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Modulation of metalloproteinase-9 in U87MG glioblastoma cells by A_3 adenosine receptors

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ABSTRACT

In this work, we investigated the biological functions of adenosine (ado) in metalloproteinase-9 (MMP-9) regulation in U87MG human glioblastoma cells. The nucleoside was able to increase both MMP-9 mRNA and protein levels through A_3 receptors activation. We revealed that A_3 receptor stimulation induced an increase of MMP-9 protein levels in cellular extracts of U87MG cells by phosphorylation of extracellular signal-regulated protein kinases (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (pJNK/SAPK), protein kinase B (Akt/PKB) and finally activator protein 1 (AP-1). A_3 receptor activation stimulated also an increase of extracellular MMP-9 in the supernatants from U87MG glioblastoma cells. Finally, the Matrigel invasion assay demonstrated that A_3 receptors, by inducing an increase in MMP-9 levels, was responsible for an increase of glioblastoma cells invasion. Collectively, these results suggest that ado, through A_3 receptors activation, modulates MMP-9 protein levels and plays a role in increasing invasion of U87MG cells.

1. Introduction

Local invasive growth is one of the key features of primary brain tumors. Glioma is the most common primary adult brain tumor with poor prognosis because of the aggressive invasion of the surrounding normal brain. Although our understanding of glioma oncogenesis has steadily improved, the molecular mechanisms that mediate glioma invasion are still poorly understood. The degradation of extracellular matrix (ECM) which exerts biochemical and mechanical barriers to cell movement has been shown to be an important biological process in tumor invasion and metastasis [1]. ECM degradation and remodelling require the action of extracellular proteinases, among which the matrix metalloproteinases (MMPs) have been shown to play an essential role. Indeed MMPs are cation-dependent endopeptidases which have been implicated in the malignancy of gliomas [2].

In particular it has been shown that MMP-9 facilitates the invasion of glioblastoma cells *in vitro* and MMP-9 overexpression correlates with the malignant progression of gliomas *in vivo* [3–6]. On the basis of reports from several different laboratories, it has been generally concluded that the basal levels of MMP-9 in most cancer cell lines are usually low and that its expression can be induced by treatment of growth factors, cytokines, tumor

Abbreviations: ADA, adenosine deaminase; Ado, adenosine; AP-1, activator protein 1; Akt/PKB, protein kinase B; CGS 21680, 2-[*p*-(carboxyethyl)-phenethylamino]-NECA; CHA, N^6-cyclohexyladenosine; Cl-IB-MECA, N^6(3-iodobenzyl)2-chloroadenosine-5′*N*-methyluronamide; DPA23, 1-deoxy-1-[6-{4-[(phenylcarbamoyl)-methoxy]phenylamino}-9H-purin-9-yl]-*N*-ethyl-β-d-ribofuranuronamide; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; ERK1/2, extracellular signal-regulated kinases; MMP-9, metalloproteinase-9; MRE 2029F20, *N*-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide]; MRE 3008F20, 5-*N*-(4-methoxyphenylcarbamoyl)-amino-8-propyl-2-(2-furyl)-pyrazolo[4,3e]-1,2,4-triazolo[1,5c]pyrimidine; NDGA, Nordihydroguaiaretic acid; pJNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; SB202190, 4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]pieno; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine; SH-5, [d-3-deoxy-2-O-methyl-myo-inositol-1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]]; siRNA, small interfering RNA; SP600125, anthra[1-9cd]pyrazol-6(2H)-one; ZM, 241385 (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,32][1,3,6]triazinyl-amino]ethyl)-phenol); U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene.

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promoters and oncogenes, through the activation of its gene promoter [7]. Several studies have identified signal transduction pathways involved in the regulation of MMP-9 expression in tumor cells [8]. A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves activation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated protein kinase (MAPK) [7,9–11]. Furthermore a critical role for PI3K/Akt signalling in the MMP-9 modulation has been described [9,12]. The human MMP-9 promoter contains several cis-acting regulatory elements that participate in the regulation of the MMP-9 gene expression, including sites that bind the transcription factors activator protein 1 (AP-1), nuclear factor-kappa B (NF-kB) and Sp1. In particular the AP-1 transcription complex appears to play an essential role in stimulating transcriptional activation of MMP-9 [10,11,13].

Adenosine (Ado) is a purine nucleoside which is released from metabolically active cells or is generated extracellularly by degradation of released ATP. It regulates a wide variety of physiological processes interacting with one or more of four known cell surface receptors named A_1, A_{2A}, A_{2B} and A_3 [14]. The development of potent A_3 agonists and selective antagonists revealed that the A_3 subtype plays a pivotal role in inflammation, in the ado-induced modulation of tumor cells biology [15–17] and the A_3 subtype has been found up-regulated in colorectal cancer [18]. Recently, we have demonstrated that ado stimulates proliferation of colon cancer cells and up-regulates under hypoxia the transcription factor hypoxia-inducible factor-1α (HIF-1α); this led to an increase in vascular endothelial growth factor (VEGF) and angiogenesis in melanoma, glioblastoma and colon carcinoma cells through A_3 receptor activation [19–22]. This effect was also observed in pro-inflammatory and pro-atherosclerotic foam cells [23]. However, the involvement of ado in the regulation of MMP-9 in tumor cells have not been investigated by now. In this study we will use U87MG human glioblastoma cells as an *in vitro* model to evaluate the role of ado in the modulation of MMP-9 and the intracellular pathways involved. The main finding of this work is that ado increases MMP-9 protein levels in cellular extracts of U87MG cells through A_3 receptors activation and phosphorylation of ERK1/2, JNK, Akt and AP-1. Furthermore it also increases extracellular MMP-9 levels in supernatants from U87MG cells; this effect is responsible for an increase of glioblastoma cell invasion.

2. Materials and methods

2.1. Materials

U87MG glioblastoma cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). [^{3}H]DPCPX (specific activity 120 Ci/mmol), was purchased by NEN Research Products, (Boston, MA, USA). [^{3}H]ZM 241385 (specific activity 20 Ci/mmol), was furnished by Tocris, (Boston, MA, USA). [^{3}H]MRE 2029F20 (specific activity 123 Ci/mmol) and [^{3}H]MRE 3008F20 (specific activity 67 Ci/mmol), were synthesized at Amersham International (Buckinghamshire, UK). MRE 2029F20, MRE 3008F20, DPA23 were synthesized by Prof. P.G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy). U0126 and SB 202190 were from Promega (Milan, Italy). SH-5 was from Vinci-Biochem (Florence, Italy). Adenosine A_3 receptors and MMP-9 small interfering RNA (siRNA) were from Santa Cruz Biotechnology, D.B.A. ITALIA s.r.l., (Milan, Italy). RNAiFect Transfection Kit was from Qiagen (Milan, Italy). NDGA was obtained from Calbiochem Inalco S.p.A. (Milan, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

2.2. Cell cultures

U87MG glioblastoma cancer cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12 medium) with 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, at 37 °C in 5% CO_2/95% air. All treatments to the cells with ado were carried out in the presence of the adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) 5 μM and those with ado agonists were performed in the presence of ADA. Cells viability was assessed through trypan blue assays and was not modified after treatments with ado or drugs.

2.3. Real-time RT-PCR

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay [19] of MMP-9 mRNA was carried out using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington Cheshire, UK). For the real-time RT-PCR of A_1, A_{2A}, A_{2B} and A_3 ado subtypes the assays-on- demand™ Gene expression Products Hs00181231_m1, Hs00169123_m1, Hs00386497_m1, Hs00181232_m1, were used, respectively (Applied Biosystems, Monza, Italy). Moreover curves of ado receptors cDNA plasmid standards with a range spanning at least six orders of magnitude (10^{-11}–10^{-16} g/μl) were generated. These standard curves displayed a linear relationship between Ct values and the logarithm of plasmid amount [19]. Quantification of ado receptor messages in cancer cells was made by interpolation from standard curve of Ct values generated from the plasmid dilution series. For the real-time RT-PCR of MMP-9 and TIMP-1 the assay-on-demand™ Gene expression Products Hs00234579_m1, Hs00171558_m1 were used, respectively. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kits was used, and the probe was fluorescent-labeled with VIC™ (Applied Biosystems, Monza, Italy).

