast, 48h-treatment with higher doses of ATP or UTP, that binds to $P2Y_{2/4}R$, or 2'(3')-*O*-(4-benzoylbenzoyl)-ATP (Bz-ATP), $P2X_7R$ agonist, decreased GSC proliferation. Such a reduction was due to apoptotic or necrotic cell death but mostly to growth arrest. Accordingly, cell re-growth and secondary neurosphere formation was observed two weeks after the end of treatment. Suramin, non-selective P2R antagonist, MRS1220 or AZ11645373, selective A_3R or $P2X_7R$ antagonists, respectively, counteracted ATP antiproliferative effects. AZ11645373 also abolished the inhibitory effect of Bz-ATP low doses on GSC growth. These findings provide important clues on the anticancer potential of ligands for A_3R, $P2Y_1R$ and $P2X_7R$, which are involved in the GSC growth control. Interestingly, ATP and BzATP potentiated the cytotoxicity of temozolomide (TMZ), currently used for GBM therapy, enabling it to cause a greater and long lasting inhibitory effect on GSCs duplication when re-added to cells previously treated with purine nucleotides plus TMZ. These are the first findings identifying purine nucleotides as able to enhance TMZ antitumor efficacy and might have an immediate translational impact.

Key words: glioblastoma stem-like cells; cancer therapy; purine receptors; purinoceptor agonists and antagonists; temozolomide.

INTRODUCTION

Grade IV astrocytoma or glioblastoma multiforme (GBM) is one of the most aggressive human cancer, characterized by high invasiveness and resistance to the current standard care [1]. Despite the progress in the therapy of this tumor, consisting in maximal safe surgical resection followed by radiotherapy and temozolomide (TMZ), the prognosis of patients suffering from GBM remains poor, with a median survival of only 1.5 years [2].

A number of papers pointed out that tumor relapse could be due to a restrict population of cells, endowed with tumor initiating potential and resistance to radio-chemotherapy [3-4], which are commonly referred to as glioma stem-like cells (GSCs). Indeed, they share some properties with the neural stem cells (NSCs), resident in specific zones of the adult brain [5-7]. Moreover, it has been suggested that GSCs could arise from the malignant transformation of NSCs, mainly deriving from the subventricular zone, from which they would migrate leading to the development of glioma in different cerebral areas [4, 8].

GSCs have been isolated from GBMs and grown as neurospheres *in vitro*, showing a high capacity for self-renewal and aberrant differentiation. Additionally, these cells are able to form tumors after grafting in immunosuppressed mice [9, 10], demonstrating that they are required for tumor development and maintenance [11]. Although the definitive role of GSCs in GBM ontology and recurrence remains elusive, these properties attracted a great interest by researchers and clinicians as new targets of the therapy of this tumor.

Extracellular purine nucleosides (adenosine, guanosine) and nucleotides (mainly ATP and GTP) have long been recognized as modulators of neurotransmission and trophic factor production in the adult central nervous system (CNS), where they contribute to regulate neuronal plasticity and glial function [12]. Accordingly, they have also been implicated in the pathophysiology of numerous pathological conditions ranging from brain trauma and ischemia to neurodegenerative and neuropsychiatric disorders [13]. More recently, purinergic signaling has been shown to be involved in embryological/postnatal brain development [14, 15] and NSC activities [16-20], and to play a role in cancer biology [21]. In this regard, some reports on purine and pyrimidine analogues, used as antileukaemic agents or experimental cytotoxics to treat solid tumors [22-25], showed that they behave as antimetabolites competing with physiologic nucleosides [26]. In contrast, many other *in vitro* and *in vivo* studies on different cancer types including brain tumors, demonstrated the antitumoral activity of purines as being linked to the selective stimulation of specific purinoceptors [21, 27-33]. While only brain specific binding sites for guanine-based compounds have been discovered [34], receptors for adenine-based purines are found in all neural cells [35]. They are divided into P1 (P1R) and P2 (P2R) receptors recognizing adenosine and ATP as the main ligands, respectively [36]. P1R family comprises 4 subtypes, namely A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, whereas P2R family is subdivided into seven ionotropic P2XR and eight metabotropic P2YR, which have currently been cloned in mammalian species and characterized [37-38]. It is to highlight that studies on purine effects are in general complicated by the presence of either P1R or P2R on the same cell, often playing opposite roles, and by the existence of ecto-enzymes rapidly transforming nucleotides into active nucleosides [39].

In the last ten years, data have been published on the activity of extracellular purines, mainly adenine-based nucleotides, acting as antitumoral drugs in human and rat brain tumor cell lines [30, 33, 40]. However, the relevance of conventional cell lines (cultured under mainly serum-based media conditions) as means to investigate GSCs is disputed, as the results may be biased by multiple new mutations induced during long-term culture in serum-containing media. On the contrary, GBM cell lines derived from freshly resected tumor specimens and cultured in serum-free medium supplemented with EGF and bFGF - conditions optimized for the growth of neural stem cells - mirror the phenotype and genotype of primary tumors more closely than serum-cultured cell lines do [41, 42]. Therefore, since GBM stem cell cultures may be a more reliable model to appreciate the efficacy of therapeutic agents, we thought to re-investigate the anticancer effects of purine compounds (natural and synthetic ligands) on these cells, checking whether they acted at extracellular levels as signaling molecules, as previously shown in conventional cell lines so far studied [30, 40, 43]. Additionally, in order to make our experimental contribution potentially useful for the improvement of the current GBM therapy, we evaluated whether purine nucleotides might increase the toxicity of TMZ and the duration of its effect on these cells.

METHODS

Chemicals

3-[[5-(2,3-Dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine hydrochloride (A438079), 3-[1-[[(3'-nitro[1,1'-biphenyl]-4-yl)oxy]methyl]-3-(4-pyridinyl)propyl]-2,4-thiazolidinedione (AZ 11645373), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), *N*-[9-Chloro-2-(2-furanyl) [1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide (MRS1220), [[(1*R*,2*R*,3*S*,4*R*, 5*S*) -4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl]diphos-phoric acid mono ester (MRS2365) trisodium salt, (1*R**,2*S**)-4-[2-iodo-6-(methyl amino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol-dihydrogen-pho-sphate ester (MRS2500) tetra-ammonium salt, 4,4'-(Carbonyl*bis*(imino-3,1-(4-methyl-phenylene)carbonylimino))*bis*(naphthalene-2,6-disulfonic acid) tetrasodium salt (NF340), 4,4'-(Carbonyl*bis* (imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)carbonylimino))-*bis*(1,3-xylene-alpha,alpha'-diphosphonic acid tetra-sodium salt (NF546), 8-[4-[4-(4-chlorophenzyl) piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB603) and 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) were purchased from Tocris (Space Import/Export, Milan, Italy); apyrase (VII grade), 2’(3’)-*O*-(4-benzoylbenzoyl)-

ATP (Bz-ATP), periodate-oxidized-ATP (oATP), suramin and TMZ were from Sigma-Aldrich as well as all other chemicals, unless differently specified (Milan, Italy).

Cell cultures

We used GSCs obtained from three different patients with a primary tumor, the molecular profile of which is reported in the Table 1. Once isolated, cells were grown in serum-free medium, supplemented with mitogens (20 ng/ml of human recombinant EGF and 10 ng/ml of human recombinant bFGF), as previously described [44, 45]. Under these culture conditions, tumor-derived cells formed classical floating aggregates called tumor neurospheres, that were used for some experiments. However, whereas the most part of them were performed on cells grown as monolayer obtained precoating culture plates with Matrigel (BD Bioscience, Buccinasco, Italy) dissolved in culture medium and then seeding the cells that were fed with the usual culture medium containing also the growth factors above mentioned (dilution 1:200). In this condition, GSCs maintained spherogenic properties [44, 45], but their use allowed a more precise quantification of cell survival in vitro in comparison with neurospheres. It is to underline that cells used in this study were previously characterized for many biologic/molecular features including the retention of stemness markers, the resistance to chemotherapeutic agents, the ability of self-renewing *in vitro* or generating a tumor identical to the human tumor in antigen expression and histological tissue organization when injected in the brain of immunocompromised mice [44-46].

Experimental protocols

Using undifferentiated GSCs grown as either monolayer or neurosphere suspension, purinergic drugs and also apyrase, the enzyme catalyzing the hydrolysis of ATP to yield AMP, were administered on the 2nd day after cell plating and renewed on the next day, whereas TMZ, either alone or in combination with purine receptor ligands, was usually administered only once, on the 2nd day after cell plating, except in the last experiment (see Fig. 7) in which it was administered a second time alone, 4 days after the first treatment. Purine receptor antagonists, when present, were administered to the cultures 30 min prior to the agonists. In the experiments with undifferentiated GSC monolayers, cell viability or apoptotic/necrotic death were measured on untreated/treated cells on the fourth and also on the seventh day after seeding. In the experiments with neurospheres in suspension, the effect of the pharmacological treatments was estimated at different intervals from the drug administration as the capability of the drugs to affect the process of neurosphere formation. The neurosphere size was evaluated the first time at day 7 after the pharmacological treatment. Then, the effect of the same pharmacological treatment was assessed on the GSC ability to form secondary neurospheres from the primary ones, that is usually considered as an index of tumor invasiveness. In this kind of experiments, untreated/treated neurospheres were allowed to recover for further 7 day (14 days from the beginning of the experiment), then they were removed, centrifuged, mechanically dissociated and re-suspended at fixed number (10,000/well). The formation of secondary neurospheres was assessed at day 21 from the beginning of the experiment.

Analysis of CD39 expression, cell cycle distribution and apoptosis by flow cytometry

To analyze the expression of CD39, a typical cell surface protein marker, 5×10^5 cells/sample were incubated with 100μl of 20 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C for 10 min. Cells were washed with 3 ml of washing buffer and centrifuged (4 °C, 400×g, 8 min). Subsequently, samples were resuspended in 100μl washing buffer containing the appropriate amount of fluorescein isothiocyanate-conjugated (FITC antibody against CD39 (Becton Dickinson, BD, San Jose, CA) and incubated for 30 min at 4 °C in the dark. Tubes were washed (3 ml of washing buffer), centrifuged (4 °C, 400×g, 8 min) and cells were resuspended with 1 ml 0.5% paraformaldehyde, incubated for 5 min at room temperature, washed, centrifuged (4 °C, 400 ×g,8 min) and stored at 4 °C in the dark until the acquisition with a FACSCalibur flow cytometer (BD), using the CellQuest™ 3.2.1.f1 software (BD). Ten min before the analysis, a specific solution, containing 7-amino-actinomycin D (BD) was added to the samples to exclude dead cells. Quality control was performed by a regular check-up with Rainbow Calibration Particles (BD Biosciences). Debris was excluded from the analysis by gating on morphological parameters; 20,000 non-debris events in the morphological gate were recorded for each sample. Data were analyzed using the FlowJo™software (TreeStar, Ashland, OR). Mean Fluorescence Intensity Ratio (MFI Ratio) was calculated dividing the MFI of positive events by the MFI of negative events.

To assess cell cycle distribution or apoptosis, $5x10^5$ cells/6-well were exposed to various concentrations of purinergic ligands for 48 h. Cells were collected, and cell cycle distribution was analyzed by propidium iodide (PI) flow cytometry. Both floating and attached cells were collected and poured together into centrifuge tubes. They were rinsed with PBS and fixed with 70% ice-cold ethanol containing 2 mg/ml RNase for 30 min. Subsequently, cells were washed 2 times with PBS and ultimately stained with PI (40 µg/ml) at room temperature for 10 min. PI fluorescence of cells was determined by the same Flow Cytometer (BD Biosciences) with excitation wavelength of 488 nm and emission of longer than 590 nm. The propidium iodide fluorescence of 20,000 cells was counted for the control and purine agonist-treated samples. The percentage of cells in G_0/G_1 phase, S phase and G_2/M phase was calculated by use of standard ModiFit and CellQuest software programs [47].

