Influence of NSAIDs and methotrexate on CD73 expression and glioma cell growth

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Abstract

Glioblastoma (GBM) is the most malignant and deadly brain tumor. GBM cells overexpress the CD73 enzyme, which controls the level of extracellular adenosine, an immunosuppressive molecule. Studies have shown that some nonsteroidal anti-inflammatory drugs (NSAIDs) and methotrexate (MTX) have antiproliferative and modulatory effects on CD73 in vitro and in vivo. However, it remains unclear whether the antiproliferative effects of MTX and NSAIDs in GBM cells are mediated by increases in CD73 expression and adenosine formation. The aim of this study was to evaluate the effect of the NSAIDs, naproxen, piroxicam, meloxicam, ibuprofen, sodium diclofenac, acetylsalicylic acid, nimesulide, and ketoprofen on CD73 expression in GBM and mononuclear cells. In addition, we sought to understand whether the effects of MTX may be mediated by CD73 expression and activity. Cell viability and CD73 expression were evaluated in C6 and mononuclear cells after exposure to NSAIDs. For analysis of the mechanism of action of MTX, GBM cells were treated with APCP (CD73 inhibitor), dipyridamole (inhibitor of adenosine uptake), ABT-702 (adenosine kinase enzyme inhibitor), or caffeine (P1 adenosine receptor antagonist), before treatment with MTX and AMP, in the presence or not of mononuclear cells. In summary, only MTX increased the expression of CD73 in GBM cells decreasing cells viability by mechanisms independent of the adenosinergic system. Further studies are needed to understand the role of MTX in the GBM microenvironment.

Keywords Glioblastoma · Anti-inflammatory drugs · NSAIDs · Methotrexate · Adenosine · CD73

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Introduction

Glioblastoma (GBM) is the most common and aggressive brain tumor and has a prognosis of about one year after diagnosis [1, 2]. GBM has complex intratumoral heterogeneity and constitutes multiple proliferating cancer cells, endothelial cells, fibroblasts, and immune cells [3]. Combined surgical resection with chemotherapy and radiation treatment is recommended for this type of tumor; however, the standard drug currently used, temozolomide, only modestly improves the survival of patients. Additionally, GBM is characterized by an immunosuppressive microenvironment due to the differentiation of regulatory cells and mechanisms that involve, among other factors, adenosine nucleoside production [4, 5].

Adenosine accumulation is observed in the extracellular environment in several pathological conditions, such as chronic inflammation and tumors, [6–8]. Depending on the

Table 1 Plasma and cytotoxic concentrations of NSAIDs

Compounds	Abbrev.	Clinically relevant plasma concentrations	Cytotoxic concentrations
Naproxen	NPX	400.0 μ M	2.0 mM
Piroxicam	PRX	20.0 μ M	1.0 mM
Meloxicam	MLX	5.0 μ M	500.0 μ M
Ibuprofen	IBF	100.0 μ M	1.0 mM
Sodium diclofenac	DCF	5.0 μ M	500.0 μ M
Acetylsalicylic acid	ASA	1.5 mM	3.0 mM
Nimesulide	NMS	20.0 μ M	500.0 μ M
Ketoprofen	KTP	50.0 μ M	3.0 mM

NSAIDs concentrations were acquired from medicine insert leaflet information and correspond to the concentration of drugs found in the patients' plasma. All plasma concentrations of NSAIDs are available from Micromedex (<https://www.micromedexsolutions.com>), Medscape (<https://www.medscape.com>) or Drugs.com (<https://www.drugs.com>) databases

extracellular concentration, adenosine can bind to P1 receptors causing either direct inhibition of effector lymphocytes or activation of regulatory cells with consequent inhibition of effector cells [9]. In the GBM microenvironment, adenosine is mostly produced through the sequential hydrolysis of ATP. Shortly, ATP is first broken down to ADP and then to AMP by E-NTPDase1 (CD39) enzyme, which is overexpressed in regulatory immune cells, and AMP is then hydrolyzed to adenosine by the ecto-5'-nucleotidase enzyme (CD73), which is overexpressed in GBM cells [10]. In fact, the coordinated roles of these enzymes in adenosine production is important for immune surveillance escape in the GBM microenvironment [11, 12].