2.4. Binding studies

Binding assays were carried out according to Gessi et al. [19]. Saturation experiments of antagonist radioligands [^{3}H]DPCPX (0.2–20 nM), [^{3}H]ZM 241385 (0.2–20 nM), [^{3}H]MRE 2029F20 (0.4–40 nM) and [^{3}H]MRE 3008F20 (0.3–30 nM) to label A_1, A_{2A}, A_{2B} and A_3 ado receptors, respectively, were carried out in U87MG cells. 100 μl of membrane homogenate (80–100 μg of protein assay^{-1}) were incubated in duplicate, in a final volume of 250 μl in test tubes containing 50 mM Tris–HCl buffer (10 mM $MgCl_2$ for A_{2A}, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine for A_{2B} and 10 mM $MgCl_2$, 1 mM EDTA for A_3) pH 7.4, with 10–12 different concentrations of each selective radioligand. Non-specific binding, defined as binding in the presence of 1 μM DPCPX, 1 μM SCH 58261, 1 μM MRE 2029F20, 1 μM MRE 3008F20 for A_1, A_{2A}, A_{2B} and A_3 ado receptors, respectively, at the K_D value for each radioligand was ≈30–35% of total binding. Bound and free radioactivity were separated, after an incubation time of 120 min at 4 °C, by filtering the assay mixture through Whatman GF/B glass-fiber filters using a cell harvester (Packard Instrument Company, CT, USA). The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

2.5. Western blotting analysis

Whole cell lysates, prepared as described previously [21], were resolved on a 10% SDS gel and transferred onto the nitrocellulose

membrane. Western blot analyses were performed as described previously using antibody against MMP-9 (Calbiochem Inalco, Milan, Italy) (1:200 dilution) in 5% non-fat dry milk in PBS 0.1% Tween-20 overnight at 4–8 °C. Recombinant active MMP-9 (83 kDa) was used as positive control (Calbiochem Inalco, Milan, Italy). Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for phosphorylated (Thr183/Tyr185) p44/p42 MAPK (1:5000 dilution) (Promega, Milan, Italy), phosphorylated (Thr180/Tyr182) p38 MAPK (1:1000 dilution) (Cell Signaling Technology, Milan, Italy), phosphorylated (Ser473) Akt/PKB (protein kinase B) (1:1000 dilution), phosphorylated (Thr183/Tyr185) SAPK/JNK, phosphorylated (Ser73) c-Jun (Cell Signaling Technology, Milan, Italy), and for A_3 receptor (Aviva Antibody Corporation, Milan, Italy) (1 mg/ml dilution). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against mouse and rabbit IgG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Amersham Corp., Arlington Heights, Ill.). Tubulin (1:250) was used to ensure equal protein loading.

2.6. Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Mean densitometry data from independent experiments were normalized to the results in control cells. The data were presented as the mean ± S.E.

2.7. Treatment of cells with small interfering RNA (siRNA)

U87MG cells were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of ado A_3 receptor or MMP-9 siRNA, was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit for 72 h [21]. A non-specific control ribonucleotide sense strand (5′-ACU CUA UCU GCA CGC UGA CdTdT-3′) and antisense strand (5′-dTdT UGA GAU AGA CGU GCG ACU G-3′) were used under identical conditions.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of MMP-9 protein secreted by the cells in the medium were determined by an ELISA kit (RayBio Elisa Kit, Tebu-bio, Milan, Italy). In brief, subconfluent cells were changed into fresh serum-free medium in the presence of solvent or various concentrations of ado and ado ligands for 24 h. The medium was collected, centrifuged for 5 min at 900 g to remove floating cells and assayed for MMP-9 content by ELISA according to the manufacturer's instructions. The data were presented as mean ± SE from three independent experiments.

2.9. Gelatin zimography

Cells were incubated in serum-free medium for 24 h and the respective supernatants were used for the experiments. Twenty micrograms were mixed with sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue) and separated on 10% SDS-polyacrylamide gels containing either gelatine from porcin skin (1 mg/ml). After electrophoresis, gels were washed for 1 h in renaturing buffer (2.5% Triton X-100) and subsequently incubated overnight at 37 °C in 50 mM Tris–HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 pH 7.5). Gels were stained with 0.3% Coomassie Brilliant blue and destained with 7% acetic acid methanol, 1% methanol and 7% isopropyl alcohol, the clear zones within the blue background indicating proteinolytic activity.

2.10. Invasion assay

Invasion was measured by assessment of the U87MG cells migration rate by using Cell Invasion Assay kit (Chemicon International, Milan, Italy). 2×10^5 U87MG cells were plated into the upper well of the chamber in serum-free medium for 72 h, while the lower well was filled up to the top with MEM plus 10% FBS as chemoattractant. Treatment of the cells with ado, Cl-IB-MECA and inhibitors was carried out for 48 h. After incubation stained invasive cells were quantitated by dissolving them in 10% acetic acid and reading OD at 560 nm according to the manufacturer's instructions.

2.11. Protein inhibitors

U0126, SB202190, SH-5 specific inhibitors of ERK 1/2, p38 and Akt were used at a concentration of 1 μM according to previous experiments showing their specificity carried out in cancer cells [22]. SP600125, the JNK inhibitor was used at a dose of 1 μM according to its affinity and selectivity data [24]. Nordihydroguaiaretic acid (NDGA), the AP-1 inhibitor was used at a dose of 10 μM according to literature data [25]. MMP-9 inhibitor I was used at a dose of 50 nM [26].

2.12. Data analysis

Binding studies were analyzed with the program LIGAND [27]. Statistical analysis was performed by means of analysis of variance (ANOVA) and the Dunnett's test. $P < 0.05$ was considered significant.

3. Results

3.1. Expression of ado receptors mRNA and protein in human U87MG glioblastoma cells

First of all we examined the pattern of expression of ado receptors in U87MG glioblastoma cells. The mRNA level of ado receptors was examined through real-time RT-PCR experiments. As shown in Fig. 1A U87MG cells expressed all ado subtypes with the following rank order $A_{2A} > A_{2B} > A_1 > A_3$. At a protein level saturation assays with the A_1 receptor antagonist [^{3}H]DPCPX revealed the presence of A_1 receptors with a K_D value of 2.5 ± 0.3 nM and a B_{max} value of 28 ± 1 fmol/mg of protein; saturation studies with the A_{2A} antagonist [^{3}H]ZM 241385 showed A_{2A} receptors with a K_D value of 2.0 ± 0.1 nM and a B_{max} value of 110 ± 13 fmol/mg of protein; saturation experiments with the A_{2B} antagonist [^{3}H]MRE2029F20 detected A_{2B} receptors with a K_D value of 3.8 ± 0.5 nM and a B_{max} value of 85 ± 9 fmol/mg of protein; saturation assays with the A_3 antagonist [^{3}H]MRE3008F20 revealed A_3 receptors with a K_D value of 2.2 ± 0.40 nM and a B_{max} value of 102 ± 10 fmol/mg of protein (Fig. 1B).

3.2. Ado effect on MMP-9 mRNA in U87MG glioblastoma cells

Initial experiments were carried out to evaluate the effect of ado on MMP-9 transcript levels in U87MG cells. After treatment of 6, 12, 24 and 48 h with the nucleoside (100 μM) we observed a time-dependent increase in MMP-9 mRNA of 1.5 ± 0.2, 1.8 ± 0.2, 2.5 ± 0.3 and 3.5 ± 0.4 fold, respectively (Fig. 2A). In order to identify which receptor subtype was involved in the ado-mediated MMP-9 increase we incubated U87MG glioblastoma cells with high affinity A_1, A_{2A}, A_{2B} and A_3 ado analogues. The A_1, A_{2A} and A_{2B} agonists, CHA, CGS 21680 and DPA23 respectively, did not affect MMP-9 increase whilst the A_3 agonist Cl-IB-MECA 100 nM was able to induce a raise of 2.0 ± 0.3 fold of MMP-9 mRNA level, after 24 h of treatment, in

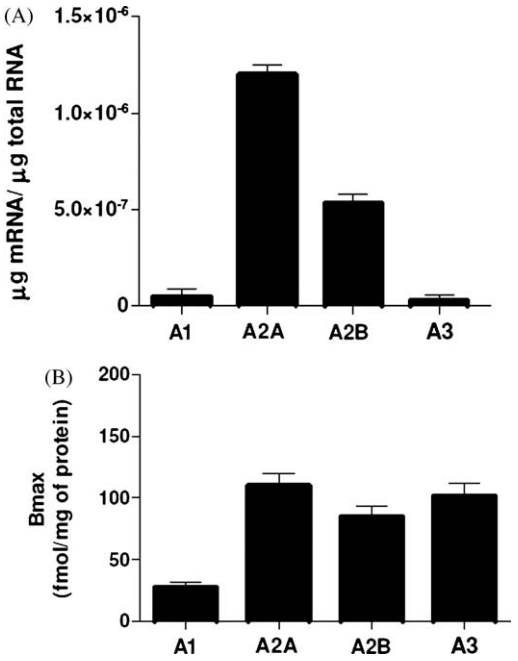

Fig. 1. mRNA and protein expression of ado receptors in human U87MG glioblastoma cells. (A) Bar graph showing µg RNA/µg total RNA of human A_1, A_{2A}, A_{2B} and A_3 ado receptors evaluated through real-time RT-PCR experiments. (B) Bar graph showing B_{max} (fmol/mg of protein) of human A_1, A_{2A}, A_{2B} and A_3 ado receptors measured by means of binding experiments. Experiments were performed as described in Section 2. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate.