Quantification of apoptosis by caspase 3/7 activity

Quantification of apoptosis in GSCs, treated with appropriate concentration of P2 agonist receptors, was performed using Caspase-Glo Assay Tecnology by providing a luminogenic caspase 3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase acitivty, the luciferase activity. Luciferase activity is proportional to the amount of caspase activity present. The assay was carried out according to the instructions of the supplier company (Promega Italia, Milan, Italy)

RNA isolation and RT-PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. The resulting RNA pellet was dissolved in 30 μl diethyl-pyrocarbonate-treated water and 5 μg were run on formaldehyde denaturing gel to confirm the integrity of the RNA. To remove any genomic DNA contaminants, RNA samples (10 μg) were treated with 1 U DNase-I RNase-free (Roche, Monza, Italy). First strand cDNA was synthesized from 1.5 μg of total RNA using the RT-PCR system RETROscript^{TM} (Ambion, Monza, Italy) with random hexamers. The resultant cDNA (2 μl) was amplified in a 100 μl reaction volume containing PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxy-dNTP, 1 μM oligonucleotide primers (MWG Biotech, Ebersberg, Germany), 2.5 U AmpliTaq Gold^{TM} DNA polymerase (Applied Biosystems, Life Technologies). The final cycle was followed by a 10-min incubation at 72°C. All primers were synthesized by MWG Biotech. PCR primers, annealing temperatures, and product sizes are shown in Table 2. Reaction was also performed without the reverse transcriptase step as control for genomic contamination. PCR products were separated by 1.5% agarose gel electrophoresis in gels containing ethidium bromide and visualized with UV light.

Western Blot Analysis

Cells were harvested at 4°C in a lysis buffer (in mM: Tris buffer 50, NaCl 150, PMSF 1.0; 1% Nonidet-P40, 5 µg/ml leupeptin, 5 µg/ml aprotinin), disrupted by sonication and centrifuged (14,000 rpm, 5 min, 4°C). Protein concentration was determined by BioRad protein assay (Bio-Rad Laboratories, Milan, Italy). Samples (50 µg), diluted in SDS-bromophenol blue buffer, were boiled (5 min) and separated on 12.5-15% SDS-polyacrylamide gels. Proteins were transferred on a polyvinylidene fluoride membrane, blocked with PBS/0.1% Tween20/5% non-fat milk (Bio-Rad Laboratories) for 2 h at 4°C, incubated overnight at 4 °C with specific primary antibodies [polyclonal rabbit anti-A_1, anti A_{2A} anti-A_{2B}, anti-A_3, anti P2Y1-2-4-6-11-12-13-14, anti-$P2X_7$, dilution 1:1000 (Alomone Labs, Jerusalem, Israel)]; polyclonal rabbit anti CD73, dilution 1:500 (Novus Biologicals, LTD) and then exposed to donkey anti-rabbit HPR-conjugated secondary antibody for 1 h at room temperature (GE Healthcare Life Sciences, Milan, Italy; final dilution 1:2500). The specificity of the antibodies used to determine the presence of adenosine receptors was previously established [48-51]. To determine the equal loading of samples, the blots were stripped and re-probed with an anti-ß-actin antibody (dilution 1:1000, incubation overnight at 4 °C ; Santa Cruz Biotechnologies). Immunocomplexes were visualised using the enhancing chemiluminescence (ECL) detection system (GE Healthcare Life Sciences) and quantified by densitometric analysis (Molecular Analyst System).

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) levels are widely used to estimate necrotic cell death since LDH is a cytoplasm enzyme that can be released following cell membrane damages. Cells, seeded ($3x10^3$ cells/well) in 96-well plates, were incubated with agonists of P1 and P2 receptors following the usual protocol. Twenty four hours after the end of the treatment, the cells were incubated at 37 ° C and 5% CO2 for 45 min with specific lysis buffer and then the plate was centrifuged at 250 g for 4 min. Then, 50 µl of supernatant from each well, transferred to a new 96-well plate, were added to 50 µl of substrate buffer consisting of 0.7 mM p-iodonitrotetrazolium Violet, 50 mM L-lactic acid, 0.3 mM phenazine methoxysulfate, 0.4 mM NAD and 0.2 M Tris-HCl pH 8.0. The plate suitably blanket was incubated in the dark at room temperature for 30 min, and finally the reaction was stopped by addition of 50 µl/well of stop solution. The absorbance was measured spectrophotometrically at 490 nm and the results were expressed as a percentage of total LDH released from the positive control consisting of cells treated with 25 µl of 10% Nonidet P-40 (NP-40) and calculated as follows: (supernatant absorbance value - white absorbance value)/ (supernatant absorbance + lysate absorbance) x 100. All reagents were from Promega Italia (Milan, Italy)

Cell proliferation

Cell proliferation was assayed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Italia, Milan, Italy), according to the manufacturer's instructions. The absorbance was measured at 490 nm, using a microtiter plate reader (Spectracount^{TM}, PerkinElmer Life, Waltham, MS, USA).

Statistical Analysis

All results are presented as mean ± SEM. Data were analyzed by the Student's t- test, with a significance level of 95%. For the analysis of difference between more than two groups one-way analysis of variance (ANOVA) was used followed by Dunnett's *post hoc* test. Difference was considered to be statistically significant at a value of $p<0.05$. Analyses were performed with GraphPad Prism 4 (GraphPad, San Diego, CA, USA).

RESULTS

GSC line characterization

We used GSCs deriving from samples of primary GBMs obtained from three patients. The primary tumors showed various molecular profiles (Table 1), that could be correlated with the patient outcome and the response to TMZ in malignant gliomas [52-53]. Notably, tumor from the patient n. 3, differently from those of the other two patients, showed an unmethylated status of the enzyme O^6-methylguanine methyltransferase (MGMT) promoter compatible with the shortest period of overall survival of that patient, as reported elsewhere [54].

Following standard protocols, GSCs were cultured in the presence of specific growth factors (see Methods section) either as cell suspension, that allows the formation of the classical neurospheres, or as adherent cells on a Matrigel substrate (Fig. 1A). In both cases, GSCs maintained an undifferentiated state, as indicated by cell morphology.

GSC express P1 and P2 purinoceptors

As first step, we ascertained the expression of P1R and P2R in GSCs, focusing on the metabotropic ones. We also looked for ionotropic $P2X_7R$, that is present in glioma cell lines [43] and causes death in a number of cells [55-56]. By either RT-PCR or Western Blot techniques, the presence of all four adenosine receptors was detected in GSCs, with the $A_{2B}R$ protein expressed to the lowest extent (Fig. 1D). In the same cells, all P2YR responsive to ATP were present, whereas, among those responsive to pyrimidine nucleotides, $P2Y_2R$ showed the greatest expression (Fig. 1E). Additionally, GSCs expressed high levels of $P2X_7R$ (Fig. 1E). We also ascertained the presence of the stem cell markers CD39 and CD73, corresponding to the enzymes ecto-nucleoside triphosphate diphosphohydrolase 1 and 5’-

ectonucleotidase (Fig. 1B-C), respectively, the activity of which is fundamental for the metabolism of extracellular nucleotides [43].

P2 receptor agonists significantly reduced GSC growth

Initially, the effect due to extracellular ATP, present as nutrient or released from cells in the culture medium, was assayed exposing cells to apyrase, that metabolizes ATP and ADP, therefore reducing the amount of extracellular active nucleotides. Apyrase alone did not modify cell growth, as shown in Fig. 2A where we reported the effect measured in GSCs from the patient n. 3, for which the results were similar to those obtained from cells of the other two patients (data not shown).

Then, the effects of the stimulation of different P2R subtypes were assayed on the proliferation of GSCs, using natural (ATP and UTP, both non selective P2R agonists), and synthetic ligands (MRS2365 and NF546, currently the most selective agonists of $P2Y_1R$ and $P2Y_{11}R$, respectively, and Bz-ATP, $P2X_7R$ agonist). We also evaluated the effect of ADPβS, a less selective agonist for $P2Y_1R$ in comparison to MRS2365, that may also interact with $P2Y_{12\text{-}13}R$. All agents were administered in a range of concentrations from 1 μM up to 1 mM, except MRS2365, employed at concentrations from 1 nM to 10 μM.

MRS2365 and, to a lesser extent, ADPβS, increased cell proliferation rate in dose-dependent fashion, most in GSCs from the patient n. 3. The same did ATP, stimulating cell duplication up to the dose of 150 μM (Fig. 2B). In contrast, when administered at greater concentrations, ATP inhibited GSC proliferation in dose-dependent manner and Bz-ATP, starting from the concentration of 100 μM onwards, did the same whereas UTP reduced cell viability only at the highest doses (500-1000 μM) and NF546 did not affect it at all (Fig. 2B). The GSCs isolated from the patient n. 3 showed a lower sensitivity to ATP or Bz-ATP cytotoxicity. Interestingly, the presence of apyrase together with sub-maximal doses of Bz-ATP further reduced cell viability and this effect was significant even in less responsive GSCs from patient n. 3 (Fig. 2A).

The effect of P2YR and $P2X_7R$ agonists on the growth of GSCs was counteracted by the respective antagonists

We investigated whether the effects caused by purine nucleotides were mediated by the interaction with P2R, using the antagonists commercially available. All compounds, when administered alone, did not affect cell viability, except o-ATP, an older $P2X_7R$ antagonist (Fig. 3A), that was no longer used in the subsequent experiments. In the presence of purinergic agonists, MRS2500, $P2Y_1R$ selective antagonist, reduced the proliferative effect of MRS2365, ADPβS and ATP (Fig. 3B-C). On the other hand, the antiproliferative effect of a submaximal dose (500 µM) of ATP was in part reversed by either suramin, non selective P2R antagonist, or AZ11645373, but not by TNP-ATP, that blocks all P2XR except the $P2X_7$ one, or by NF340, blocking the $P2Y_{11}R$ (Fig. 3 B). AZ11645373 (Fig. 3C) and A438079 (data not shown) also inhibited the effect of low (100 µM) but not of high (500 µM) doses of BzATP, whereas suramin did not counteract the effect of 500 µM UTP. As expected, NF340 did not alter GSC growth in the presence of the $P2Y_{11}R$ agonist NF546 (Fig. 3C).

Inhibitory effect of adenosine on the growth of GSCs

Next, the activity of nucleosides, such as adenosine, inosine and uridine, again administered in the range from 1 µM to 1 mM for two consecutive days, was assayed to verify if they might be responsible for the effect caused by purine/pyrimidine nucleotides. Only adenosine, at the highest doses, reduced the cell survival by about 40-60% (Table 3), whereas the other assayed nucleosides did not significantly affect GSC growth (data not shown). To prove the involvement of one or more adenosine receptors (AR), we tested the adenosine effect in the presence of DPCPX (100 nM), ZM241385 (100 nM), PSB603 (300 nM) and MRS1220 (1 µM), selective antagonists of A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, respectively. Among these, MRS1220 (Table 3), but not the other compounds (data not shown), counteracted the adenosine effect. MRS1220 was also able to limit the inhibitory activity of ATP high doses on cell growth (Table 3), thus suggesting the nucleotide effect could in part be mediated by the formation of extracellular adenosine that in turn, interacting with A_3R receptor, may cause cell arrest.

Synergistic antitumoral effect of the combined treatment of GSCs with TMZ, a classic antitumoral agent for GBM therapy, and purinoceptor ligands

In another set of experiments, we evaluated the anti-proliferative activity of purine compounds in the presence of TMZ, an agent currently used in the chemotherapy of GBM [2]. TMZ, administered alone to the cultures (Fig. 4A), caused a dose-dependent reduction of cell survival, which was still evident after 7 days. When it was administered at 50 and 75 µM in the presence of submaximal doses of ATP or UTP or BzATP, the antiproliferative effect of these nucleotides was significantly enhanced (Fig. 4B), except in the GSCs from the patient n. 3, less sensitive to the cytotoxicity induced by all compounds.

We wondered whether the reduction of cell viability by purine compounds, alone or combined with TMZ, was due to cell death or cell growth impairment. All assayed nucleotides significantly increased cell necrosis as revealed by the measurement of LDH release (Fig. 5A); ATP and UTP remarkably enhanced also the cell apoptotic death as pointed out by the assay for the caspase 3/7 levels and/or by the propidium iodide assay (Fig. 5 B-C). Additionally, ATP diminished the number of cells in the G_2/M phase, whereas Bz-ATP accumulated them in the S phase (Table 4). Thus, the decrease in cell viability was in part due to a cytotoxic effect by purine nucleotides, which caused also an arrest of cell proliferation acting at intracellular level, likely on DNA duplication process.

