

# Potential 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<sub>7</sub> receptors. GSC exposure for 48 h to 10–150 μM ATP, P2R ligand, or to ADPβS or MRS2365, P2Y<sub>1</sub>R agonists, enhanced cell expansion. This effect was counteracted by the P<sub>Y</sub><sub>1</sub>R antagonist MRS2500. In contrast, 48-h treatment with higher doses of ATP or UTP, which binds to P2Y<sub>2/4</sub>R, or 2'(3')-O-(4-benzoylbenzoyl)-ATP (Bz-ATP), P2X<sub>7</sub>R agonist, decreased GSC proliferation. Such a reduction was due to apoptotic or necrotic cell death but mostly to growth arrest. Accordingly, cell regrowth and

secondary neurosphere formation were observed 2 weeks after the end of treatment. Suramin, nonselective P2R antagonist, MRS1220 or AZ11645373, selective A<sub>3</sub>R or P2X<sub>7</sub>R 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<sub>3</sub>R, P2Y<sub>1</sub>R, and P2X<sub>7</sub>R, 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 GSC duplication when readded 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.

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## 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 radiochemotherapy [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 analogs, used as antileukemic 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<sub>1</sub>R, A<sub>2A</sub>R, A<sub>2B</sub>R, and A<sub>3</sub>R, 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 ectoenzymes rapidly transforming nucleotides into active nucleosides [39].

In the last 10 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 reinvestigate 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]diphosphoric acid mono ester (MRS2365) trisodium salt, (1*R*\*,2*S*\*)-4-[2-iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonoxy)bicyclo[3.1.0]hexane-1-methanol-dihydrogen-phosphate ester (MRS2500) tetra-ammonium salt, 4,4'-(Carbonylbis(imino-3,1-(4-methylphenylene)carbo-nylimino))bis(naphthalene-2,6-disulfonic acid) tetrasodium salt (NF340), 4,4'-(Carbonylbis(imino-3,1-phenylene-carbonylimino))-bis(1,3-xylene-alpha,alpha'-diphosphonic acid tetra-sodium salt (NF546), 8-[4-[4-(4-chlorophenyl) piperazine-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 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, which 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 was 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, which is usually considered as an index of tumor invasiveness. In this kind of experiments, untreated/treated neurospheres were allowed to recover for further 7 days (14 days from the beginning of the experiment); then, they were removed, centrifuged, mechanically dissociated, and resuspended 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,  $5 \times 10^5$  cells/sample, was incubated with 100  $\mu$ l 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 $\times$ g, 8 min). Subsequently, samples were resuspended in 100- $\mu$ l 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 $\times$ g, 8 min), and cells were resuspended with 1-ml 0.5 % paraformaldehyde, incubated for 5 min at room

**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	EGFRvIII	VEGF	PTEN
1	40	80	20	6.0	12.5	M	-	hyper	-
2	72	90	5	6.0	11.5	M	-	hyper	-
3	52	70	40	3.0	8.0	UM	+	hyper	+

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

*EGFRvIII* epidermal growth factor receptor variant III, *KPS* Karnofsky performance status, *MGMT* O<sup>6</sup>-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

temperature, washed, centrifuged (4 °C, 400×g, 8 min), and stored at 4 °C in the dark until the acquisition with a FACS Calibur flow cytometer (BD), using the CellQuest™ 3.2.1.fl software (BD). Ten minutes 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 nondebris 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,  $5 \times 10^5$  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<sub>0</sub>/G<sub>1</sub> phase, S phase, and G<sub>2</sub>/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 Technology by providing a luminogenic caspase 3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, 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-pyrocyanate-treated water, and 5 µg was 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™ (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<sub>2</sub>, 0.2 mM each deoxy-dNTP, 1 µM oligonucleotide primers (MWG Biotech, Ebersberg, Germany), 2.5 U AmpliTaq Gold™ 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 % nonfat milk (Bio-Rad Laboratories) for 2 h at 4 °C, incubated overnight at 4 °C with specific primary antibodies [polyclonal rabbit anti-A<sub>1</sub>, anti A<sub>2A</sub> anti-A<sub>2B</sub>, anti-A<sub>3</sub>, anti P2Y<sub>1-2-4-6-11-12-13-14</sub>, anti-P2X<sub>7</sub>, dilution 1:1000 (Alomone Labs, Jerusalem, Israel)]; polyclonal rabbit anti CD73, dilution 1:500 (Novus Biological Europe, Cambridge, UK) and then exposed to donkey anti-rabbit HRP-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 reprobed with an anti-β-actin antibody (dilution 1:1000, incubation overnight at 4 °C; Santa Cruz Biotechnologies). Immunocomplexes were visualized 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 ( $3 \times 10^3$  cells/well) in 96-well plates, were incubated with agonists of P1 and P2 receptors following the usual