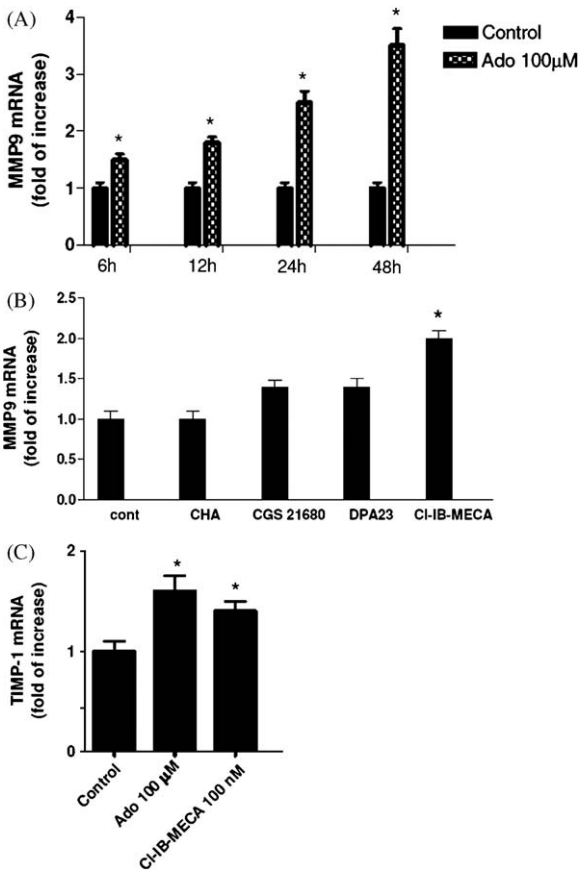

Fig. 2. Modulation of MMP-9 mRNA by ado in U87MG glioblastoma cells. (A) Time course of MMP-9 mRNA expression in U87MG cells after treatment with ado 100 µM. (B) Effect of ado receptor agonists, 100 nM CHA, 100 nM CGS 21680, 100 nM DPA23, 100 nM Cl-IB-MECA on MMP-9 mRNA induction in U87MG cells. (C) Effect of ado and Cl-IB-MECA on TIMP-1 mRNA expression in U87MG glioblastoma cells. Experiments were performed as described in Section 2. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate. $P < 0.05$ compared with the control (cells incubated in the absence of treatment). MMP-9 mRNA control values at 6, 12, 24 and 48 h were arbitrarily defined as 1.

U87MG glioblastoma cells. These results indicated that ado may increase MMP-9 mRNA through the involvement of A_3 receptors (Fig. 2B). Furthermore ado 100 µM and the A_3 agonist Cl-IB-MECA 100 nM induced a modest stimulation of 1.6 ± 0.2 and 1.4 ± 0.2 fold of increase, respectively, of tissue inhibitor metalloproteinase-1 (TIMP-1) (Fig. 2C). The relative affinities of adenosine agonists as well as antagonists used in this study for the different adenosine receptor subtypes are provided in Table 1.

3.3. Ado effect on MMP-9 protein levels in U87MG glioblastoma cells

Using an anti-MMP-9 antibody, that recognized both the pro and active forms of MMP-9 as bands migrating at 92 kDa and ≈78 kDa respectively, we found that Ado 100 µM induced a time-dependent increase of both pro and active MMP-9 levels starting from 6 h and reaching a peak after 12–24 h of incubation (Fig. 3A and B). U87MG glioblastoma cells showed constitutive expression of the ≈78 kDa form of MMP-9, that was the active MMP-9 protein, as further verified by comparison with the recombinant active MMP-9, used as positive control (Fig. 3C). As the effect induced by ado on active MMP-9 was most evident we focused our attention on it. Treatment of the cells with ado in the range 0.01–100 µM for 24 h resulted in a dose-dependent increase of active MMP-9 with an EC_{50} of 3.5 ± 0.2 µM (Fig. 4A and B). By using a series of selective antagonists of A_1, A_{2A}, A_{2B} and A_3 ado receptors we observed that the nucleoside effect was abrogated in the presence of the A_3 blocker MRE 3008F20, whilst was unaffected by 100 nM DPCPX, SCH 58261 and MRE 2029F20, A_1, A_{2A}, and A_{2B} antagonists, respectively (Fig. 4C and D). To further verify the involvement of A_3 receptors in the modulation of MMP-9 protein expression, we treated U87MG glioblastoma cells with the high affinity A_3 receptor agonist Cl-IB-MECA, in western blotting experiments (Fig. 5A). A_3 receptor activation by Cl-IB-MECA, in the range of concentration 1–500 nM, produced a stimulatory effect on MMP-9 protein levels, with an EC_{50} of 10 ± 1 nM (Fig. 5B). Furthermore increasing concentrations of MRE 3008F20 (0.05–100 nM) were able to inhibit MMP-9 protein increase, induced by a maximal dose (100 nM) of Cl-IB-MECA, with an IC_{50} of 1.3 ± 0.1 nM (Fig. 5C). To demonstrate more conclusively a role for A_3 receptor in the adenosine-induced MMP-9 protein accumulation, we tried to knockdown A_3 receptor expression using small interfering-(si)-RNA leading to a transient knockdown of the A_3 receptor gene (siRNAA_3).

Table 1
Affinity (K_i, nM) of selected adenosine receptor agonists and antagonists to A_1, A_{2A}, A_{2B} and A_3 adenosine receptors.

	A_1	A_{2A}	A_{2B}	A_3	Ref.
Agonists					
CHA	3.5	812	>1000	83	[60]
CGS 21680	289	27	>10,000	67	[61]
DPA23	8.5	>1000	7.3	38.4	[62]
Cl-IB-MECA	220	5360	>100,000	1.4	[61]
Antagonists					
DPCPX	3.9	129	51	1100	[15]
SCH 58261	549	1.1	>10,000	>10,000	[15]
MRE 2029F20	200	>1000	5.5	>1000	[15]
MRE 3008F20	1200	141	2100	0.82	[15]
ZM 241385	774	1.6	75	0.82	[61]