Also the inhibitory effect of TMZ alone on GSC growth was due in part to the increase of the percentage of either apoptotic or necrotic cells and to a substantial arrest of the cell growth with a consequent increase of their number in the S and G2/M phases of the cell cycle (Table 4 and Fig. 5B-C). When TMZ was administered together with ATP, the number of apoptotic cells significantly increased, even though there was no evident involvement of the caspase 3/7 activity (Fig. 5B-C). Moreover, this combined treatment further enhanced the percentage of GSCs arrested in the G_2/M phase of the cell cycle, although this effect did not reach a statistical significance when compared to the effect of TMZ alone (Table 4). In contrast, the contemporaneous presence of Bz-ATP with TMZ determined a more relevant necrotic process (Fig. 5A), whereas the changes in the amount of GSCs in the G2/M phase or in the S phase of the cell cycle were equivalent to the effect of Bz-ATP alone, the effect of which was prevalent over that exerted by TMZ alone (Table 4).

Since GSCs are provided with many mechanisms for DNA repair or against apoptosis [57], we checked whether the effect caused by nucleotides in combination or not with TMZ on cell growth was long-lasting. As expected, cell proliferation evaluated by MTS assay was restored 14 days after the pharmacological treatment (data not shown).

Effect of purine/pyrimidine compounds and TMZ on the formation of secondary neurospheres following different period from the initial treatment of primary neurospheres.

We tested the effect of purine/pyrimidine nucleotides and/or TMZ, added to the cells following the usual protocol, on the formation of neurospheres that develops when GSCs are cultured as cell suspension and could result more resistant to any pharmacological treatment. The size of surviving neurospheres, measured 7 days from the beginning of the experiment, was significantly reduced by cell exposure to nucleotides (Fig. 6A-C). In contrast, the size of secondary neurospheres, considered as an index of tumor invasiveness, was similar to that of control, except for GSCs exposed to Bz-ATP or TMZ alone, that significantly reduced it. The administration of TMZ in the presence of purine/pyrimidine nucleotides did not cause further significant reduction in the size of primary or secondary neurospheres, measured after 7 or 21 days, respectively, in comparison to the effect of nucleotides alone (Fig. 6B-D).

As the last experiment, we assayed a further experimental protocol, administering TMZ alone a second time, that is 4 days after the initial treatment performed with TMZ in combination with nucleotides. Also in this case, the effect of TMZ was greater in cells previously treated also with ATP and Bz-ATP but the effect was still evident after 14 days even in GSCs from the patient n. 3 (Fig. 7).

DISCUSSION

GBM is the most common and aggressive primary tumor of the adult CNS, against which the current therapy is largely ineffective [1, 2]. In the attempt to find novel druggable targets to combat this tumor, we investigated the antitumoral effects of purine/pyrimidine nucleotides that are able to interact with specific own receptors. These agents were administered alone or in combination with a specific anti-tumoral agent, the TMZ, in GSCs deriving from primary human tumors, which are believed to be responsible of GBM relapse/invasiveness [3, 4]. We had the opportunity to use cells isolated from primary tumors with different biological characteristics and various expression of molecular prognosticators (Table 1) [52-54]. Interestingly, GSCs from the tumor of the patient n. 3, who had a shorter period of overall survival in comparison with the other two patients, showed a decreased response to the antiproliferative effects induced by TMZ and also by purine nucleotides. It is reasonable to hypothesize that this could be due to intrinsic mechanisms of greater resistance of these cells

(i. e. processes related to DNA repair) or to a dysfunction of some of the investigated receptors and not to differences in their expression, that was similar in all three kinds of cells, in agreement with levels reported for glioma cells lines [43, 58]. Of course, this aspect should deserve a deeper investigation.

Noteworthy, the expression of $P2X_7R$ in our GSCs resulted more pronounced than that of other purinergic receptors. In agreement with these data, we here demonstrated that $P2X_7R$ plays a pivot role in inhibiting GSC growth. In fact, our cells were sensitive to the cytotoxic effects caused by Bz-ATP, $P2X_7R$ agonist, even when it was administered to GSCs grown as neurospheres. A similar effect was found also in cells exposed to high concentrations of ATP, which are known to stimulate the same receptor. Finally, Bz-ATP and ATP effects were in part counteracted by the $P2X_7$ antagonist. Similar findings were reported in glioma cell lines in which a toxic effect was induced by purine nucleotides coupled to a prevalent involvement of $P2X_7R$ [reviewed in reference 43], whereas our results differ from those obtained by Ledur et al. [40], who showed a toxic effect of ATP low concentrations on the growth of tumor spheres obtained from those cell lines, especially in the absence of serum supply. We hypothesize that this difference may be due to a greater resistance of GSCs deriving from freshly resected human glioblastoma to nucleotide toxic insults.

ATP antitumor activity, in part likely due to its conversion into adenosine by ectoenzymes present in the GCS membrane, was reduced also by cell pre-treatment with MRS1220, an A_3R antagonist that restrained also the citotoxicity caused by high doses of adenosine. The A_3R anticancer effect, like that of $P2X_7R$, has been described in other malignancies [59-62], even though other papers argued the opposite [63-66]. Of note, there was no involvement in the control of GSC growth by other AR, such as A_1R and $A_{2B}R$, the stimulation of which caused a prominent antiproliferative effect on stem cells from human GBM cell lines [67]. Such a discrepancy may depend on differences in receptor expression and function in tumor cells, in turn depending also on the source of GSCs, that in our case derive from primary tumors.

Our findings indicate that purine effects are only in part receptor-mediated. Indeed, the cell cycle analysis suggested that GSC growth impairment was due also to their direct interference with DNA duplication, as previously reported [26]. Also this aspect should be taken into due consideration for future clinical application of these compounds, as the failure of cell duplication in normal tissues might cause possible broader side-effects. So far, ATP has been used as a compassionate drug in terminally ill patients [68, 69]. The systemic administration did not cause important side effects indicating that the therapy with this compound may be tolerated [70]. Nothing is known as for the administration of Bz-ATP or other compounds acting on $P2X_7R$ in humans and the investigation in animals is just at the beginning [71, 72].

Noteworthy, ATP may also favor GSC proliferation. The trophic role of ATP, reported by other authors in human glioma cell lines [30], is also suggested by the effect of apyrase, able to metabolize extracellulare ATP, thus reducing its presence in the growth medium. In this way, although ineffective when administered alone, apyrase likely potentiated the inhibitory activity of moderate doses of the synthetic $P2X_7R$ agonist, Bz-ATP, on CGS duplication. $P2Y_1R$ are involved in the ATP-induced tumor growth as MRS2365 and ADPβS, $P2Y_1R$ agonists, showed an effect similar to that of a low dose of ATP whereas MRS2500, $P2Y_1R$ specific antagonist, counteracted the trophic effect of all agonists cited above. A tumorigenic role of $P2Y_1R$ was reported for embryonic carcinoma cells [32], in which, differently from our findings, the stimulatory effect on cell growth was shared with the activation of $P2Y_2R$, responsive to UTP. However, in other tumor cells, i.e. in prostate cancer cells, $P2Y_1R$ stimulation caused apoptosis [73].

Other ATP P2 receptors do not seem to be involved in the control of GSC proliferation. In fact, TNP-ATP, able to block all P2XR except $P2X_7R$, did not counteract ATP effects; also the involvement of $P2Y_{11}R$ could also be excluded as well as that of $P2Y_{12\text{-}13}R$, as the proliferative effect of ADPβS, agonist also for these receptors, was completely reversed by the $P2Y_1R$ antagonist. These findings are different from that obtained in murine and human cell lines, in which $P2Y_{12}R$ appear to increase cell proliferation, whereas $P2Y_1R$ have opposite effects in different cell lines, causing either an increase or a decrease in the cell population [43]. Altogether, our results confirm the need to evaluate the pattern of purinoceptor expression and function, which is specific for each tumor.

Finally, in our opinion, the effects obtained by the cell exposure to TMZ in combination with cytotoxic doses of purinergic compounds are remarkable. For the first time, we demonstrated that this combined pharmacological treatment resulted in a potentiated antiproliferative effect, at least in cells derived from primary GBMs grown as monolayer, which likely were more easily accessible by the drugs. In particular, cell exposure to ATP or BzATP plus TMZ significantly enhanced the percentage of apoptotic or necrotic cells. Apart a possible increased cytotoxicity induced by the nucleotides, a tentative explanation of the potentiated effect might be that ATP or BzATP, by opening the pore associated to the $P2X_7R$, favor TMZ entry into the cells, counteracting the drug extrusion through the membrane ABC protein, a mechanism through which TMZ loses its efficacy [74]. Moreover, since BzATP significantly reduced GSC accumulation in the G2/M phase of the cell cycle caused by TMZ, a period during which DNA repair may occur, a mitotic catastrophe might be favored, like that induced by resveratrol [75].

Of interest, the potentiated effect due to the combined administration of nucleotides plus TMZ to GSCs was long-lasting when TMZ was re-administered. Likely, TMZ cytotoxicity was increased as it acted after a short period on cells damaged by the previous combined treatment with nucleotides and TMZ. This aspect, in our opinion, deserves further investigation and we hypothesize that repeated treatments could be successful also in GSCs grown as neurospheres.

Thus, a lot of work remains to be done to assess the most appropriate doses and times of drug administration in order to optimize the antitumoral effect of purines either if administered alone or in combination with a classic antineoplastic agent. However, based on our results, we think that the investigation on purinoceptor ligands is promising and may open the way to further studies on *in vivo* tumor models.

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REFERENCES

1. Wen PY, Kesari S. (2008) Malignant gliomas in adults. N Engl J Med 359: 492-507.
2. Clarke J, Butowski N, Chang S. (2010) Recent Advances in Therapy for Glioblastoma. Arch Neurol 67: 279-283.
3. Deleyrolle LP, Harding A, Cato K, Siebzehnrubl FA, Rahman M, Azari H, Olson S, Gabrielli B, Osborne G, Vescovi A, Reynolds BA. (2011) Evidence for label-retaining tumour-initiating cells in human glioblastoma. Brain 134(Pt 5): 1331-1343.
4. Chen J, Li Y, Yu TS, McKay RM, Burns DK, Kernie SG, Parada LF. (2012) A restricted cell population propagates glioblastoma growth after chemotherapy. Nature 488 (7412): 522-526.

5. Götz M, Huttner WB. (2005) The cell biology of neurogenesis. Nat Rev Mol Cell Biol. 6:777–788.

6. Lois C, Alvarez-Buylla A. (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci. USA. 90: 2074–2077.

7. Morshead CM, Craig CG, van der Kooy D. (1998) In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. Development 125:2251–2261.

8. Berger F, Gay E, Pelletier L, Tropel P, Wion D. (2004) Development of gliomas: potential role of asymmetrical cell division of neural stem cells. Lancet Oncol. 5(8): 511-514.

9. Singh SK, Clarke ID, Hide T, Dirks PB. Cancer stem cells in nervous system tumors. Oncogene 2004; 23:7267-7273.

10. Pérez Castillo A, Aguilar-Morante D, Morales-García JA, Dorado J. (2008) Cancer stem cells and brain tumors. Clin Transl Oncol. 10:262-267.

11. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. (2004) Identification of human brain tumour initiating cells. Nature 432:396-401.

12. Rathbone MP, Middlemiss PJ, Gysbers JW, Andrew C, Herman MAR, Reed JK, Ciccarelli R, Di Iorio P, Caciagli F. (1999) Trophic effects of purines in neuron and glial cells. Prog Neurobiol 59: 663-690.

13. Burnstock G. (2008) Purinergic signalling and disorders of the central nervous system. Nat Rev Drug Discov. 7(7): 575-590.

14. Burnstock G, Ulrich H. (2011) Purinergic signaling in embryonic and stem cell development. Cell Mol Life Sci. 68(8): 1369-1394.

15. Zimmermann H. (2006) Nucleotide signaling in nervous system development. Pflugers Arch. 452: 573–588.

16. Rishton GM. (2008) Small molecules that promote neurogenesis in vitro. Recent Patents CNS Drug Discov. 3(3): 200-8.

17. Resende, RR, Majumder P, Gomes KN, Britto LRG, Ulrich H. (2007) P19 embryonal carcinoma cells as in vitro model for studying purinergic receptor expression and modulation of N-methyl-D-aspartate-glutamate and acetylcholine receptors during neuronal differentiation. Neuroscience 146: 1169–1181.