**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	AGGGCTAAGGCATCATTG	GGATACGGTAGGCGTAGATGA	58	519
hA2b	NM_000676	TGACTTCTACGGTGCCTCTT	TGACTTGGCTGCATGGATCT	55	487
hA3	NM_000677	TTTGCTGGCTGGTGTCATT	AGGCATAGACGATAGGGTTCA	55	452
hP2Y1	BC074785	TCATTGTGGTGGTGCGA	TGCTGGGGTCTGAAAATCAA	55	353
hP2Y2	BC012104	CCTTTGCCGTCATCCTTGT	CAGTTCTGTCGGATCTGCG	55	405
hP2Y4	BC096067	TGCCACCCTCATCTACTATT	TGACAATGTTCACTACTCGGC	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	TGAAAAGTGAACCTGGGACGG	TTCTTTGACTGGCAGCTGT	55	590
hP2X7	NM_002562	ATTCTGGACAACCAGAGGAG	TGCCTGGCTTCAGTAAGGACT	58	497
h5'-NT (CD73)	NM_002526	GCACTATCTGGTTCACCGTGT	ATGAATGGGTACTTCCCAGCA	58	532
hGAPDH	NM_002046	GAGTCCACTGGCGTCTTCAC	GGTGCTAAGCAGTTGGTGGT	55	190

protocol. Twenty-four hours after the end of the treatment, the cells were incubated at 37 °C and 5 % CO<sub>2</sub> 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, was 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) × 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<sup>®</sup> AQueous 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<sup>™</sup>, 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