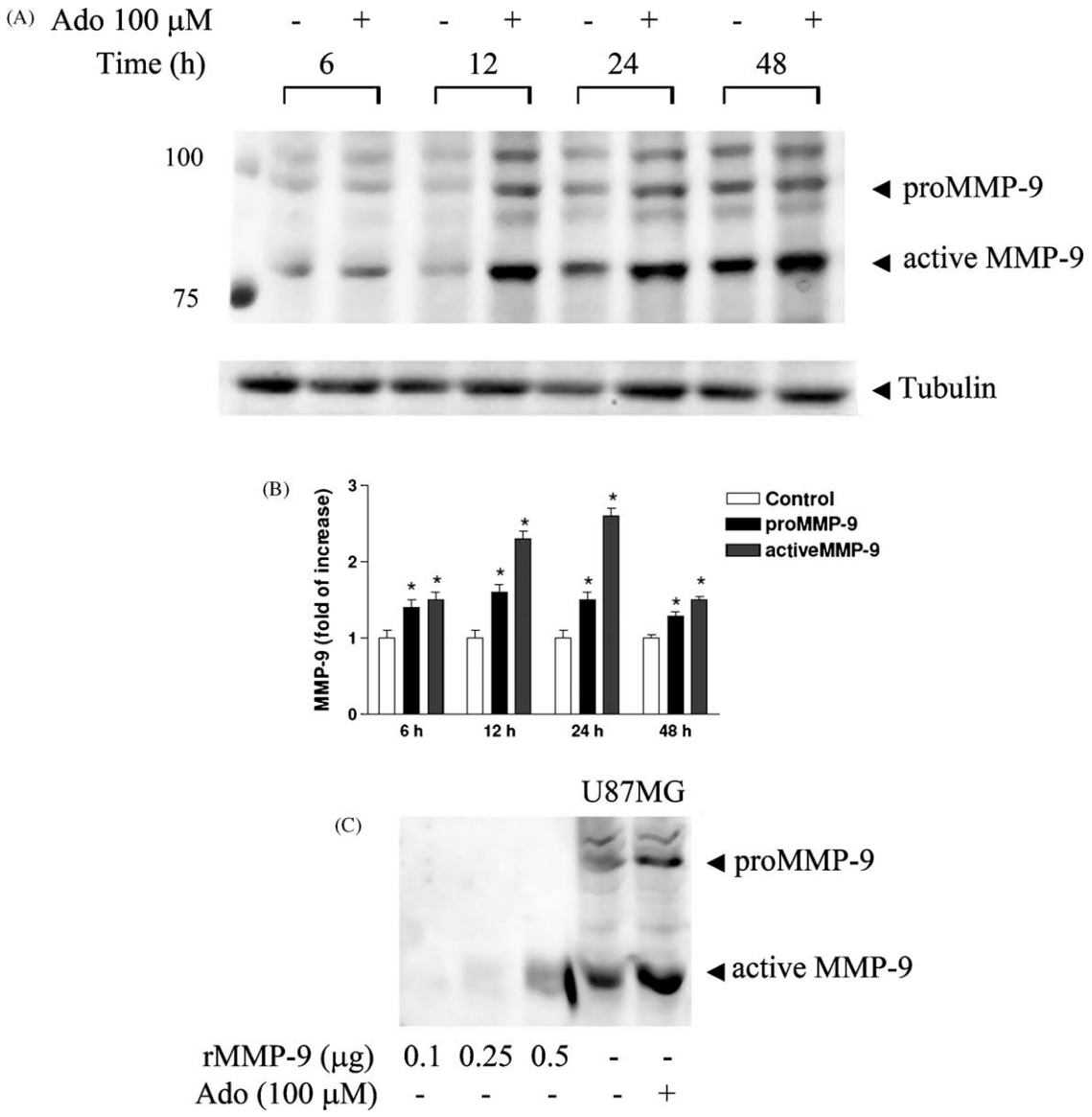

Fig. 3. Modulation of pro and active MMP-9 protein levels by ado in U87MG glioblastoma cells. (A) Time course of MMP-9 protein levels in U87MG cells after treatment with ado 100 μM. On the left the 75 and 100 kDa bands of the molecular weight marker are shown. (B) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control. MMP-9 protein control values at 6, 12, 24 and 48 h were arbitrarily defined as 1. (C) Increasing concentrations (0.1, 0.25, 0.5 μg) of recombinant active MMP-9 (83 kDa) and endogenous pro and active MMP-9 in U87MG cells in the absence or presence of ado.

U87MG glioblastoma cells were transfected with non-specific random control ribonucleotides or with siRNAA_3. As expected 72 h post transfection, A_3 receptor mRNA and protein levels of U87MG glioblastoma cells were significantly reduced (Fig. 5D and E, respectively). Therefore, at 72 h from the siRNA transfection we found that the stimulatory effect induced by a maximal dose of the A_3 agonist was strongly reduced, suggesting that inhibition of A_3 receptor expression is responsible for the block of Cl-IB-MECA-mediated MMP-9 increase (Fig. 5F and G).

3.4. Intracellular pathways activated by ado A_3 receptor to increase MMP-9 levels in U87MG glioblastoma cells

To establish the intracellular pathways triggered by A_3 receptors to stimulate MMP-9 protein increase in U87MG cells the effect of the A_3 receptor agonist on MAPK and Akt activation was investigated. To this aim glioblastoma cells were pretreated for 30 min with 1 μM U0126, SB202190, SP600125, SH-5, specific inhibitors of ERK1/2, p38, JNK and Akt, respectively and subsequently stimulated with Cl-IB-MECA. As reported in Fig. 6A and B, U0126, SP600125 and SH-5 inhibitors at a concentration of 1 μM were able to inhibit the effect induced by the A_3 agonist Cl-IB-MECA on MMP-9 protein levels whilst SB202190 did not, suggesting that ERK1/2, JNK and Akt but not p38 pathway were required for MMP-9 protein increase induced by A_3 receptor activation. Furthermore, we observed that treatment of glioblastoma cells with Cl-IB-MECA for 6, 12 and 24 h induced a time-dependent increase of ERK1/2, JNK and Akt phosphorylation (Fig. 6C–E). Similar results were obtained with ado (Fig. 7A–C) and its effect was antagonized by MRE 3008F20 and siRNAA_3 supporting a role for A_3 receptors in kinases phosphorylation (Fig. 7D–F).

3.5. AP-1 involvement in the A_3 receptor-mediated increase of MMP-9 levels in U87MG glioblastoma cells.

The activator protein-1 (AP-1) transcription complex appears to play an essential role in stimulating transcriptional activation

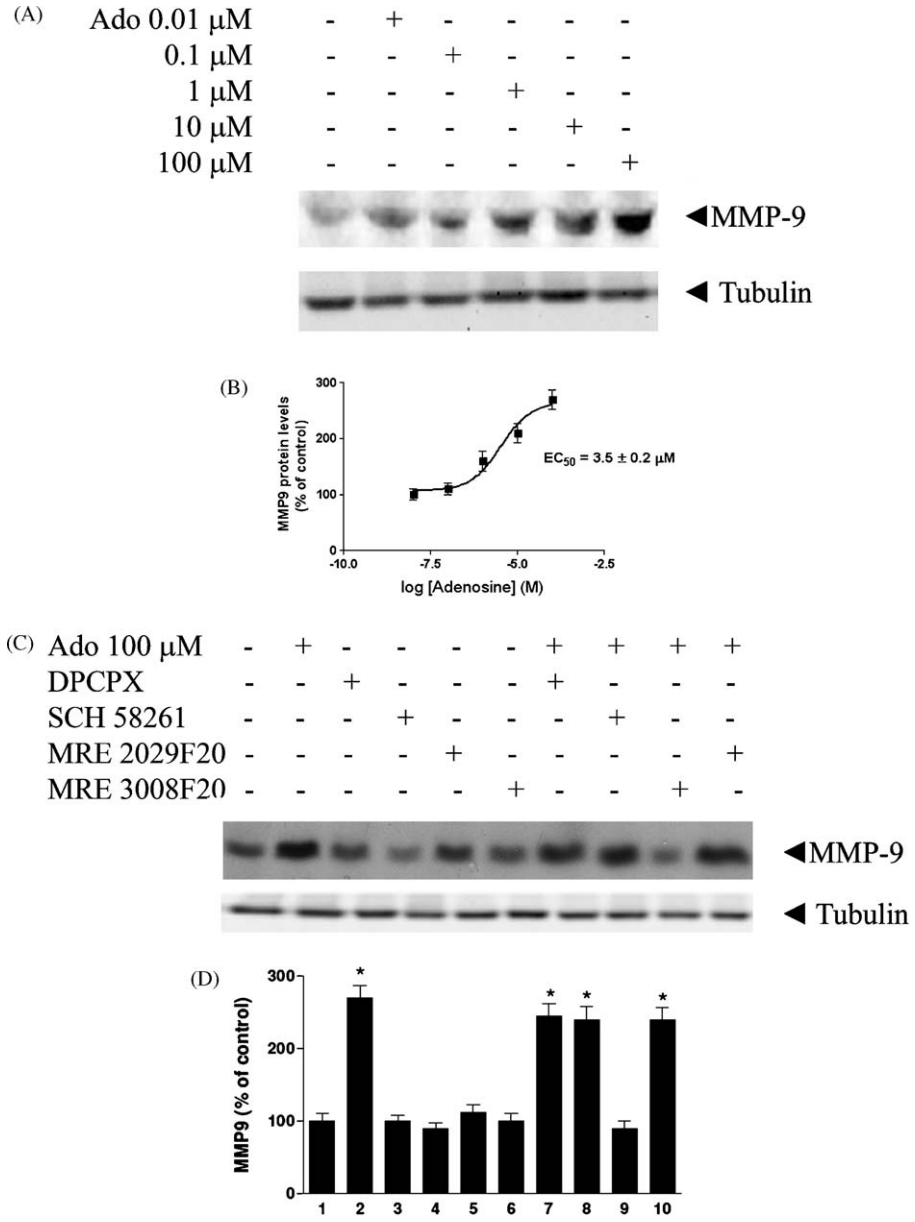

Fig. 4. Effect of ado and ado antagonists on MMP-9 protein levels in U87MG glioblastoma cells. (A and B) Immunoblot and relative dose-response curve of ado (0.01–100 μM) on MMP-9 protein levels, respectively. (C) Effect of ado 100 μM on MMP-9 protein levels and antagonism by 100 nM DPCPX, SCH 58261, MRE 3008F20 and MRE 2029F20. (D) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control (cells incubated in the absence of treatment).

of MMP-9. In order to verify the involvement of AP-1 in the A_3 ado receptor-induced MMP-9 protein increase, we performed western blotting experiments in the presence of ado 100 μM and the A_3 agonist Cl-IB-MECA 500 nM, in combination with the AP-1 antagonist NDGA 10 μM. As reported in Fig. 8A and B the AP-1 inhibitor was able to reduce the ado A_3 receptor-mediated effect on MMP-9 levels. Furthermore, to evaluate in more detail the effect of A_3 receptor activation on the phosphorylation of c-Jun, a major subunit of AP-1, glioblastoma cells were treated with increasing concentrations of ado and Cl-IB-MECA. As shown in Fig. 8C-F both ado and Cl-IB-MECA were able to raise p-c-Jun protein levels in a dose-dependent way with an EC_{50} of 4.5 ± 0.3 μM and 9.0 ± 0.8 nM, respectively. When the A_3 receptor subtype was blocked with the A_3 antagonist MRE 3008F20, the stimulatory effect induced by Cl-IB-MECA on p-c-Jun was reverted, suggesting again the involvement of A_3 receptors in this effect (Fig. 8G and H).