18. Migita H, Kominami K, Higashida M, Maruyama R, Tuchida N, McDonald F, Shimada F, Sakudara K. (2008) Activation of adenosine A_1 receptor-induced neural stem cells proliferation via MEK/ERK and Akt signaling pathway. J Neurosci Res. 86: 2820-2828.

19. Jagasia R, Song H, Gage FH, Lie DC. (2006) New regulators in adult neurogenesis and their potential role for repair. Trends Mol Med. 12: 400-405.

20. Zang Y, Yu L-F, Pang T, Fang L-P, Feng X, Wen T-Q, Nan F-J, Feng L-Y, Li J. (2008) AICAR induces astroglial differentiation of neural stem cells via activating the JAK/STAT3 pathway independently of AMP-activated protein kinase. J. Biol. Chem. 283: 6201-08.

21. White N, Burnstock G. (2006) P2 receptors and cancer. Trends Pharmacol Sci. 27: 211-217.

22. Robak T, Robak P. (2012) Purine nucleoside analogs in the treatment of rarer chronic lymphoid leukemias. Curr Pharm Des. 18(23): 3373-3388.

23. Miano M, Pistorio A, Putti MC, Dufour C, Messina C, Barisone E, Ziino O, Parasole R, Luciani M, Lo Nigro L, De Rossi G, Varotto S, Bertorello N, Petruzziello F, Calvillo M, Micalizzi C. (2012) Clofarabine, cyclophosphamide and etoposide for the treatment of

relapsed or resistant acute leukemia in pediatric patients. Leuk Lymphoma. 53(9): 1693-1698.

24. Holowiecki J, Grosicki S, Giebel S, Robak T, Kyrcz-Krzemien S, Kuliczkowski K, Skotnicki AB, Hellmann A, Sulek K, Dmoszynska A, Kloczko J, Jedrzejczak WW, Zdziarska B, Warzocha K, Zawilska K, Komarnicki M, Kielbinski M, Piatkowska-Jakubas B, Wierzbowska A, Wach M, Haus O. (2012) Cladribine, but not fludarabine, added to daunorubicin and cytarabine during induction prolongs survival of patients with acute myeloid leukemia: a multicenter, randomized phase III study. J Clin Oncol. 30 (20): 2441-2448.
25. Bellezza I, Tucci A, Minelli A. (2008) 2-Chloroadenosine and human prostate cancer cells. Anticancer Agents Med Chem. 8(7): 783-789.
26. Galmarini CM, Popowycz F, Joseph B. (2008) Cytotoxic nucleoside analogues: different strategies to improve their clinical efficacy. Curr Med Chem. 15(11): 1072-82.
27. Kim H, Kang JW, Lee S, Choi WJ, Jeong LS, Yang Y, Hong JT, Yoon do Y. (2010) A_3 adenosine receptor antagonist, truncated thio-Cl-IB-MECA, induces apoptosis in T24 human bladder cancer cells. Anticancer Res. 30(7): 2823-2830.
28. Bar-Yehuda S, Stemmer SM, Madi L, Castel D, Ochaion A, Cohen S, Barer F, Zabutti A, Perez-Liz G, Del Valle L, Fishman P. (2008) The A3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-kappaB signal transduction pathways. Int J Oncol. 33(2): 287-295
29. Schafer R, Sedehizade F, Welte T, Reiser G. (2003) ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. Am J Physiol Lung Cell Mol Physiol. 285(2): L376-385.

30. Morrone FB, Jacques-Silva MC, Horn AP, Bernardi A, Schwartsmann G, Rodnight R, Lenz G. (2003) Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. J. Neurooncol. 64: 211–218.

31. Sellers LA, Simon J, Lundahl TS, Cousens DJ, Humphrey PP, Barnard EA. (2001) Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. J Biol Chem. 276: 16379–16390

32. Resende RR, Britto LRG, Ulrich H. (2008) Pharmacological properties of purinergic receptors and their effects on proliferation and induction of neuronal differentiation of P19 carcinoma cells. Int J Dev Neurosci. 26(7): 763-777.

33. Guarnieri S, Pilla R, Morabito C, Sacchetti S, Mancinelli R, Fanò G, Mariggiò MA. (2009) Extracellular guanosine and GTP promote expression of differentiation markers and induce S-phase cell-cycle arrest in human SH-SY5Y neuroblastoma cells. Int. J. Dev. Neurosci. 27(2): 135-147.

34. Traversa U, Bombi G, Di Iorio P, Ciccarelli R, Werstiuk ES, Rathbone MP. (2002) Specific [(3)H]-guanosine binding sites in rat brain membranes. Br J Pharmacol. 135(4): 969-76.

35. Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. (2008) Purinergic signalling in the nervous system: an overview. Trends Neurosci. 32: 19-29.

36. Burnstock G. (2002) Potential therapeutic targets in the rapidly expanding field of purinergic signalling. Clin Med. 2(1): 45-53

37. Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE. (2011) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors: an update. Pharmacol Rev. 63(1): 1-34.

38. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. (2006) International

Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 58(3): 281-341.

39. Mishra SK, Braun N, Shukla V, Fullgrabe M, Schomerus C, Korf HW, Gachet C, Ikehara Y, Sevigny J, Robson SC, Zimmermann H. (2006) Extracellular nucleotide signaling in adult neural stem cells: synergism with growth factor-mediated cellular proliferation. Development 133: 675–684.

40. Ledur PF, Villodre ES, Paulus R, Cruz LA, Flores DG, Lenz G. (2012) Extracellular ATP reduces tumor sphere growth and cancer stem cell population in glioblastoma cells. Purinergic Signal. 8(1): 39-48.

41. Li A, Walling J, Kotliarov Y, Center A, Steed ME, Ahn SJ, Rosenblum M, Mikkelsen T, Zenklusen JC, Fine HA. (2008) Genomic changes and gene expression profiles reveal that established glioma cell lines are poorly representative of primary human gliomas. Mol Cancer Res 6: 21-30.

42. Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, Pastorino S, Purow BW, Christopher N, Zhang W, Park JK, Fine HA. (2006) Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9: 391-403.

43. Braganhol E, Wink MR, Lenz G, Battastini AM. (2013) Purinergic signaling in glioma progression. Adv Exp Med Biol. 986: 81-102.

44. Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, Pilozzi E, Larocca LM, Peschle C, De Maria R. (2006) Chemotherapy resistance of glioblastoma stem cells. Cell Death Differ. 13(7): 1238-1241.

45. Pallini R, Ricci-Vitiani L, Banna GL, Signore M, Lombardi D, Todaro M, Stassi G, Martini M, Maira G, Larocca LM, De Maria R. (2008). Cancer stem cell analysis and

clinical outcome in patients with glioblastoma multiforme. Clin Cancer Res. 14(24): 8205-8212.

46. Griffero F, Daga A, Marubbi D, Capra MC, Melotti A, Pattarozzi A, Gatti M, Bajetto A, Barbieri F, Favoni RE, Lo Casto M, Zona G, Spaziante R, Florio T, Corte G. (2009). Different response of human glioma tumor-initiating cells to Epidermal Growth Factor receptor kinase inhibitors. J Biol Chem 284 (11): 7138-7148.

47. Angelucci S, Marchisio M, Di Giuseppe F, Pierdomenico L, Sulpizio M, Eleuterio E, Lanuti P, Sabatino G, Miscia S, Di Ilio C. (2010) Proteome analysis of human Wharton's jelly cells during in vitro expansion. Proteome Sci. 8: 18.

48. Vindeirinho J, Costa GN, Correia MB, Cavadas C, Santos PF. (2013) Effect of diabetes/hyperglycemia on the rat retinal adenosinergic system. PLoS One. 8(6): e67499.

49. Sim JA, Young MT, Sung HY, North RA, Surprenant A. (2004) Reanalysis of P2X7 receptor expression in rodent brain. J Neurosci. 24(28): 6307-6314.

50. Alvarenga EC, Rodrigues R, Caricati-Neto A, Silva-Filho FC, Paredes-Gamero EJ, Ferreira AT. (2010) Low-intensity pulsed ultrasound-dependent osteoblast proliferation occurs by via activation of the P2Y receptor: role of the P2Y1 receptor. Bone 46(2): 355-362.

51. Zhu CB, Lindler KM, Campbell NG, Sutcliffe JS, Hewlett WA, Blakely RD. (2011) Colocalization and regulated physical association of presynaptic serotonin transporters with A_3 adenosine receptors. Mol Pharmacol. 80(3): 458-465.

52. Montano N, Cenci T, Martini M, D'Alessandris QG, Pelacchi F, Ricci-Vitiani L, Maira G, de Maria R, Larocca LM, Pallini R. (2011) Expresion of EGFRvIII in glioblastoma: prognostic significance revisited. Neoplasia 13(12): 1113-1121.

53. Heimberger AB, Hlatky R, Suki D, Yang D, Weinberg J, Gilbert M, Sawaya R, Aldape K (2005). Prognostic effect of epidermal growth factor receptor and EGFRvIII in glioblastoma multiforme patients. Clin Cancer Res 11:1462–1466.

54. Hegi ME, Diserens AC, Gorlia T,Hamou MF, deTribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. N. Engl J Med 352: 997–1003.

55. Roger S, Pelegrin P. $P2X_7$ receptor antagonism in the treatment of cancers. (2011) Expert Opin Investig Drugs 20(7): 875-880.

56. Souza CO, Santoro GF, Figliuolo VR, Nanini HF, de Souza HS, Castelo-Branco MT, Abalo AA, Paiva MM, Coutinho CM, Coutinho-Silva R. (2012) Extracellular ATP induces cell death in human intestinal epithelial cells. Biochim Biophys Acta 1820(12): 1867-1878.

57. Vlachostergios PJ, Hatzidaki E, Papandreou CN. (2013) MGMT repletion after treatment of glioblastoma cells with temozolomide and O6-benzylguanine implicates NFκB and mutant p53. Neurol Res. 35(8): 879-882.

58. Ceruti S, Abbracchio MP. (2013) Adenosine signaling in glioma cells. Adv Exp Med Biol. 986: 13-30.

59. Tafani M, Di Vito M, Frati A, Pellegrini L, De Santis E, Sette G, Eramo A, Sale P, Mari E, Santoro A, Raco A, Salvati M, De Maria R, Russo MA. (2011) Pro-inflammatory gene expression in solid glioblastoma microenvironment and in hypoxic stem cells from human glioblastoma. J Neuroinflammation. 8: 32.

60. Madi LOA, Rath-Wolfson L, Bar-Yehuda S, Erlanger A, Ohana G, Harish A, Merimski O, Barer F, Fishman P. (2004) The A_3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. Clin Cancer Res 10(13): 4472–4479.

61. Anccasi RM, Ornelas IM, Cossenza M, Persechini PM, Ventura AL. (2013) ATP induces the death of developing avian retinal neurons in culture via activation of $P2X_7$ and glutamate receptors. Purinergic Signal. 9(1): 15-29.

62. Kim TH, Kim YK, Woo JS. (2012) The Adenosine A3 Receptor Agonist Cl-IB-MECA Induces Cell Death Through Ca(2+)/ROS-Dependent Down Regulation of ERK and Akt in A172 Human Glioma Cells. Neurochem Res. 37(12): 2667-2677.

63. Fishman P, Bar-Yehuda S, Liang BT, Jacobson KA. Pharmacological and therapeutic effects of A3 adenosine receptor agonists. Drug Discov Today. 2012; 17(7-8): 359-366.

64. Adinolfi E, Raffaghello L, Giuliani AL, Cavazzini L, Capece M, Chiozzi P, Bianchi G, Kroemer G, Pistoia V, Di Virgilio F. (2012) Expression of P2X7 receptor increases in vivo tumor growth. Cancer Res. 72(12): 2957-2969.

65. Glaser T, Cappellari AR, Pillat MM, Iser IC, Wink MR, Battastini AM, Ulrich H (2012). Perspectives of purinergic signaling in stem cell differentiation and tissue regeneration. Purinergic Signal. 8(3): 523-537.