Anti-inflammatory drugs are effective therapeutic agents that share the common characteristic of inhibiting the activity of the cyclooxygenase enzymes (COX), making them useful for treating inflammatory disorders, autoimmune diseases, and solid tumors [13]. Anti-inflammatory drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs), are reported

to have the potential to increase apoptosis and decrease migration and tumor angiogenesis. Experimental, epidemiological and clinical studies suggest that NSAIDs may contribute to tumorigenesis prevention or cancer treatment by inhibiting the overexpressed enzyme, COX2, although the precise mechanism behind this anticancer activity remains elusive [14, 15]. Other studies suggest that the antiproliferative effect of NSAIDs is, at least in part, independent of COX inhibition and prostaglandin synthesis [16]. For instance, a previous study conducted by our group showed that indomethacin increases the levels of CD73 enzyme and, consequently, adenosine formation. Thus, adenosine, when produced extracellularly, may contribute to decrease GBM cell proliferation via a possible activation of A3 receptors [17]. In addition to NSAIDs, other classes of anti-inflammatory drugs have demonstrated potential modulation of tumor cell proliferation. Dexamethasone, a corticosteroid drug, contributes to the efficacy of chemotherapeutic agents in glioma, breast, lung, and colon cancer [18–21]. Our laboratory has previously demonstrated that dexamethasone upregulates CD73 expression in GBM cells, contributing to the hypothesis that adenosine may be involved in the antitumor role of this drug [22].

Another drug that has also been tested is methotrexate (MTX), which is an antimetabolic drug with anti-inflammatory and antiproliferative actions that is widely used in the clinic as an adjuvant for several types of cancer therapies [23–25]. Structurally, MTX is analogous to folic acid and acts by competitively inhibiting the dihydrofolate reductase (DHFR) enzyme. This inhibition results in the depletion of tetrahydrofolates, which are required for the synthesis of purines and thymidylate. Consequently, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) synthesis, as well as other metabolic reactions, are interrupted [26, 27]. The mechanism of action of MTX is most striking in cell populations during the exponential growth phase, which explains the greater activity of this molecule in tumor cells and proliferating tissues

Table 2 Concentrations of MTX, AMP, inhibitors, and combined treatments

Compounds	Concentrations
MTX	1.0 μ M
Dipyridamole (DIP)	5.0 μ M
DIP + MTX	5.0 μ M + 1.0 μ M
ABT-702 (ABT)	0.1 μ M
ABT + MTX	0.1 μ M + 1.0 μ M
APCP	10 μ M
APCP + MTX	10.0 μ M + 1.0 μ M
Caffeine (CAF)	30 μ M
CAF + MTX	30.0 μ M + 1.0 μ M
AMP	20.0 μ M

Fig. 1 C6 cell viability and CD73 expression after NSAIDs treatment. **a** C6 cells were treated with plasma concentrations of NSAIDs for 48 h, and cell viability was measured by Trypan Blue exclusion dye test, $n = 5$. **b** C6 cells were treated with plasma concentrations of NSAIDs for 48 h, and CD73 immunocontent was measured by flow cytometry; data are presented as mean fluorescence intensity (MFI), $n = 3$. **c** C6 cells were

treated with cytotoxic concentrations of NSAIDs for 48 h, and cell viability was measured by Trypan Blue exclusion dye test, $n = 3$. **d** C6 cells were treated with cytotoxic concentrations of NSAIDs for 48 h, and CD73 immunocontent was measured by flow cytometry; data are presented as MFI, $n = 5$. Data are shown as means $\pm$ SD (** $p < 0.01$, *** $p < 0.001$)

[28]. We have shown that MTX can increase CD73 expression in GBM cells and T lymphocytes present in the tumor microenvironment. The indiscriminate increase in CD73 expression and, consequently, in the concentration of adenosine culminates in a low frequency of T lymphocytes, with Treg cells being practically abolished. The decrease in Treg cells in tumor tissue may relieve the immunosuppression imposed on T-effector and NK cells, leading to an apoptosis-mediated decreased tumor size [29].