3.6. MMP-9 levels in supernatants from U87MG glioblastoma cells by ELISA and gelatin zimography assays

In order to investigate whether A_3 receptor activation was also able to increase MMP-9 protein levels in the supernatants from human U87MG glioblastoma cells, ELISA assays, recognizing both the latent and active MMP-9 forms, were performed. After 24 h of treatment ado and the A_3 agonist Cl-IB-MECA, were able to induce a stimulatory effect of 162 ± 14% and 175 ± 15% on MMP-9 levels in cancer cells that was antagonized by both A_3 antagonist and siRNA treatment (Fig. 9A). The A_1, A_{2A} and A_{2B} adenosine analogues did not modulate MMP-9 levels.

Furthermore, MMP-9 activity was evaluated in the conditioned medium using zymography. A band corresponding to the active 82 kDa MMP-9 form was detected and was increased after exposure of U87MG glioblastoma cells to the A_3 agonist Cl-IB-MECA 100–500 nM and Ado 10–100 μM for 24 h (Fig. 9B and C).

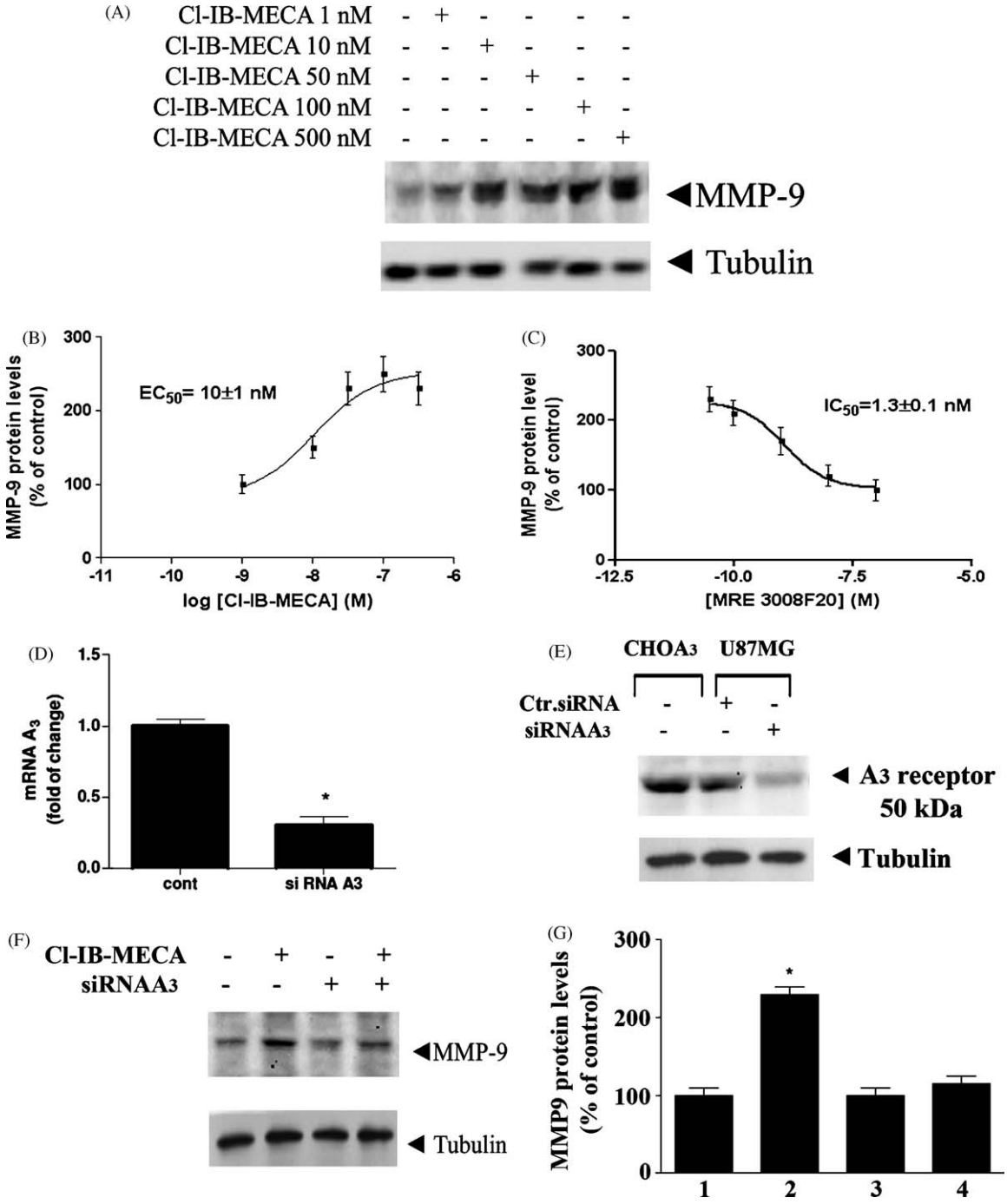

Fig. 5. Involvement of A_3 receptor on ado-induced MMP-9 protein levels modulation in U87MG glioblastoma cells. (A and B) Immunoblot and relative dose-response curve of Cl-IB-MECA (1–500 nM) on MMP-9 protein levels, respectively. (C) Dose-response curve of antagonism by MRE 3008F20 (0.05–100 nM) on 100 nM Cl-IB-MECA effect. Values are the means and vertical lines SE of the mean of three separate experiments performed in triplicate. (D) Relative A_3 ado receptor mRNA quantification, related to β-actin mRNA, and (E) A_3 receptor protein level in U87MG cells after transfection with A_3 siRNA for 72 h. (F) Effect of A_3 receptors siRNA treatment on Cl-IB-MECA (100 nM) induction of MMP-9 in U87MG cells. (G) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control.

3.7. A_3 receptor effect on invasiveness of U87MG glioblastoma cells

To assess whether increased production of MMP-9 protein by A_3 receptor activation resulted also in increased invasion of tumor cells, an *in vitro* cell invasion assay was done. We cultured U87MG glioblastoma cells on invasion chambers in the absence and in the presence of Ado 100 μM and the A_3 agonist Cl-IB-MECA 100 nM. After 48 h of cultures in the presence of Ado and the A_3 agonist, the invasive capacity of U87MG cells was highly increased (160 ± 20% and 180 ± 30% of control, respectively) and the effect was reduced by the addition of the A_3 antagonist MRE 3008F20, A_3 silencing, MMP-9 inhibitor I and MMP-9 silencing. This suggests that A_3 receptors and MMP-9 play a role in modulating glioblastoma invasiveness (Fig. 10A). The stimulatory effect induced by both Ado and Cl-IB-MECA was also antagonized by U0126, SH-5 and SP600125 indicating the involvement of ERK1/2, Akt and JNK in the invasion of U87MG cells (Fig. 10B).