66. Taliani S, La Motta C, Mugnaini L, Simorini F, Salerno S, Marini AM, Da Settimo F, Cosconati S, Cosimelli B, Greco G, Limongelli V, Marinelli L, Novellino E, Ciampi O, Daniele S, Trincavelli ML, Martini C. (2010) Novel N2-substituted pyrazolo[3,4-d]pyrimidine adenosine A3 receptor antagonists: inhibition of A3-mediated human glioblastoma cell proliferation. J Med Chem. 53(10): 3954-3963.

67. Daniele S, Zappelli E, Natali L, Martini C, Trincavelli ML. (2014) Modulation of A1 and A2B adenosine receptor activity: a new strategy to sensitise glioblastoma stem cells to chemotherapy. Cell Death Dis. 5: e1539.

68. Leij-Halfwerk S, Agteresck KJ, Sijens PE, Dagnelie PC. (2002) Adenosine triphosphate infusion increases liver energy status in advanced lung cancer patients: an in vivo 31P magnetic resonance spectroscopy study. Hepatology 35 (2): 421-424.

69. Beijer S, van Rossum E, Hupperets PS, Spreeuwenberg C, van den Beuken M, Winkens RA, van den Borne BE, de Graeff A, Dagnelie PC. (2007) Application of adenosine 5'-triphosphate (ATP) infusions in palliative home care: design of a randomized clinical trial. BMC Public Health 7: 4.

70. Shabbir M, Burnstock G. (2009) Purinergic receptor-mediated effects of adenosine 5'-triphosphate in urological malignant diseases. Int. J. Urol. 16: 143- 150.

71. Barberà-Cremades M, Baroja-Mazo A, Gomez AI, Machado F, Di Virgilio F, Pelegrin P. (2012) P2X7 receptor-stimulation causes fever via PGE2 and IL-1β release. FASEB J. 26 (7): 2951-2962.

72. Peng W, Cotrina ML, Han X, Yu H, Bekar L, Blum L, Takano T, Tian GF, Goldman SA, Nedergaard M. (2009) Systemic administration of an antagonist of the ATP-senstive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci USA 106(30): 12489-12493.

73. Wei Q, Costanzi S, Liu QZ, Gao ZG, Jacobson KA. (2011) Activation of the P2Y1 receptor induces apoptosis and inhibits proliferation of prostate cancer cells. Biochem Pharmacol. 82(4): 418-425.

74. Peigñan L, Garrido W, Segura R, Melo R, Rojas D, Cárcamo JG, San Martín R, Quezada C. (2011) Combined use of anticancer drugs and an inhibitor of multiple drug resistance-associated protein-1 increases sensitivity and decreases survival of glioblastoma multiforme cells in vitro. Neurochem Res. 36(8): 1397-1406.

75. Filippi-Chiela EC, Thome MP, Bueno e Silva MM, Pelegrini AL, Ledur PF, Garicochea B, Zamin LL, Lenz G. (2013) Resveratrol abrogates the temozolomide-induced G2 arrest leading to mitotic catastrophe and reinforces the temozolomide-induced senescence in glioma cells. BMC Cancer 13: 147.

LEGENDS

Fig. 1 Human GSCs express different subtypes of purine receptors and two stemness markers such as CD39 and CD73, corresponding to the enzymes ecto-nucleoside triphosphate diphosphohydrolase 1and 5'-ectonucleotidase. **A**) GSCs, isolated from human primary GBM and used for experiments, were cultured either as cell suspension leading to the formation of classical neurosperes or as cell monolayer when seeded on plates coated with a suitable substrate (Matrigel). The panels show cultures obtained from the patient n. 3 and are representative of similar cultures obtained from the other two patients selected for this study. **B-E**) The presence of metabotropic receptors belonging to the P1 family (panel D), responsive to adenosine, and to the P2 family (panel E) responsive to ATP/ADP ($P2Y_{1,11\text{-}14}$) and UTP ($P2Y_{2,4,6}$) as well as the expression of the ionotropic $P2X_7$ receptor (panel E) and of CD73 (panel B) have been evaluated by either RT-PCR or western blot analysis whereas the expression of CD39 was assayed by cytofluorimetric analysis (panel C). Bands or histograms were obtained from GSCs deriving from surgical tissue samples of three different patients identified as # 1, 2 and 3. In the panel C, a semi-quantitative evaluation of antigen expression, obtained dividing the mean fluorescence ratio (MFI) of positive events by the MFI of negative events, is reported. In the panels B, D and E, for RT-PCR assay, a lane is reported on the left of each panel in which standard molecules (M) were run and within brackets it has been reported the indication of the base pairs (bp) of the products that are those currently indicated in literature for each receptor subtype. Expression of GAPDH or β-actin was used as an internal control. The bands obtained by Western blotting were analyzed by densitometry and the obtained values were normalized for those evaluated for β-actin bands and are reported in the tables. Data are representative of three independent experiments with very similar results.

Fig. 2 Effect of purine receptor agonists and apyrase on the GSC viability. Undifferentiated cells grown on Matrigel were exposed for two consecutive days to: **A**) apyrase, administered alone or in combination with different doses of Bz-ATP. Values are the mean±SEM of 3 separate experiments using cells from the patient # 3. Similar values were obtained using GSCs from the other two patients. *$p < 0.05$, ***$p < 0.001$: statistical significance vs. control cells; §§ $p < 0.01$, §§§ $p < 0.001$: statistical significance vs. cells exposed to Bz-ATP alone (one way ANOVA plus Dunnett's test); **B**) different doses of non selective agonists for P2 receptors (ATP and UTP) or of more selective agonists for $P2Y_1$ (MRS2365 and ADPβS), $P2Y_{11}$ (NF546) and $P2X_7$ (Bz-ATP) receptor subtypes. Data reported in the panel B are representative of six independent experiments, carried out using cells derived from three different patients. In all figures, cell viability evaluated by the MTS assay as the absorbance detected at 490 nm was measured after two further days following cell exposure to drugs.

Fig. 3 Effect of nucleotides, administered alone or in the presence of different purine receptor antagonists, on the growth of GSCs. **A**) Cells grown on Matrigel were exposed for two consecutive days to selective antagonists of $P2Y_1R$ (MRS2500), $P2Y_{11}R$ (NF340) and $P2X_7R$ (A438079, AZ116453, o-ATP) and to non selective antagonists of P2R (suramin) and P2XR (TNP-ATP). **B-C**) The effect caused by cell exposure for two consecutive days to selective agonists for $P2Y_1R$ (MRS2365 and ADPβS) and $P2X_7R$ (Bz-ATP) or to non selective agonists (ATP or UTP) for P2 receptors, was evaluated in GSCs, in the presence or not of the antagonists reported above. When present, the antagonists were administered 30 min prior to the agonists. Cell viability evaluated by the MTS assay as the absorbance detected at 490 nm was measured after two further days following cell exposure to drugs. Values are the mean±SEM of 6 separate experiments in which different cell samples were used. *$p < 0.05$, *** $p < 0.001$: statistical significance vs. control cells; § $p < 0.05$, §§§ $p < 0.001$: statistical significance vs. cells exposed to purine nucleotides alone (one way ANOVA plus Dunnett's test).

Fig. 4 Effect of temozolomide (TMZ), administered alone or in combination with purine receptor agonists on the growth of GSCs. **A**) Undifferentiated GSCs derived from samples of different patients and grown on Matrigel were exposed to TMZ, administered only once at different concentrations on the 2nd day after cell seeding. Cell viability, evaluated by MTS assay as the absorbance detected at 490 nm in untreated cultures (control, CTRL) and in cultures exposed to TMZ, was detected at 4 and 7 days from the beginning of the experiments. The results are expressed as the mean ± S.E.M. of three independent experiments, in which different cell samples were used and evaluated in triplicate; **B**) GSCs derived from the same three patients and grown on Matrigel, were exposed for two consecutive days to 500 μM ATP or UTP, non selective agonists for P2 receptors, or Bz-ATP, selective agonist for $P2X_7R$, alone or in combination with to 50 or 75 μM TMZ, administered once on the second day together with purine/pyrimidine nucleotides. Cell viability, evaluated again by MTS assay as the absorbance detected at 490 nm, was measured at the day 4 from the beginning of the experiments. $*p < 0.05$, $***p < 0.001$: statistical significance of vs. untreated (control) cells; § $p < 0.05$, §§§ $p < 0.001$: statistical significance vs. cells exposed to TMZ alone (one way ANOVA plus Dunnett's test).

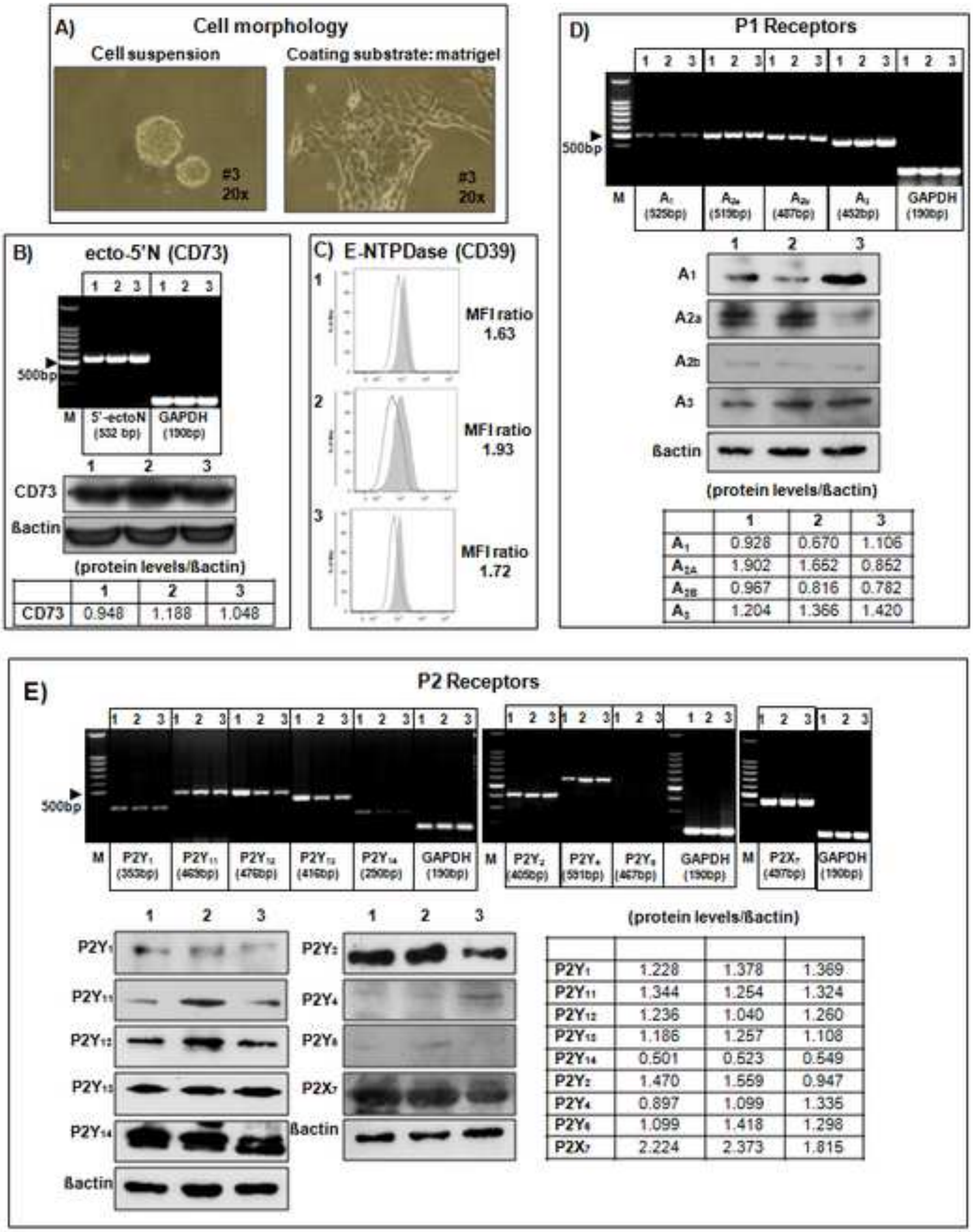
Fig. 5 Evaluation of cell necrotic or apoptotic death as a consequence of GSC exposure to purine/pyrimidine nucleotides or TMZ, administered alone or in combination.