Several NSAIDs demonstrate anticancer activity, and some of these have the ability to modulate the adenosinergic system. However, the mechanisms by which these drugs contribute to chemotherapy in GBM are not fully understood. Therefore, the present study sought to investigate whether a range of NSAIDs may also interfere in CD73 expression. In addition, we evaluated whether the modulation of the adenosinergic system by MTX is directly related to its cytotoxic effect in GBM cells.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Fungizone®, penicillin/streptomycin, and

trypsin/EDTA solution were obtained from Gibco (USA). Dimethylsulfoxide (DMSO), ABT-702 (4-amino-5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido(2,3-d)pyrimidine), AMP, APCP (adenosine 5'-(α , β -methylene) diphosphate), dipyrindamole (DIP), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium), and Trypan Blue were purchased from Sigma-Aldrich (USA). MTX was supplied by Pharma Nostra (Brazil). Nimesulide (NMS), naproxen (NPX), and meloxicam (MLX) were obtained from All Chemistry (Brazil). Ketoprofen (KTP), acetylsalicylic acid (ASA), and sodium diclofenac (DCF) were from SM enterprises (Brazil). Piroxicam (PRX) was obtained from Galena (Brazil), and ibuprofen (IBF) was from Valdequímica (Brazil). MTS was bought from Promega (USA). The NSAIDs concentrations shown in Table 1 are determined using Micromedex (<https://www.micromedexsolutions.com/>), Medscape (<https://www.medscape.com/>), and Drugs.com (<https://www.drugs.com/>) databases. All other chemicals and solvents used were of analytical or pharmaceutical grade.

Maintenance of cell lines

The C6 rat glioma cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA).

Fig. 2 Mononuclear cell viability and CD73 expression after NSAIDs treatment. **a** Mononuclear cells were treated with plasma concentrations of NSAIDs for 48 h, and cell viability was measured by Trypan Blue exclusion dye test, $n = 5$. **b** Gate strategy for CD73 expression on mononuclear cells. For CD73 immunocontent analysis, mononuclear

cells were treated with plasma concentrations of NSAIDs for 48 h and analyzed by flow cytometry as total mononuclear cells (**c**), lymphocytes CD4⁺ (**d**), lymphocytes CD8⁺ (**e**), and lymphocytes CD4⁺CD25^{high} (**f**); data are presented as % cells, $n = 3$. Data are shown as means $\pm$ SD (** $p < 0.01$, *** $p < 0.001$)

Cells, up to the 30 passage, were grown and maintained in DMEM containing antibiotics (0.5 U/mL penicillin/streptomycin) and supplemented with 5% FBS. Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO₂ in air.

Mononuclear cell isolation

Adult male Wistar rats (220–300 g) were used for isolation of monocytes and lymphocytes from the mesenteric lymph node,

as previously described [30] and with approval from the Ethics Committee of the Universidade Federal do Rio Grande do Sul (Protocol #26389). Briefly, the mesenteric lymph nodes were removed and dissociated through a metal mesh in 0.9% saline and filtered with 40- μ m mesh. Cells were then washed three times with saline and centrifuged at 400 $\times$ g for 10 min. Mononuclear cells were counted with a Neubauer chamber and plated according to the respective assay, as described in later sections. Mononuclear cells were maintained in RPMI containing antibiotics (0.5 U/mL penicillin/

Fig. 3 C6 or mononuclear cell viability and CD73 expression after treatment with MTX. **a.** C6 cells were treated with MTX for 48 h, and cell viability was measured by Trypan Blue exclusion dye test, $n = 3$. **b.** Histogram of CD73 expression on C6 cells and **c** quantitative analysis of CD73 immunocontent on C6 cells after treatment with MTX for 48 h and analysis by flow cytometry. Data are presented as MFI, $n = 3$. **d.** Mononuclear cells were treated with MTX for 48 h, and cell viability

was measured by Trypan Blue exclusion dye test, $n = 3$. For CD73 immunocontent analysis, mononuclear cells were treated with MTX for 48 h and analyzed by flow cytometry as total mononuclear cells (**e**), lymphocytes $CD4^+$ (**f**), lymphocytes $CD8^+$ (**g**), and lymphocytes $CD4^+CD25^{high}$ (**h**); data are presented as % cells, $n = 3$. Gate strategy for CD73 expression is the same as presented in Fig. 2b. Data are shown as means $\pm$ SD (** $p < 0.01$, *** $p < 0.001$)

streptomycin) and 10% FBS and kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