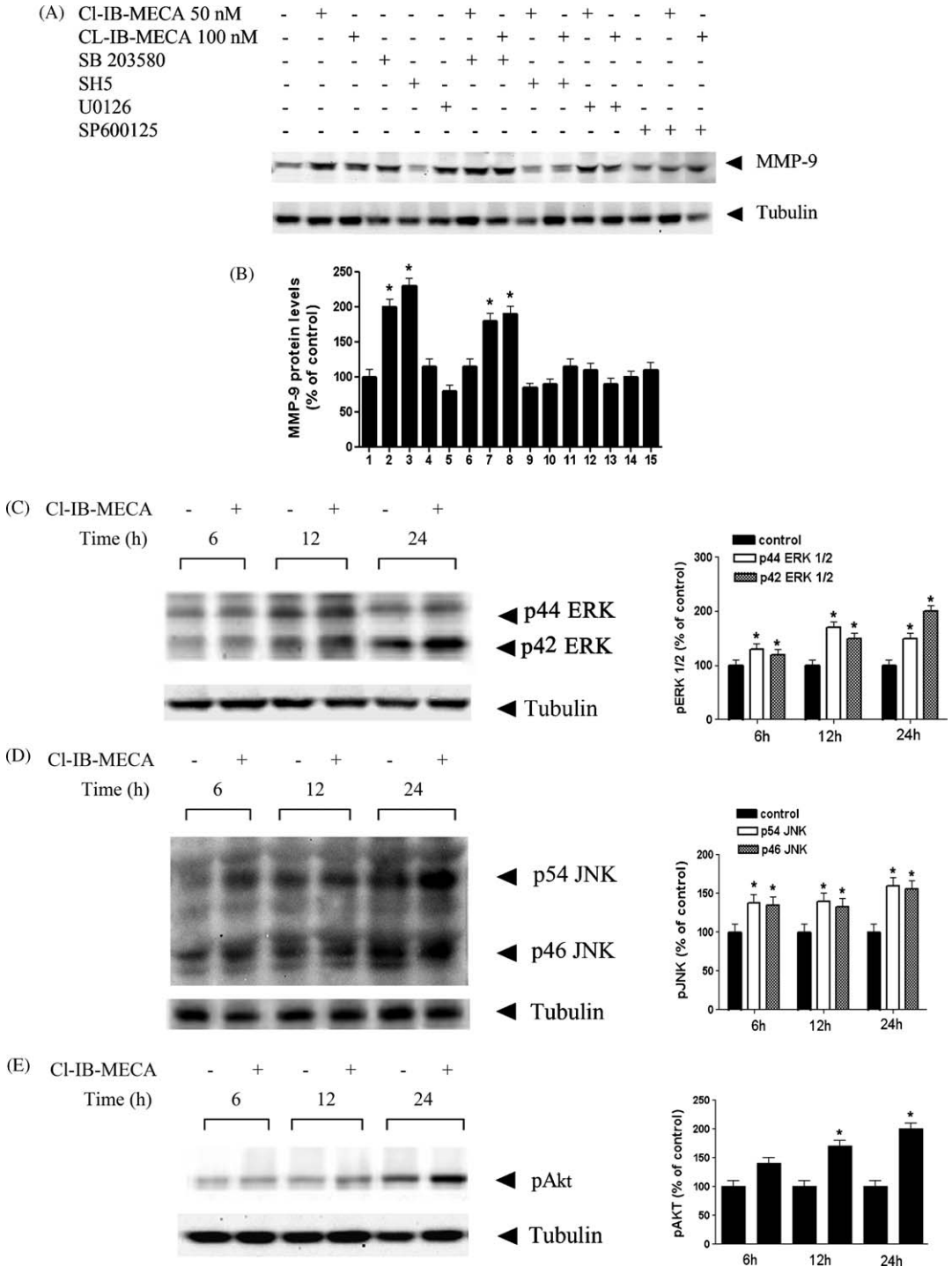

Fig. 6. Involvement of MAPK and Akt kinases in A_3 receptor-induced MMP-9 protein levels in U87MG cells. (A) Role of p38, Akt, ERK 1/2 and JNK inhibitors in MMP-9 modulation induced by Cl-IB-MECA 50–100 nM. (B) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); (C–E) Time course of Cl-IB-MECA 100 nM on ERK1/2 (C), JNK (D) and Akt (E) phosphorylation. Densitometric quantification of western blots is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control (cells incubated in the absence of treatment).

4. Discussion

MMPs play a major role in promoting tumor metastasis. In particular overexpression of MMP-9 has been shown to be associated with the progression and invasion of several tumors including gliomas [3]. Although malignant gliomas rarely metastasize outside of the central nervous system, they are extremely invasive tumors. Several works have reported that glioblastomas produce significantly higher levels of MMP-9 than do lower-grade tumors and normal brain tissue [7,28].

Ado depending on the extracellular concentration and expression of different ado receptor subtypes is known as a modulator of cell proliferation, apoptosis and angiogenesis (for review see Ref. [16]). Only few experimental works have focused on the effects of ado in MMP modulation and have been performed in cells of the immune system. In fact it has been reported that ado inhibits

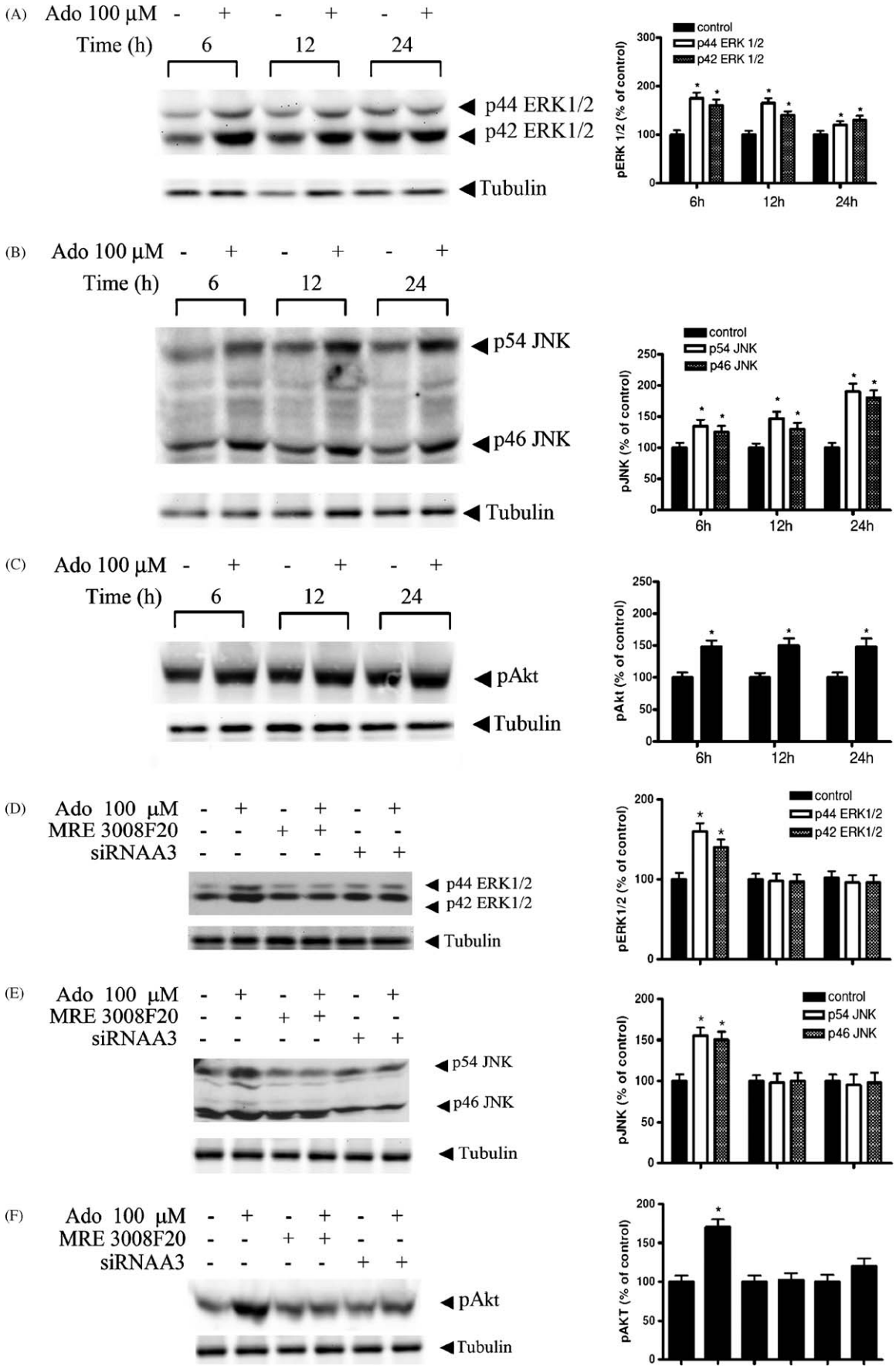

Fig. 7. Time course of ado 100 μM on ERK1/2 (A), JNK (B) and Akt (C) phosphorylation and antagonism by MRE 3008F20 and siRNA of A_3 receptors on the ado effect (12 h) on ERK1/2 (D), JNK (E) and Akt (F) phosphorylation. Densitometric quantification of western blots is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control (cells incubated in the absence of treatment, or with MRE 3008F20, or with siRNAA_3 alone, defined as 100).