The effect caused by cell exposure for two consecutive days to purine nucleotides (ATP, UTP or BzATP) and for one day to TMZ, administered alone or in combination (see the experimental protocols reported in the text of the paper) was evaluated in GSCs from the patient # 3. Similar values were obtained using GSCs from the other two patients. **A**) LDH release from GSCs, assumed as an index of necrotic death, was measured as reported in the Methods section. Values are expressed as the percentage of the total amount of the enzyme released in the medium from the cells after their lysis and are the mean±SEM of 6 separate experiments in triplicate. **B-C**) Apoptic death was assessed by two methods. The first one evaluated the release of caspase 3 and 7, the most involved in this process, by luminescence using a commercial kit and following the manufacturer's instruction. Cell apoptosis was also measured by incubating cells with propidium iodide and revealing the incorporated fluorescence by FACS. The values in the panels B and C are the mean±S.E.M. of four independent experiments in which each sample was tested in triplicate. $*p < 0.05$, $***p < 0.001$: statistical significance vs. untreated cells; §§ $p < 0.01$, §§§ $p < 0.001$: statistical significance vs. cells treated with TMZ (one way ANOVA plus Dunnett's test).

Fig. 6 Evaluation of the effect of purine receptor agonists on the formation of neurospheres by GSCs. GSCs grown as cell suspension were exposed for two consecutive days to ATP or UTP or Bz-ATP or TMZ, administered alone or in combination as reported in the experimental protocol. **A)** The formation of neurospheres was first evaluated after 7 days from the cell exposure to the drugs. Then the cells were allowed to recover for further 7 days and, subsequently, after a recovery period of 7 days, the surviving neurospheres were submitted to dissociation. **B)** Cells obtained from this procedure were re-seeded and evaluated after further 7 days following dissociation for their ability to form secondary neurospheres. The panels A-B are related to experiments performed using cells from the patient # 3 and are representative of six independent experiments performed using GSC deriving from different patients and which gave similar results. The cells were observed with a Nikon Eclipse TS100 phase contrast microscope and images were acquired with the Zoom Browser EX software.

Original magnification: 20X. In the graphs below the panels (C-D), values related to the size of primary and secondary neurospheres are reported. They were obtained using cells from three patients. The values are the mean±S.E.M. of three independent experiments in which each sample was tested in triplicate. $*p < 0.05$, $**p < 0.01$: statistical significance vs. untreated cells (control) (one way ANOVA plus Dunnett's test).

Fig. 7 Effect of TMZ, administered twice (the first time in combination with purine receptor agonists and the second time alone) on the growth of GSCs. Using GSCs grown on Matrigel, cells were exposed for two consecutive days to 500 µM ATP or UTP or BZ-ATP and to 50 or 75 µM TMZ, administered on the second day together with purine nucleotides. After three further days, cells were re-exposed to TMZ alone. Cell viability was detected at the day 14 from the beginning of the experiments. Values reported in the graph are the mean±SEM of 6 separate experiments in triplicate using cells from all patients. $***p < 0.001$: statistical significance of vs. untreated (control) cells; §§§$p < 0.001$: statistical significance vs. cells exposed to purine nucleotides alone (one way ANOVA plus Dunnett's test) .



(protein levels/ßactin)

	1	2	3
CD73	0.948	1.188	1.048

(protein levels/ßactin)

	1	2	3
A_1	0.928	0.670	1.106
A_{2A}	1.902	1.652	0.852
A_{2B}	0.967	0.816	0.782
A_3	1.204	1.366	1.420

(protein levels/ßactin)

$P2Y_1$	1.228	1.378	1.369
$P2Y_{11}$	1.344	1.254	1.324
$P2Y_{12}$	1.236	1.040	1.260
$P2Y_{13}$	1.186	1.257	1.108
$P2Y_{14}$	0.501	0.523	0.549
$P2Y_2$	1.470	1.559	0.947
$P2Y_4$	0.897	1.099	1.335
$P2Y_6$	1.099	1.418	1.298
$P2X_7$	2.224	2.373	1.815

Figure 1

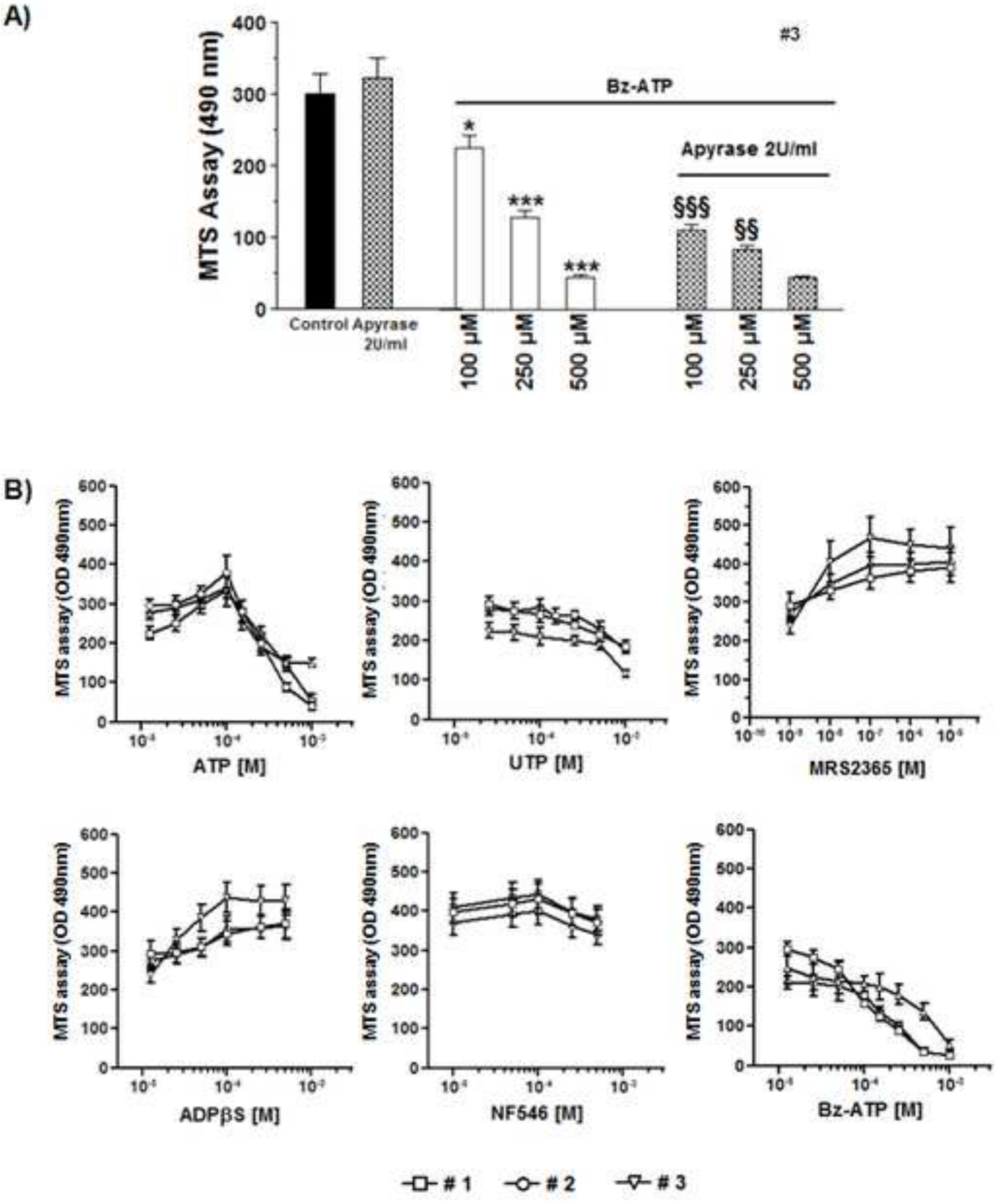

A)
MTS Assay (490 nm)
400
300
200
100
0
#3
Bz-ATP
*
***
***
Apyrase 2U/ml
§§§
§§
Control
Apyrase 2U/ml
100 µM
250 µM
500 µM
100 µM
250 µM
500 µM
B)
MTS assay (OD 490nm)
600
500
400
300
200
100
0
10⁻⁵
10⁻⁴
10⁻³
ATP [M]
UTP [M]
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
MRS2365 [M]
ADPβS [M]
NF546 [M]
Bz-ATP [M]
1
2
3

Figure 2

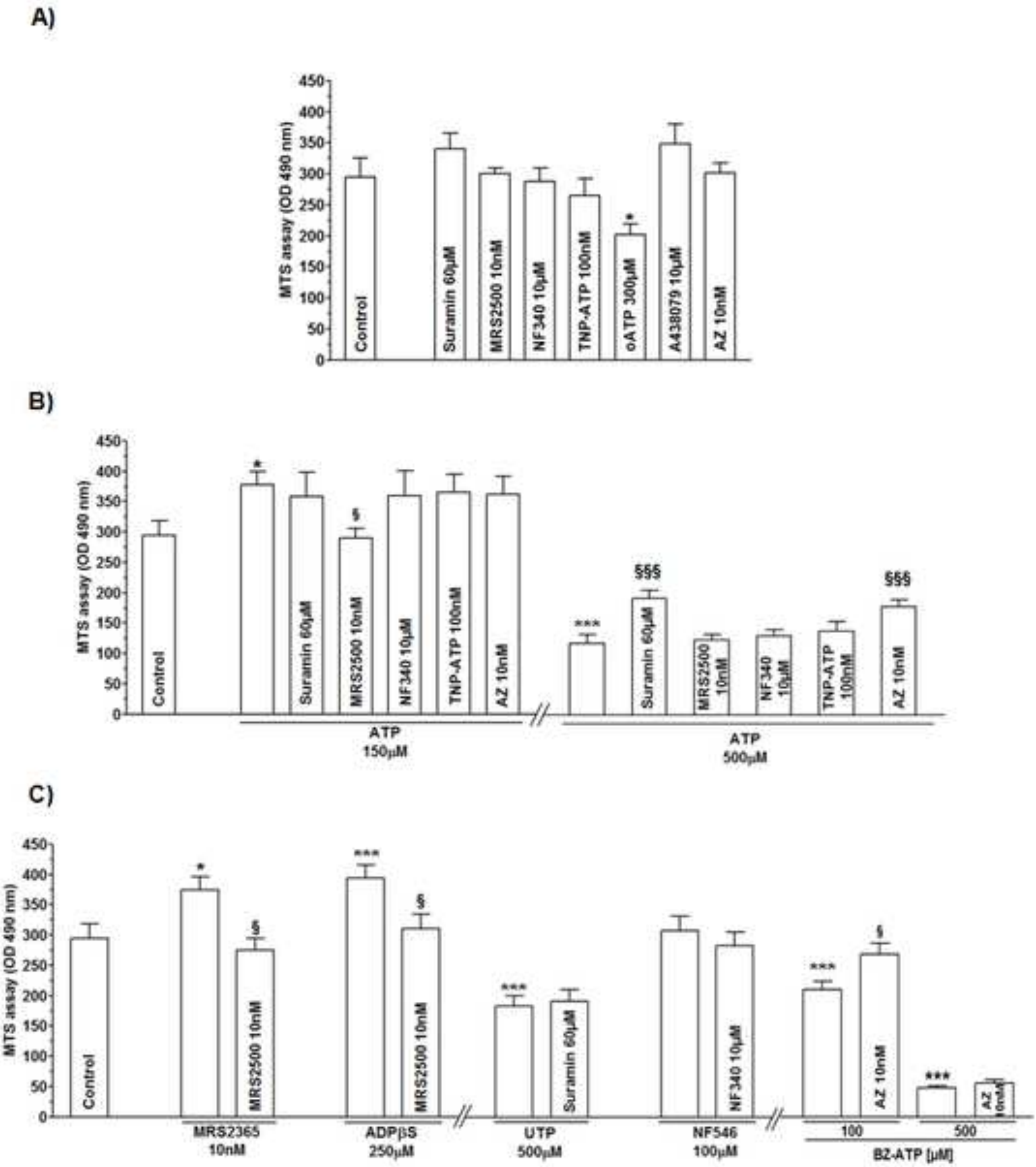

A)
MTS assay (OD 490 nm)
0
50
100
150
200
250
300
350
400
450
Control
Suramin 60µM
MRS2500 10nM
NF340 10µM
TNP-ATP 100nM
oATP 300µM
*
A438079 10µM
AZ 10nM
B)
MTS assay (OD 490 nm)
Control
*
Suramin 60µM
§
MRS2500 10nM
NF340 10µM
TNP-ATP 100nM
AZ 10nM
ATP
150µM
***
§§§
Suramin 60µM
MRS2500 10nM
NF340 10µM
TNP-ATP 100nM
§§§
AZ 10nM
ATP
500µM
C)
MTS assay (OD 490 nm)
Control
*
§
MRS2500 10nM
MRS2365
10nM
***
§
MRS2500 10nM
ADPβS
250µM
***
Suramin 60µM
UTP
500µM
NF340 10µM
NF546
100µM
***
§
AZ 10nM
100
***
AZ 10nM
500
BZ-ATP [µM]