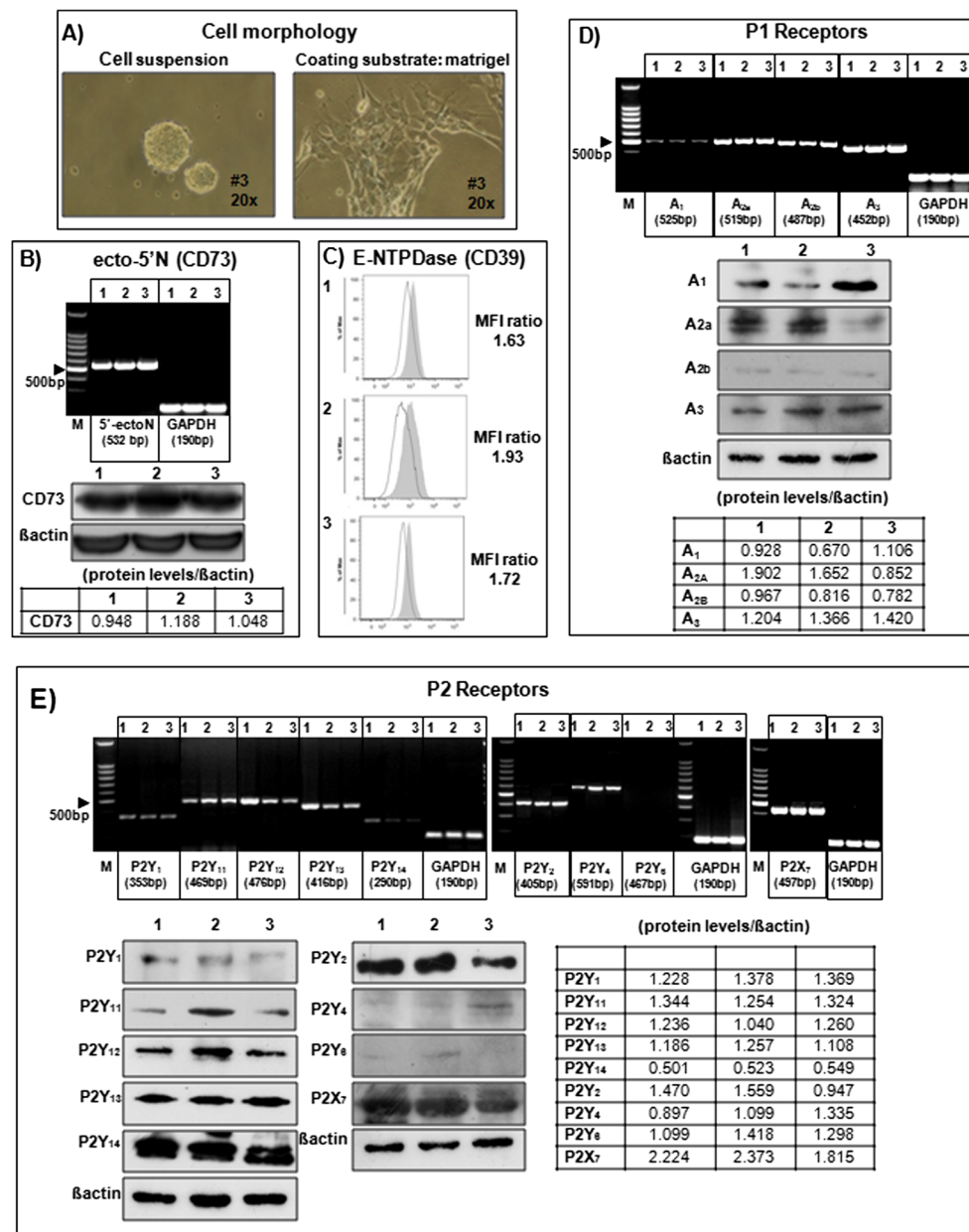
### GSC line characterization

We used GSCs deriving from samples of primary GBMs obtained from three patients. The primary tumors showed various molecular profiles (Table 1), which could be correlated with the patient outcome and the response to TMZ in malignant gliomas [52, 53]. Notably, tumor from the patient no. 3, differently from those of the other two patients, showed an unmethylated status of the enzyme O<sup>6</sup>-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, which 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



**Fig. 1** Human GSCs express different subtypes of purine receptors and two stemness markers such as CD39 and CD73, corresponding to the enzymes ectonucleoside triphosphate diphosphohydrolase 1 and 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 neurospheres or as cell monolayer when seeded on plates coated with a suitable substrate (Matrigel). The panels show cultures obtained from the patient no. 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 (**d**), responsive to adenosine, and to the P2 family (**e**) responsive to ATP/ADP (P2Y<sub>1,11–14</sub>) and UTP (P2Y<sub>2,4,6</sub>) as well as the expression of the ionotropic P2X<sub>7</sub> receptor (**e**) and of CD73 (**b**) have been evaluated by either RT-PCR or Western blot analysis whereas the expression of CD39

was assayed by cytofluorimetric analysis (**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 **c**, a semiquantitative evaluation of antigen expression, obtained dividing the mean fluorescence ratio (MFI) of positive events by the MFI of negative events, is reported. In **b–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

ionotropic P2X<sub>7</sub>R, which 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<sub>2B</sub>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<sub>2</sub>R showed the greatest expression (Fig. 1e). Additionally, GSCs expressed high levels of P2X<sub>7</sub>R (Fig. 1e). We also ascertained the presence of the stem cell markers CD39 and CD73, corresponding to the enzymes ectonucleoside 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, which 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 no. 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 nonselective P2R agonists), and synthetic ligands (MRS2365 and NF546, currently the most selective agonists of P2Y<sub>1</sub>R and P2Y<sub>11</sub>R, respectively, and Bz-ATP, P2X<sub>7</sub>R agonist). We also evaluated the effect of ADPβS, a less selective agonist for P2Y<sub>1</sub>R in comparison to MRS2365, which may also interact with P2Y<sub>12-13</sub>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 no. 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 no. 3 showed a lower sensitivity to ATP or Bz-ATP cytotoxicity. Interestingly, the presence of apyrase together with submaximal doses of Bz-ATP further reduced cell viability, and this effect was significant even in less responsive GSCs from patient no. 3 (Fig. 2a).

### The effect of P2YR and P2X<sub>7</sub>R 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<sub>7</sub>R antagonist (Fig. 3a), which was no longer used in the subsequent experiments. In the presence of purinergic agonists, MRS2500, P2Y<sub>1</sub>R 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, nonselective P2R antagonist, or AZ11645373, but not by TNP-ATP, which blocks all P2XR except the P2X<sub>7</sub> one, or by NF340, blocking the P2Y<sub>11</sub>R (Fig. 3b). 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<sub>11</sub>R agonist NF546 (Fig. 3c).