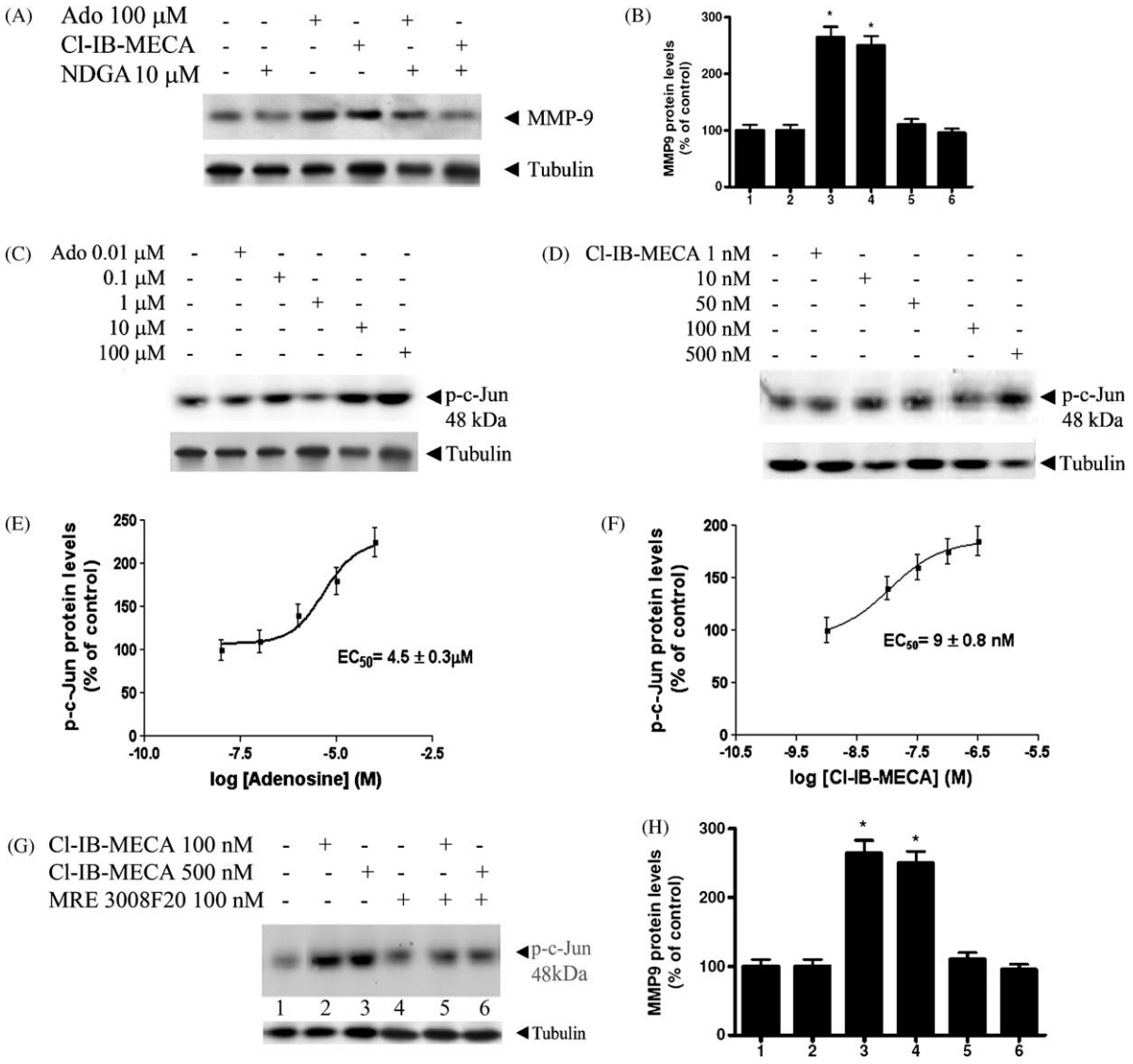

Fig. 8. Involvement of AP-1 in A_3 receptor-induced MMP-9 protein levels in U87MG cells. (A) Detection of MMP-9 protein by western blotting experiment in the presence of ado (100 μM) or the A_3 agonist Cl-IB-MECA (500 nM) in combination with the AP-1 antagonist NDGA 10 μM. (B) Densitometric quantification of western blots is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control. (C–F) Immunoblots and relative dose-response curves of ado (0.01–100 μM) and Cl-IB-MECA (1–500 nM) on p-c-Jun protein levels, respectively. (G) Effect of Cl-IB-MECA 100 and 500 nM on p-c-Jun levels and antagonism by MRE 3008F20 100 nM on Cl-IB-MECA effect. (H) Densitometric quantification of MMP-9 western blot is the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control.

neutrophil-produced MMP-9 via A_{2A} receptors, it increases MMP-9 in macrophages through A_3 subtypes [29,30] and it suppresses MMP-9 production under hypoxic conditions in human monocyte-derived dendritic cells via A_{2B} receptors [31]. In the current study, we sought to determine the involvement of ado in the regulation of MMP-9 production in U87MG glioblastoma cells. The main finding of this work is that ado increases MMP-9 levels through activation of A_3 receptors, ERK 1/2, JNK, Akt, AP-1 and is responsible for an increase in cell invasion. First of all we evaluated mRNA and protein levels of ado receptors in U87MG cells. Our results from real-time RT-PCR experiments showed that all ado receptors were expressed in glioblastoma cells with the following order $A_{2A} > A_{2B} > A_1 > A_3$. This mRNA order of expression did not match exactly the protein order found by binding assays that was $A_{2A} > A_3 > A_{2B} > A_1$. This was due to the low mRNA level of A_3 receptors in comparison to its high protein amount, already observed in different cell systems [19,32]. This supports the emerging evidence that mRNA expression patterns are necessary but are by themselves insufficient for the quantitative description of biological systems. This evidence includes discoveries of post-transcriptional mechanisms controlling the protein translation rate or the half-lives of specific proteins or mRNAs [33,34]. However it is also known that the gene promoter region of A_3 receptors is rich in putative binding sites for ubiquitous transcription factors including AP-1, SP-1, EF-1A. These ubiquitous factors may be responsible for the widespread, low level of A_3 mRNA expression observed [35]. In this particular case it could be speculated that c-Jun, stimulated by A_3 receptors in U87MG cells, may be involved in negative feedback mechanisms to regulate A_3 mRNA expression in response to high MMP-9 and cell invasion levels. Other transcription factors such as NF-kB and CREB interacting with the A_3 gene promoter have been demonstrated to be involved in the overexpression of A_3 receptors in peripheral blood cells of patients with rheumatoid artritis [36].

As uncontrolled activation of MMP-9 is potentially dangerous in a cellular environment, enzyme expression is tightly regulated at the transcriptional and/or translational level. Therefore we started to evaluate the effect of ado on MMP-9 transcription in U87MG

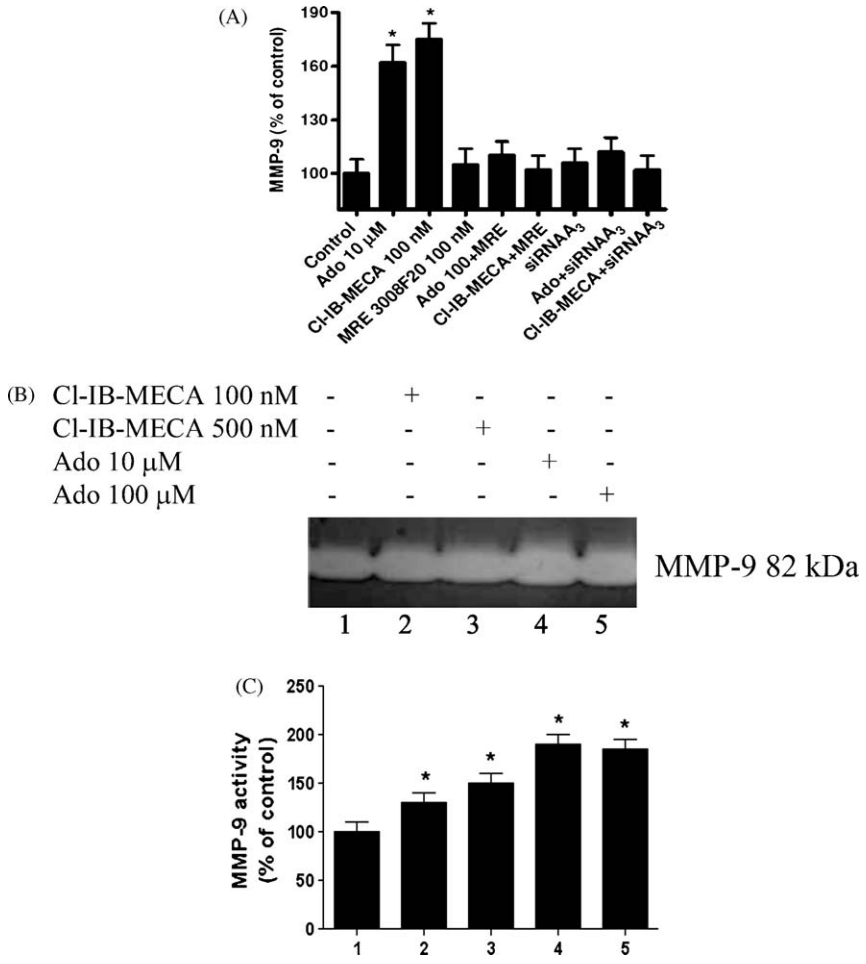

Fig. 9. A_3 receptor-mediated increase of MMP-9 in supernatants of U87MG cells. (A) Effect of ado, Cl-IB-MECA, A_3 antagonists and A_3 silencing on MMP-9 levels by ELISA assay. Values are the mean ± S.E. values ($N = 3$); $P < 0.05$ compared with the control. (B) Effect of 100, 500 nM Cl-IB-MECA and 10, 100 μM ado on MMP-9 activity by gelatin zymography. Experiments were performed as described in Section 2. (C) Densitometric quantification of gelatine zimography. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate. $P < 0.05$ compared with the control.