Figure 3

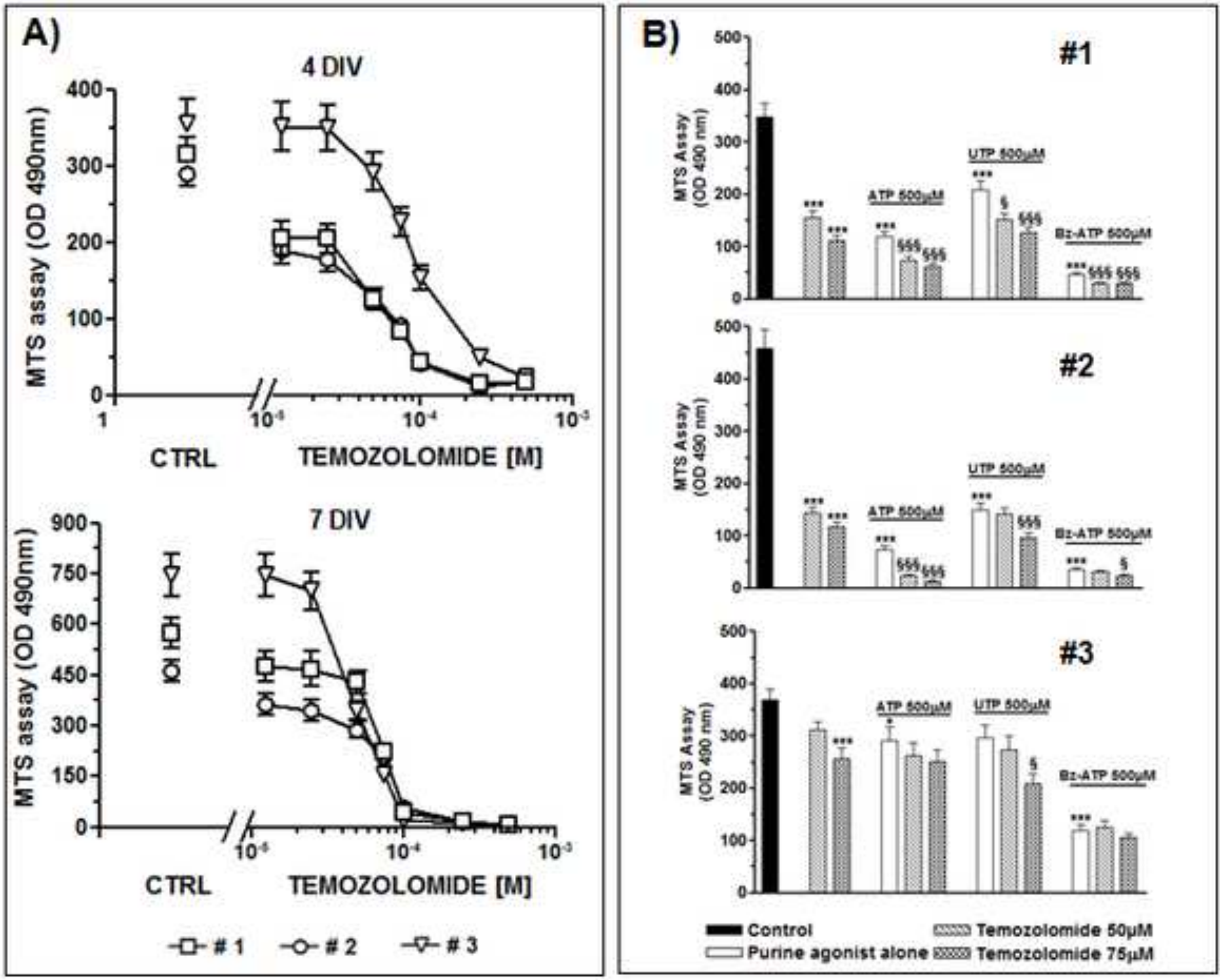

A)
4 DIV
MTS assay (OD 490nm)
400
300
200
100
0
1
10⁻⁵
10⁻⁴
10⁻³
CTRL
TEMOZOLOMIDE [M]
7 DIV
MTS assay (OD 490nm)
900
750
600
450
300
150
0
10⁻⁵
10⁻⁴
10⁻³
CTRL
TEMOZOLOMIDE [M]
1
2
3
B)
#1
#2
#3
MTS Assay (OD 490 nm)
500
400
300
200
100
0
ATP 500µM
UTP 500µM
Bz-ATP 500µM
***
§
§§§
*
Control
Purine agonist alone
Temozolomide 50µM
Temozolomide 75µM

Figure 4

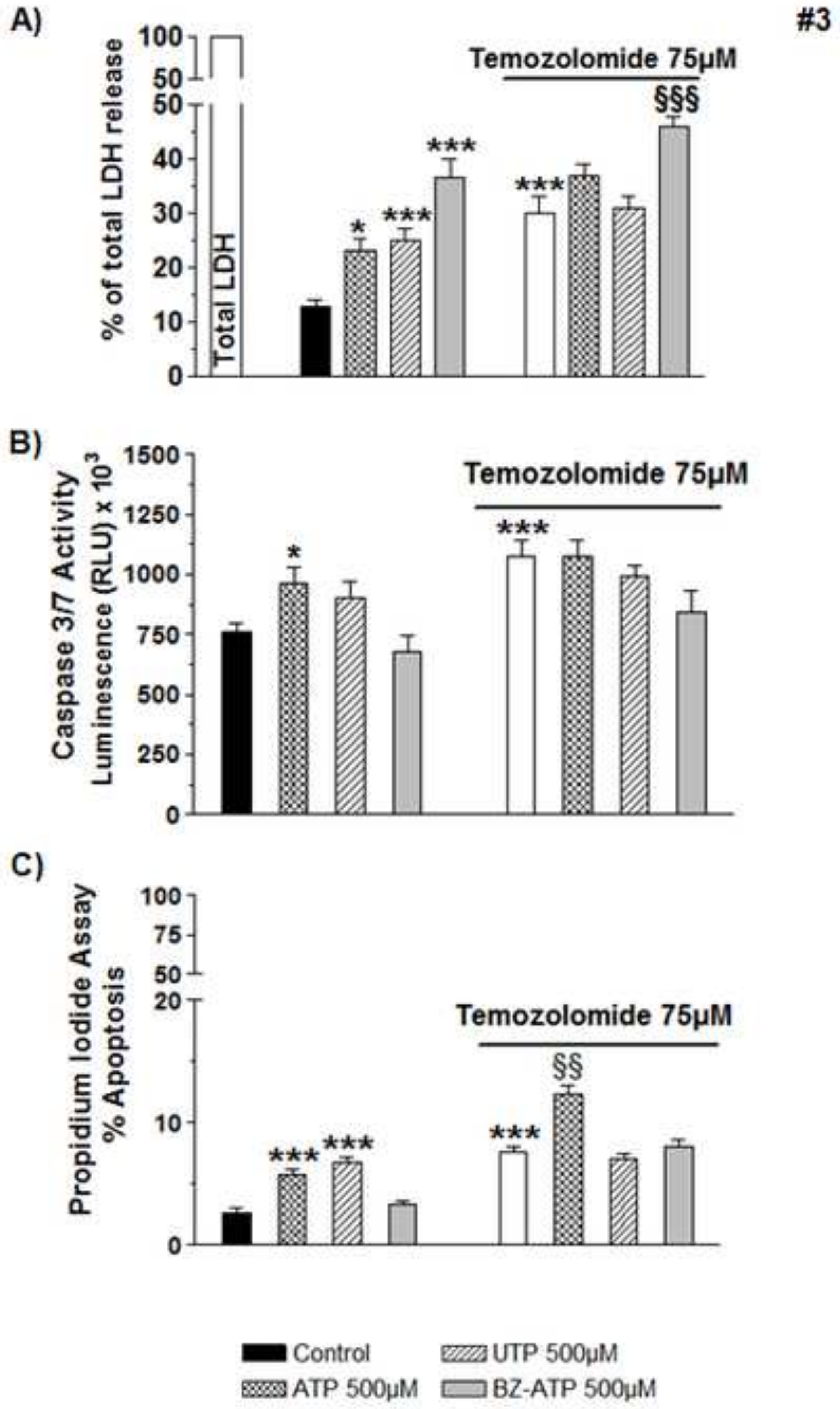

A)
#3
% of total LDH release
100
50
50
40
30
20
10
0
Total LDH
Temozolomide 75µM
*
***
***
***
§§§
B)
Caspase 3/7 Activity
Luminescence (RLU) x 10^3
1500
1250
1000
750
500
250
0
Temozolomide 75µM
*
***
C)
Propidium Iodide Assay
% Apoptosis
100
75
50
20
10
0
Temozolomide 75µM
***
***
***
§§
■ Control
▨ UTP 500µM
▩ ATP 500µM
▭ BZ-ATP 500µM