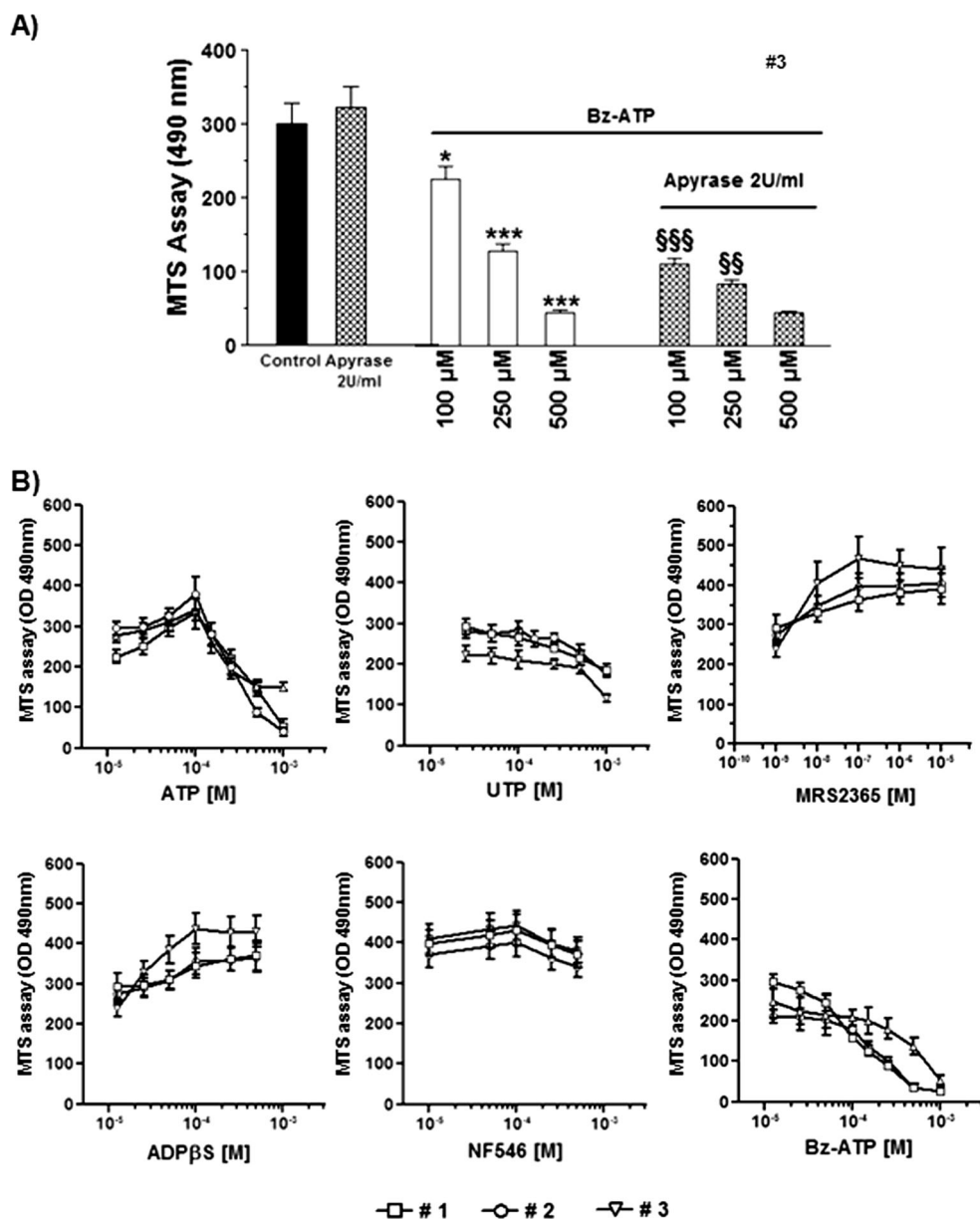
### Inhibitory effect of adenosine on the growth of GSCs

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<sub>1</sub>R, A<sub>2A</sub>R, A<sub>2B</sub>R, and A<sub>3</sub>R, 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 that the nucleotide effect could in part be mediated by the formation of extracellular adenosine that in turn, interacting with A<sub>3</sub>R 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 antiproliferative 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 no. 3, less sensitive to the cytotoxicity induced by all compounds.

**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  $\pm$  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 nonselective agonists for P2 receptors (ATP and UTP) or of more selective agonists for P2Y<sub>1</sub> (MRS2365 and ADP $\beta$ S), P2Y<sub>11</sub> (NF546), and P2X<sub>7</sub> (Bz-ATP) receptor subtypes. Data reported in **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