cells. We found that the nucleoside was able to increase in a time-dependent manner the expression of MMP-9 mRNA starting from 6 h, with the maximal effect observed after 12–24 h. The effect of ado was replicated by the A_3 ligand Cl-IB-MECA, but not by A_1, A_{2A} and A_{2B} agonists suggesting the involvement of A_3 receptors. Furthermore we found that both ado and the A_3 agonist induced a modest increase of TIMP-1 expression levels, suggesting the existence of a regulatory loop to compensate for MMP-9 production. This was observed also in THP-1 macrophages by Velot et al. [30]. However like in THP-1 cells also in U87MG cells its low increase is probably insufficient to counterbalance the raised expression of MMP-9, induced by A_3 receptor activation [30]. A reduced level of both MMP-9 and TIMP-1 levels have been detected also in lungs and in the bronchoalveolar lavage fluid of A_3 receptors deficient mice suggesting that the A_3 plays a regulatory role in their production [37]. Like most MMPs, MMP-9 is secreted as a latent zymogen, requiring activation in the extracellular milieu or on the cell surfaces to be catalytically competent [38,39]. However it has been also reported that some cellular types e.g. microvascular endothelial cells or tumor cells are capable of accumulating active gelatinase B in the cytoplasm or in membrane vesicles [40–42] or that specific cell-MMP interactions may occur such as binding of MMP-9 to CD44 [43]. Evidence of constitutive activation of MMP-9 in U87MG cells was suggested by expression of the active form of MMP-9 in cellular extracts of glioblastoma cells. Similar results have been obtained in different types of cancer cell lines including DU145 (prostate androgen-independent carcinoma), SK-NEP-1 (adult-derived anaplastic Wilms' tumor), A431 (squamous cell carcinoma), Wit 49 (Wilms' tumor cell line), A549 (non-small cell lung carcinoma) and LNCaP (prostate androgen-dependent carcinoma) [42]. Ado increased, in glioblastoma cells, the intracellular levels of both latent and active MMP-9 with a major effect on the second one. The conclusion that the effects of ado on MMP-9 protein were mediated through A_3 receptors was supported by the observation that the stimulatory effects of this nucleoside on MMP-9 protein were mimicked by the A_3 receptor agonist Cl-IB-MECA, and inhibited by the A_3 receptor antagonist MRE 3008F20. In particular, the potencies of these drugs were in agreement with their inhibitory equilibrium binding constants (K_i) observed in binding experiments for the ado A_3 receptor [44]. Furthermore, the A_3 receptor-induced MMP-9 protein increase was blocked following the inhibition of A_3 receptor expression at the mRNA and protein levels, by the RNA interference approach.

MMP-9 gene expression can be activated via a number of signal transduction pathways including those involving ERK1/2, p38, JNK and PKB/Akt [7,9]. Ado has been demonstrate to activate some of these signalling molecules in different systems and cellular models through the activation of A_3 receptors (for review see Ref. [45]). In particular an increase in ERK1/2 phosphorylation has been reported initially by Schulte and Fredholm [46] in transfected CHO cells and then it has been confirmed in other systems like microglial and colon carcinoma cells [19,47]. Several studies have also indicated the

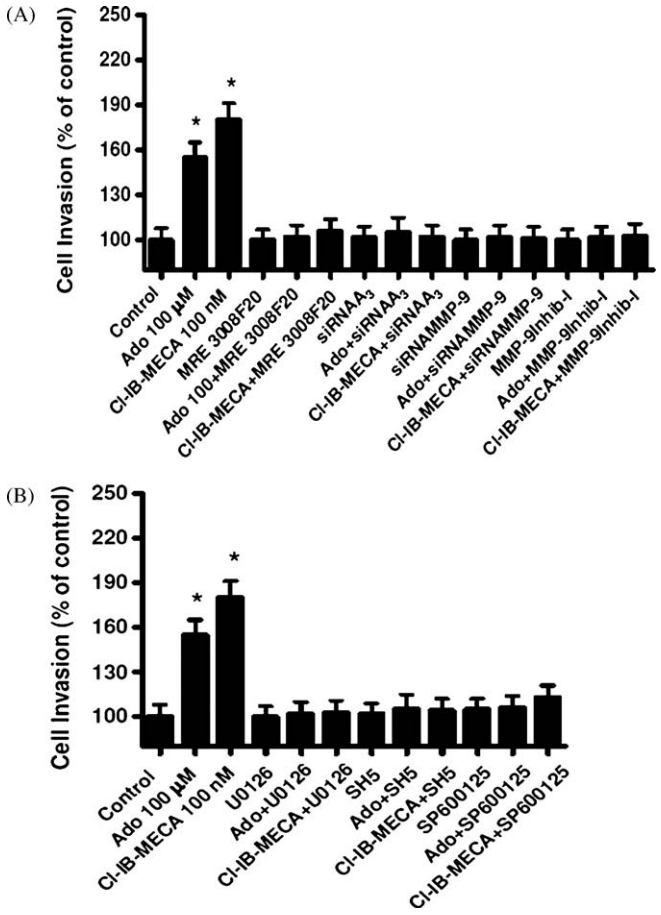

Fig. 10. Modulation of U87MG cell invasion. (A) Effect of 100 μM ado and 100 nM Cl-IB-MECA on U87MG cell invasion and antagonism by 100 nM MRE 3008F20, A_3 receptor silencing, MMP-9 silencing and 50 nM MMP-9 inhibitor I. (B) Effect of ERK 1/2, Akt and JNK inhibitors in A_3 receptor-mediated cell invasion. Experiments were performed as described in Section 2. Values are the means and vertical lines S.E. of the mean of three separate experiments performed in triplicate. $P < 0.05$ compared with the control.

ability of A_3 receptors to activate PKB/Akt. This has been observed in rat basophilic leukemia 2H3 mast cells, in cardiomyocytes, in melanoma and colon carcinoma cells [20,22,48,49]. As for p38 a link with ado A_3 receptors has been observed in rat basophilic leukemia 2H3 cells, colon carcinoma cells and rat hearts [22,50,51]. In contrast the effect of A_3 receptor activation on JNK has not been investigated. Our results show that stimulation of MMP-9 by A_3 receptor activation was abrogated by inhibitors of ERK1/2, JNK and Akt but not of p38, suggesting that more than one signalling pathway is involved in the MMP-9 modulation induced by A_3 receptor activation. Indeed, Cl-IB-MECA and ado-mediated an increase in the phosphorylation of ERK1/2, JNK and Akt kinases confirming that they belong to the signaling pathways utilized by the nucleoside following A_3 receptor stimulation. In particular it has been reported that JNK is necessary to phosphorylate c-Jun proteins and it is well known that the AP-1 transcription complex appears to play an essential role in stimulating transcriptional activation of MMP-9 [7,13]. In its active form AP-1 complex may comprise homodimers of c-Jun or heterodimers between c-Fos, c-Jun and ATF2 [52]. Furthermore activation of ERK1/2 has been shown to induce c-Jun expression and phosphorylation, indicating cross-talk between ERK1/2 and JNK pathways in the regulation of c-Jun activity [8,53]. We found that MMP-9 regulation by A_3 ado receptor activation was blocked after treatment with the AP-1 inhibitor; c-Jun, was also increased by ado and Cl-IB-MECA and this effect was antagonized by MRE 3008F20 suggesting the involvement of A_3 ado receptor. We demonstrated that A_3 receptor activation induced an increase of active MMP-9 as shown by ELISA and gelatine zymography experiments carried out on the supernatants obtained from U87MG cells, according to data showed by Velot et al. in macrophages [30].

Finally, as for the physiological relevance of the A_3 receptor-mediated stimulation of MMP-9 we found that Cl-IB-MECA was responsible for an increase of the invasive ability of U87MG cells. This effect was dependent by modulation of MMP-9, exerted through A_3 receptors activation and ERK1/2, JNK, Akt and AP-1 phosphorylation. Interestingly it was reported that MMP-9 inhibition might be useful for treating the invasiveness of brain tumors. Furthermore the antisense MMP-9 vector or adenovirus expressing antisense MMP-9 decreased the PMA-induced migration and invasion of glioblastoma cells [54]. Interestingly the increase induced by adenosine in cell invasion has been previously reported also in breast cancer cells [55,56].

It is well known that the A_3 receptor plays an important role in regulating normal and tumor cell growth [16]. Elegant *in vivo* studies by Fishman's group showed the efficacy of A_3 agonists in various tumor-bearing animals, supporting the utilization of A_3 agonists to treat cancer [57]. In particular melanoma, colon, prostate and hepatocellular carcinomas were reduced in *in vivo* animal models by IB-MECA or Cl-IB-MECA [17,57,58]. Importantly in these studies, the combined treatment of IB–MECA with the specific agents cyclophosphamide, 5-fluorouracil and taxol respectively, resulted in an enhanced antitumor effect. On the other hand, it has been reported that in U87MG glioblastoma cells under hypoxic conditions, Cl-IB-MECA induced up-regulation of hypoxia-inducible factor 1 (HIF-1) alpha and VEGF supporting a role for A_3 antagonists as a novel approach for the treatment of glioblastomas [21]. A possible explanation for these different results may be that cell response to a given A_3 agonist is determined by a plethora of factors, including agonist concentration and affinity, receptor density, interaction between different adenosine receptors expressed on the cell surface, cell type and the cell microenvironment. Furthermore ado effects on MMP-9 and cell invasion not necessarily shall to occur during carcinogenesis. For example, it has been demonstrated that the *in vivo* administration of Ado reversed cirrhosis and liver dysfunction and that one of the mechanisms involved in this effect was through an increase in collagenolytic activity [59]. Accordingly the results of this work, revealing a role of A_3 receptor in the increase of glioblastoma cell invasion by stimulation of MMP-9, adds a new function to the complex role of A_3 receptors in tumor biology and need to be verified in *in vivo* experiments.

Acknowledgements

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