Figure 5

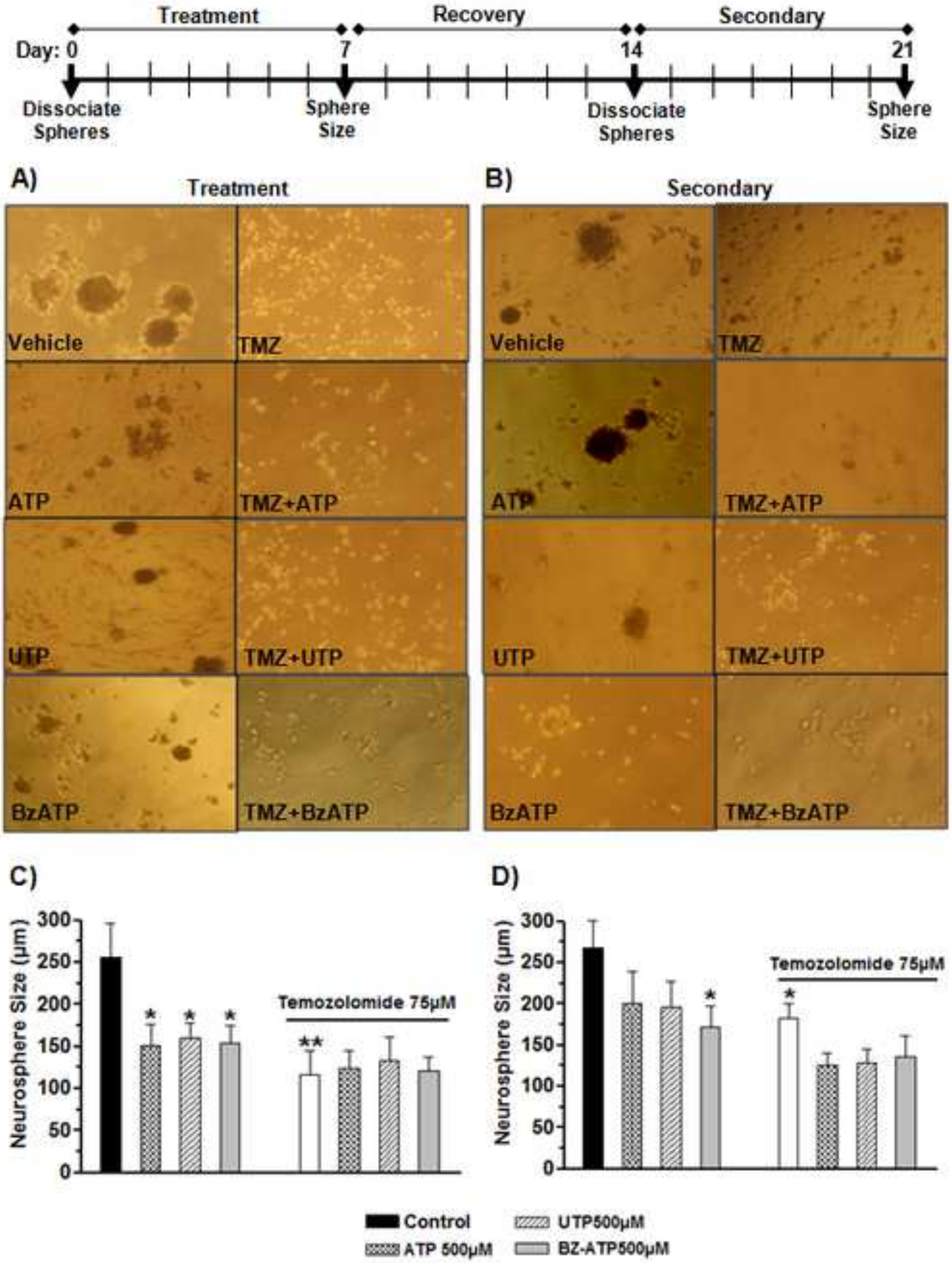

Treatment
Recovery
Secondary
Day: 0
7
14
21
Dissociate Spheres
Sphere Size
Dissociate Spheres
Sphere Size
A)
Treatment
Vehicle
TMZ
ATP
TMZ+ATP
UTP
TMZ+UTP
BzATP
TMZ+BzATP
B)
Secondary
Vehicle
TMZ
ATP
TMZ+ATP
UTP
TMZ+UTP
BzATP
TMZ+BzATP
C)
Neurosphere Size (µm)
300
250
200
150
100
50
0
*
*
*
**
Temozolomide 75µM
D)
Neurosphere Size (µm)
300
250
200
150
100
50
0
*
*
Temozolomide 75µM
Control
ATP 500µM
UTP500µM
BZ-ATP500µM

Figure 6

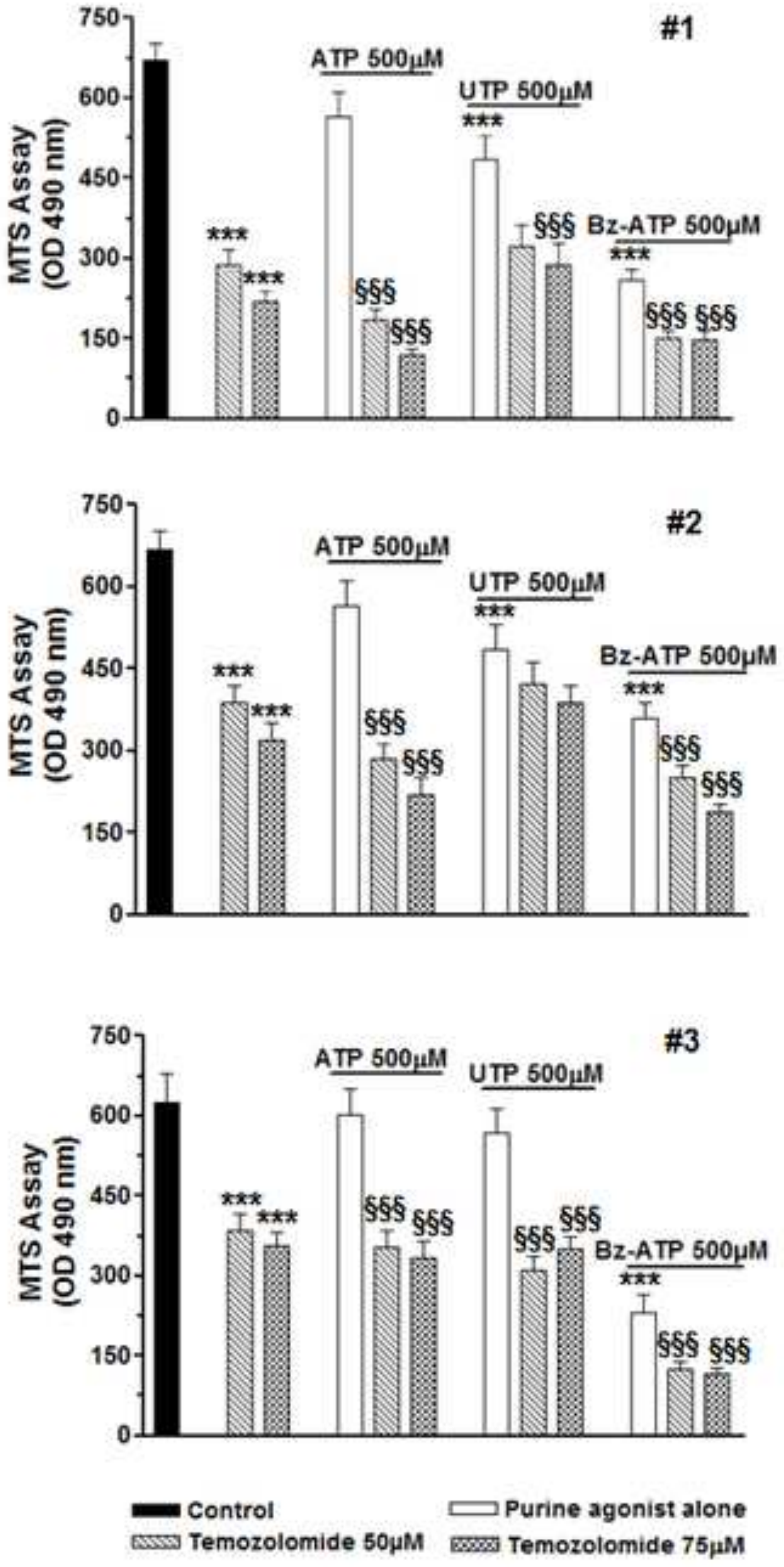

#1
#2
#3
MTS Assay (OD 490 nm)
750
600
450
300
150
0
ATP 500µM
UTP 500µM
Bz-ATP 500µM
***
§§§
Control
Purine agonist alone
Temozolomide 50µM
Temozolomide 75µM

Figure 7

Table 1: Clinical and biological characteristics of the primary tumor (GBM) obtained from three different patients

GBM #	Age (yr)	KPS (score)	Ki 67 (%)	PFS (mo.)	OS (mo.)	MGMT	EGFR vIII	VEGF	PTEN
1	40	80	20	6.0	12.5	M	-	iper	-
2	72	90	5	6.0	11.5	M	-	iper	-
3	52	70	40	3.0	8.0	UM	+	iper	+

GBM tissue specimens were collected at surgery from adult patients who had undergone craniotomy at the Institute of Neurosurgery, Catholic University School of Medicine, Rome. Abbreviations: EGFRvIII, Epidermal Growth Factor Receptor variant III; KPS, Karnofsky Performance Status; MGMT, O^6-methylguanine methyltransferase (M, methylated; UM, unmethylated); OS, overall survival (mo, months); PTEN, Phosphatase and TENsin homolog; PFS, progression-free survival (mo, months); VEGF: Vascular Endothelial Growth Factor; yr, years.

Table 2: Primer sequences used for RT-PCR analysis

Gene	***Accession Number***	***Forward primer***	***Reverse primer***	***T (°C) annealing***	***Product (bp)***
hA1	**NM_000674**	**TCTTCCTCTTTGCCCTCAGCT**	**GCTCAGAACACTGTTGCCTCT**	**55**	**525**
hA2a	**NM_000675.4**	**AGGGCTAAGGGCATCATTG**	**GGATACGGTAGGCGTAGATGA**	**58**	**519**
hA2b	**NM_000676**	**TGACTTCTACGGCTGCCTCTT**	**TGACTTGGCTGCATGGATCT**	**55**	**487**
hA3	**NM_000677**	**TTTGCTGGCTGGTGTCATT**	**AGGCATAGACGATAGGGTTCA**	**55**	**452**
hP2Y1	**BC074785**	**TCATTGTGGTGGTGGCGA**	**TGCTGGGGTCTGAAAATCAA**	**55**	**353**
hP2Y2	**BC012104**	**CCTTTGCCGTCATCCTTGT**	**CAGTTCTGTCGGATCTGCG**	**55**	**405**
hP2Y4	**BC096067**	**TGCCCACCCTCATCTACTATT**	**TGACAATGTTCAGTACTCGGC**	**55**	**591**
hP2Y6	**BC000571**	**TTCCTCTTCTATGCCAACCTG**	**TTGGTGATGTGAAAAGGCAG**	**55**	**467**
hP2Y11	**BC073827**	**TGGTTGAGTTCCTGGTGGC**	**AGACACTTGATGCAGGCCTC**	**55**	**469**
hP2Y12	**BC017898**	**CATGATTCTGACCAACAGGC**	**AATTGGGGCACTTCAGCATA**	**55**	**476**
hP2Y13	**BC041116**	**AAGCTACCATGTATGCAAGGG**	**AGGGGAGGTTTGTAGGGATAT**	**55**	**416**
hP2Y14	**BC034989**	**TGAAAAGTGAACTGGGACGG**	**TTCTTTTGACTGGCAGCTGT**	**55**	**590**
hP2X7	**NM_002562**	**ATTCCTGGACAACCAGAGGAG**	**TGCCTGGCTTCAGTAAGGACT**	**58**	**497**
h5’-NT (CD73)	**NM_002526**	**GCACTATCTGGTTCACCGTGT**	**ATGAATGGGTACTTCCCAGCA**	**58**	**532**
hGAPDH	**NM_002046**	**GAGTCCACTGGCGTCTTCAC**	**GGTGCTAAGCAGTTGGTGGT**	**55**	**190**

Table 3: GSC viability measured by MTS assay in cultured cells at day 4 after two consecutive days of pharmacological treatment.

Treatment	OD 490 nm
None	443±45
MRS1220 1 µM	398±33
Ado 500 µM	266±20 ***
Ado 500µM+MRS1220 1µM	335±19 §
Ado 1 mM	175±15 ***
Ado 1Mm + MRS1220 1µM	247±19 §
ATP 500 µM	202±19 ***
ATP 500µM + MRS1220 1 µM	283±22 §

The effect caused by cell exposure for two consecutive days to the nucleoside adenosine or to ATP was evaluated in GSC deriving from the patient # 3, in the presence or not of the A_3 selective antagonist MRS1220. When present, the A_3 antagonist was administered 30 min prior to the agonists. The values of measured optical density (OD) related to MTS assay are the mean±S.E.M. of four independent experiments in which each sample was tested in triplicate. Similar values were obtained using cells from other two patients. ***$p < 0.001$: statistical significance vs. untreated cells; §$p < 0.05$: statistical significance vs. cells treated with the respective agonist (adenosine, Ado, or ATP) alone (one way ANOVA plus Dunnett’s test).

TABLE 4: Cytofluorimetric analysis of the cells cycle

	Cell cycle phases		
Treatment	**G0/G1**	**S**	**G2/M**
None	67.75±6.4	8.3±0.9	23.95±2.8
ATP 500 µM	79.51±8.1	9.35±1.0	11.14±0.9 ***
UTP 500 µM	62.29±6.4	8.92±0.7	28.79±3.0
Bz-ATP 500 µM	60.58±6.2	18.6±1.5 ***	22.45±2.5
TZM 75 µM	55.76±8.0	12.5±1.1 *	33.00±2.0 *
ATP 500 µM + TZM 75 µM	56.8±6.1	10.8±0.9	35.40±2.1***
UTP 500 µM + TZM 75 µM	59.37±6.2	11.2±1.2	29.53±3.0
Bz-ATP 500µM + TZM 75 µM	60.8±8.0	17.9±1.3§§	21.33±2.4§

The effect caused by cell exposure for two consecutive days to purine nucleotides (ATP, UTP or BzATP) and for one day to TMZ, administered alone or in combination (see the experimental protocols reported in the text of the paper) was evaluated in GSCs from the patient # 3. The values measured by incubating cells with PI and revealing the incorporated fluorescence by FACS are the mean±S.E.M. of four independent experiments in which each sample was tested in triplicate. Similar values were obtained using cells from other two patients. *$p < 0.05$, ***$p < 0.001$: statistical significance vs. untreated cells; §$p < 0.05$, §§$p < 0.01$: statistical significance vs. cells treated with TMZ (one way ANOVA plus Dunnett's test).