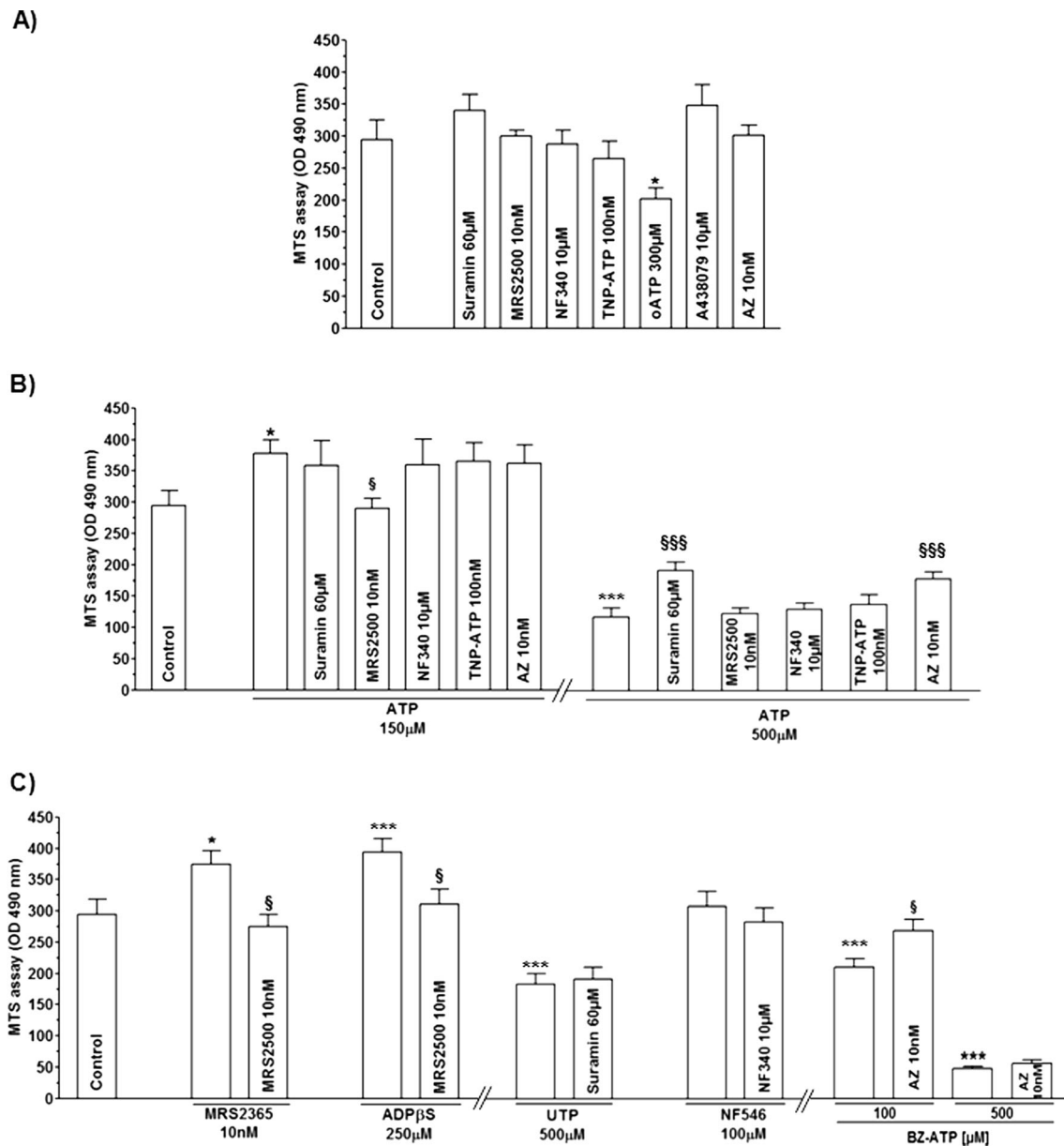


We wondered whether the reduction of cell viability by purine compounds, alone or combined with TMZ, was due to cell death or cell growth impairment. All assayed nucleotides significantly increased cell necrosis as revealed by the measurement of LDH release (Fig. 5a); ATP and UTP remarkably enhanced 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. 5b, c). Additionally, ATP diminished the number of cells in the G<sub>2</sub>/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 G<sub>2</sub>/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<sub>2</sub>/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 G<sub>2</sub>/M phase or in the S phase of the cell cycle were equivalent to the effect of Bz-ATP alone,





**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<sub>1</sub>R (MRS2500), P2Y<sub>11</sub>R (NF340), and P2X<sub>7</sub>R (A438079, AZ116453, o-ATP) and to nonselective 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<sub>1</sub>R (MRS2365 and ADPβS) and P2X<sub>7</sub>R (Bz-ATP) or to nonselective 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)

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

**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 500 μM + MRS1220 1 μM	335±19 §
Ado 1 mM	175±15 ***
Ado 1 mM + MRS1220 1 μM	247±19 §
ATP 500 μM	202±19 ***
ATP 500 μM + 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 no. 3, in the presence or not of the A<sub>3</sub> selective antagonist MRS1220. When present, the A<sub>3</sub> 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)

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, which 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, which 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 no. 3 (Fig. 7).

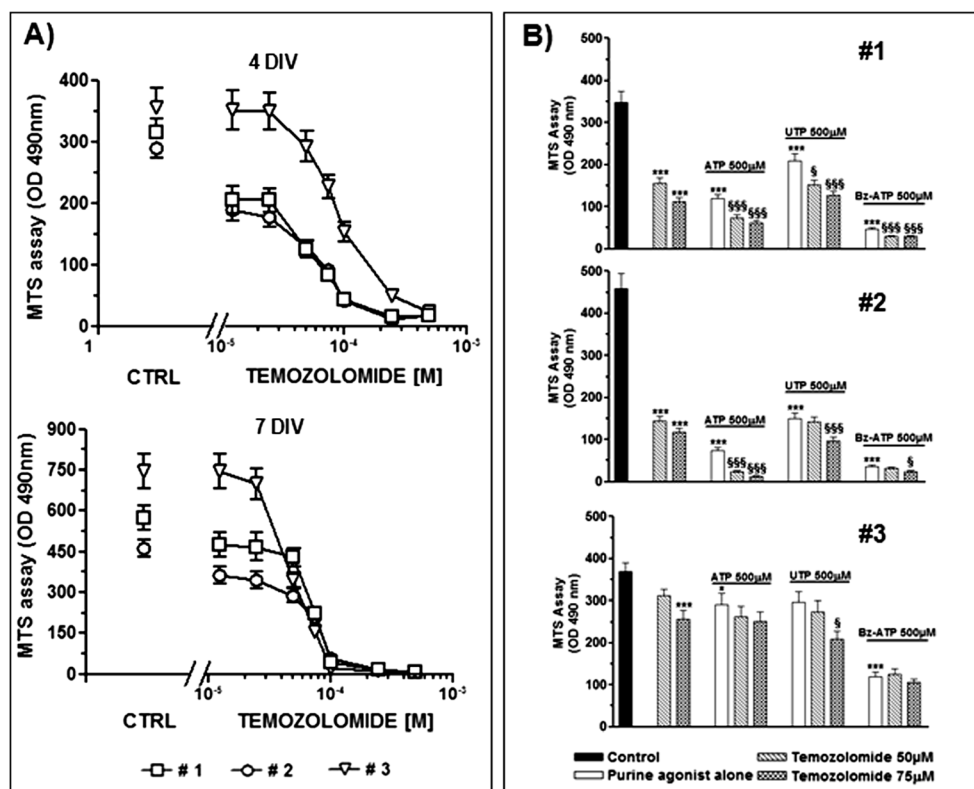
## Discussion

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 antitumoral 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 no. 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, which was similar in all three kinds of cells, in agreement with levels reported for glioma cell lines [43, 58]. Of course, this aspect should deserve a deeper investigation.

Noteworthy, the expression of P2X<sub>7</sub>R in our GSCs resulted more pronounced than that of other purinergic receptors. In agreement with these data, we here demonstrated that P2X<sub>7</sub>R plays a pivot role in inhibiting GSC growth. In fact, our cells were sensitive to the cytotoxic effects caused by Bz-ATP, P2X<sub>7</sub>R 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<sub>7</sub> 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<sub>7</sub>R [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 GSC membrane, was reduced also by cell pretreatment with MRS1220, an A<sub>3</sub>R antagonist that restrained also the cytotoxicity caused by high doses of adenosine. The A<sub>3</sub>R anticancer effect, like that of P2X<sub>7</sub>R, 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<sub>1</sub>R and A<sub>2B</sub>R, the stimulation of which caused a prominent antiproliferative effect on stem cells from



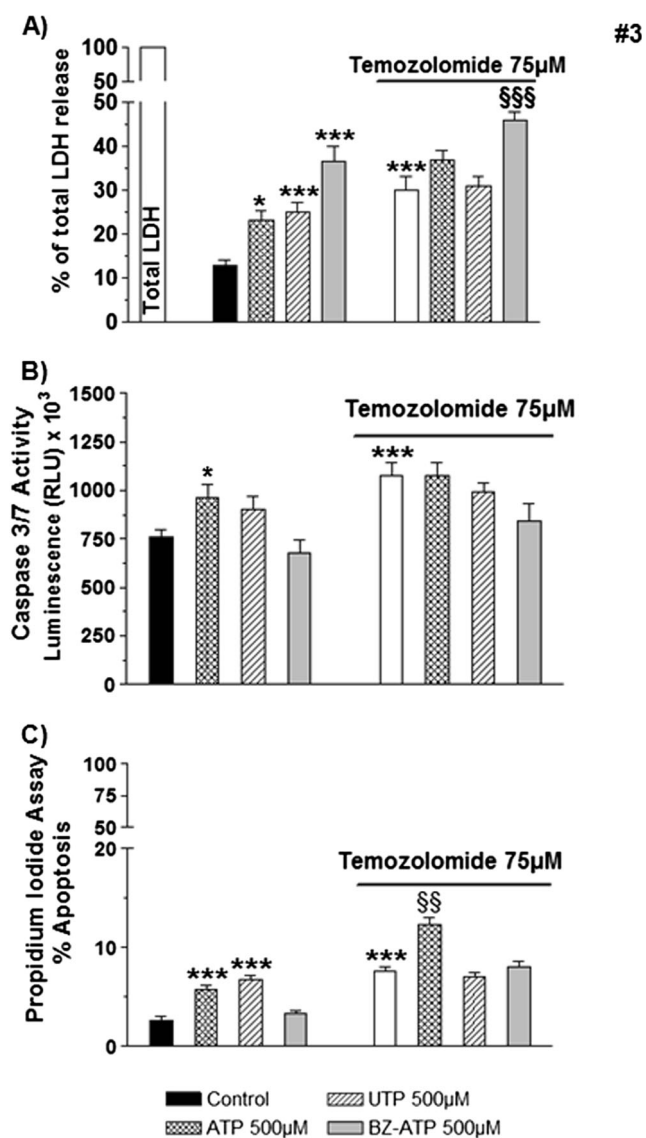
**Fig. 4** Effect of temozolomide (TMZ), administered alone or in combination with purine receptor agonists on the growth of GSCs. **a** Undifferentiated GSCs derived from samples of different patients and grown on Matrigel were exposed to TMZ, administered only once at different concentrations on the 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  $\pm$  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  $\mu$ M ATP or UTP, nonselective agonists for P2 receptors, or Bz-ATP, selective agonist for P2X<sub>7</sub>R, alone or in combination with 50 or 75  $\mu$ M 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)

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, which 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<sub>7</sub>R 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 extracellular 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<sub>7</sub>R agonist, Bz-ATP, on CGS duplication. P2Y<sub>1</sub>R are involved in the ATP-induced tumor growth as MRS2365 and ADP $\beta$ S, P2Y<sub>1</sub>R agonists, showed an effect similar to that of a low dose of ATP whereas MRS2500, P2Y<sub>1</sub>R specific antagonist, counteracted the trophic effect of all agonists cited above. A tumorigenic role of P2Y<sub>1</sub>R 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<sub>2</sub>R, responsive to UTP. However, in other tumor cells, i.e., in prostate cancer cells, P2Y<sub>1</sub>R stimulation caused apoptosis [73].



**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 1 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 no. 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** Apoptotic 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 **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)

#3

**Table 4** Cytofluorimetric analysis of the cells cycle

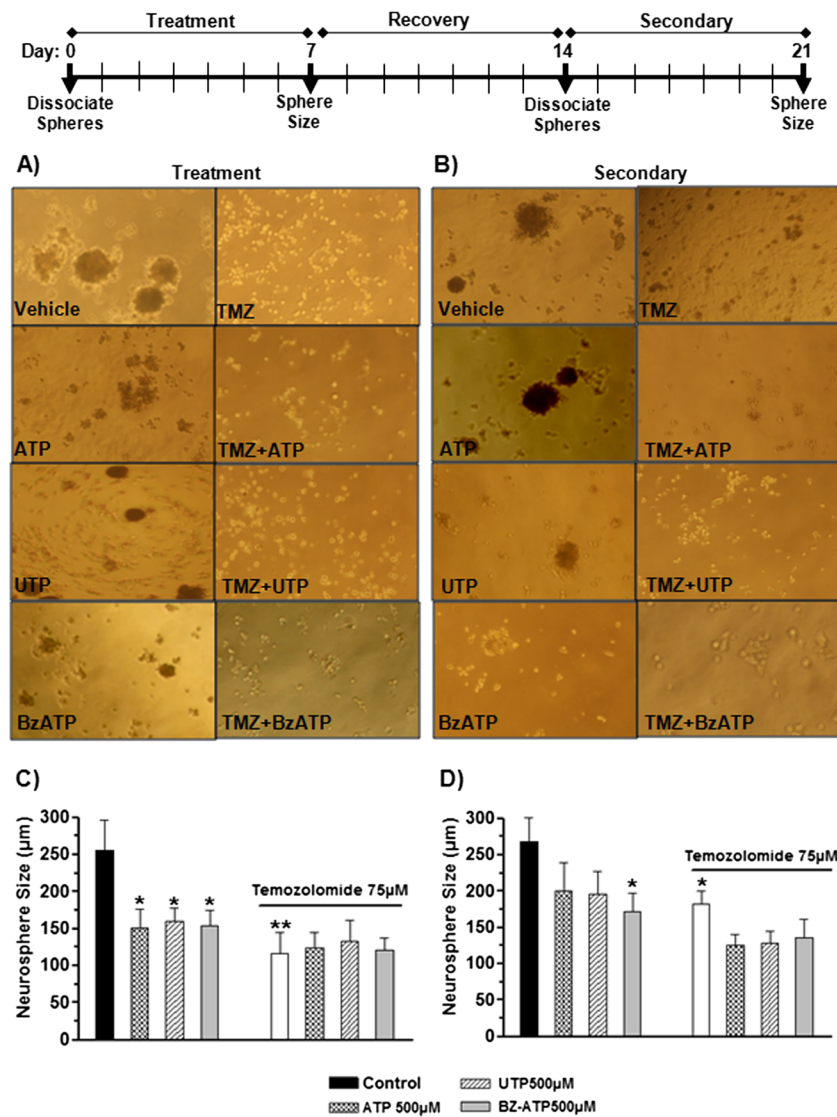
Treatment	Cell cycle phases		
	G0/G1	S	G2/M
None	67.75±6.4	8.3±0.9	23.95±2.8
ATP 500 µM	79.51±8.1	9.35±1.0	11.14±0.9 ***
UTP 500 µM	62.29±6.4	8.92±0.7	28.79±3.0
Bz-ATP 500 µM	60.58±6.2	18.6±1.5 ***	22.45±2.5
TZM 75 µM	55.76±8.0	12.5±1.1 *	33.00±2.0 *
ATP 500 µM + TZM 75 µM	56.8±6.1	10.8±0.9	35.40±2.1***
UTP 500 µM + TZM 75 µM	59.37±6.2	11.2±1.2	29.53±3.0
Bz-ATP 500 µM + 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 1 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 no. 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)

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<sub>7</sub>R, did not counteract ATP effects; also, the involvement of P2Y<sub>11</sub>R could also be excluded as well as that of P2Y<sub>12-13</sub>R, as the proliferative effect of ADPβS, agonist also for these receptors, was completely reversed by the P2Y<sub>1</sub>R antagonist. These findings are different from that obtained in murine and human cell lines, in which P2Y<sub>12</sub>R appear to increase cell proliferation, whereas P2Y<sub>1</sub>R 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



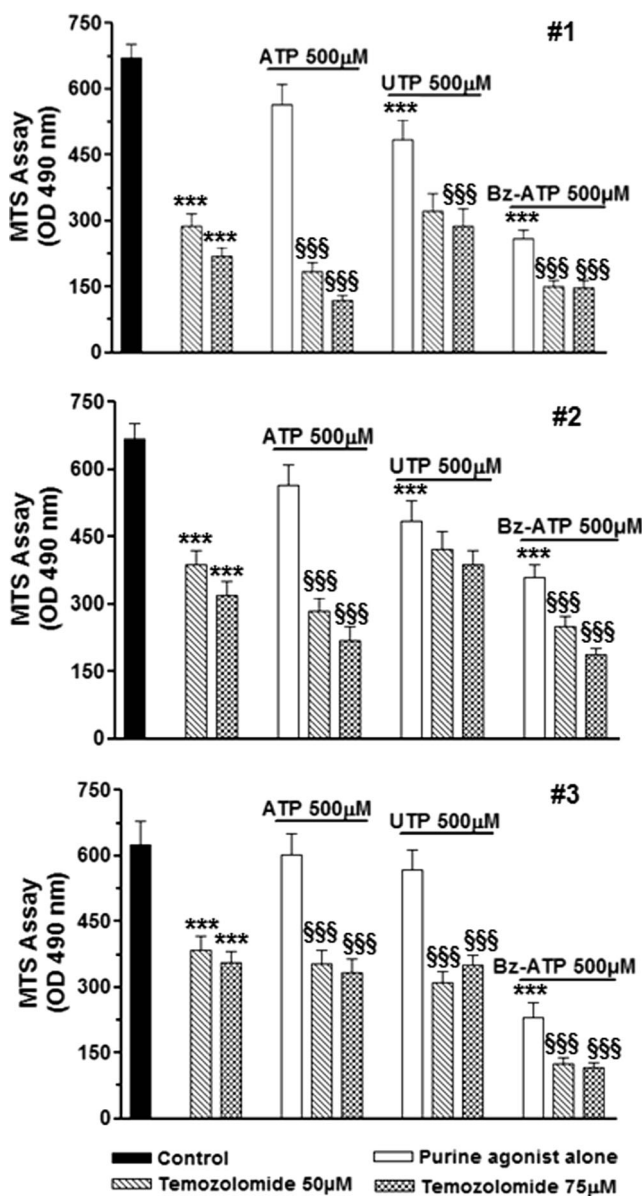


**Fig. 6** Evaluation of the effect of purine receptor agonists on the formation of neurospheres by GSCs. GSCs grown as cell suspension were exposed for two consecutive days to ATP or UTP or Bz-ATP or TMZ, administered alone or in combination as reported in the experimental protocol. **a** The formation 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 reseeded 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 no. 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,  $\times 20$ . 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  $\pm$  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)

BzATP, by opening the pore associated to the P2X<sub>7</sub>R, 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 readministered. 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.



**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 reexposed 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)

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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