Cell viability assays

C6 cell line

C6 cells were seeded in 24- or 96-well plates (10×10^3 or 2.5×10^3 cells/well, respectively). Cells were treated with plasma or cytotoxic concentrations of NSAIDs, as represented in Table 1 for 48 h, or treated with adenosinergic interventions, as described in Table 2, for 24 h or 48 h. Cell viability was assessed using the Trypan Blue exclusion dye test or the MTS method. Briefly, for the Trypan Blue exclusion dye test, at the end of the treatment, C6 cells were washed twice with PBS (pH 7.4) and trypsinized with 0.5% trypsin/EDTA solution, and Trypan Blue (0.1%) was added; cells were then counted immediately in a hemocytometer. For analysis by the MTS method, at

the end of the treatments, 20 μ L of MTS was added directly to each well. After 2 h, absorbance was read at 490 nm with a plate reader (Spectramax M5, Molecular Devices, USA).

Mononuclear cells

Mononuclear cells were seeded in 24-well plates (500×10^3 cells/well). Cells were treated immediately after seeding with plasma concentrations of NSAIDs, as represented in Table 1, or treated with adenosinergic interventions, as described in Table 2, for 24 h or 48 h. After the treatment, cell viability was determined using the Trypan Blue exclusion dye test by collecting cells from the supernatant, staining with Trypan Blue, and counting immediately in a hemocytometer.

C6 cells co-cultured with mononuclear cells

C6 cells were seeded in 96-well plates (2.5×10^3 cells/well) and allowed to grow until semi-confluence, before co-

Fig. 4 C6 cell viability after treatment with ABT-702, DIP, and MTX for 24 h or 48 h. C6 cells were treated with 1.0 μ M MTX for 24 h (a) and 48 h (b), with or without previous treatment with 0.1 μ M ABT-702 or 10.0 μ M DIP. Cell viability was determined by Trypan Blue exclusion dye test, $n = 3$. Data are shown as means $\pm$ SD (** $p < 0.01$, *** $p < 0.001$)

culturing with mononuclear cells (5×10^3 cells/well). Cells were treated with adenosinergic interventions, as described in Table 2, for 24 h or 48 h. Cell viability was assessed using the MTT method. After treatment, mononuclear cells were discarded, and the C6 cells were washed twice with PBS (pH 7.4), and 10 μ L MTT diluted in DMEM (5 mg/mL^{-1} , 100 μ L) were added to each well. The cells were incubated for 2 h, and the absorbance was read at 570–630 nm with a plate reader (Spectramax M5, Molecular Devices, USA).

CD73 immunocontent

C6 or mononuclear cells were seeded in 24-well plates (10×10^3 or 500×10^3 cells/well, respectively) and maintained under standard culture conditions. Cells were treated with plasma or cytotoxic concentrations of NSAIDs, as described in Table 1, for 48 h. After treatment, mononuclear cells were collected and centrifuged for $400 \times g$ for 6 min, and C6 cells were trypsinized and centrifuged at $400 \times g$ for 6 min. Subsequently, C6 or mononuclear cells were independently suspended in buffer (2% FBS in PBS) containing polyclonal rabbit anti-rat ecto-5'-nucleotidase/CD73 (1:200, cat. rNu-9 L, J. Sevigny's research lab, CA) and incubated for 30 min at

room temperature. The cells were then washed twice and incubated with FITC-conjugated (ex:488/em:530) goat anti-rabbit IgG secondary Ab (1:100, cat. 65-6111, Invitrogen, USA) for 30 min in the absence of light. Antibodies for CD4⁺ (APC-conjugated, 1:80, cat. 550057), CD8⁺ (PerCP-conjugated, 1:80, cat. 558824), and CD4⁺CD25^{high} (PE-conjugated, 1:200, cat. 554866), all from BD Bioscience, were used to distinguish the T mononuclear cells subsets. Data acquisition for the C6 cell line was performed using a flow cytometer (FACSCalibur, BD Biosciences, USA). Mononuclear cells were analyzed using an Accuri flow cytometer (BD Biosciences, USA). Results were analyzed using FlowJo® 7.5.6 software (USA) or BD Accuri C6 software (USA), respectively. An isotype control was used as a nonspecific binding control.

Statistical analysis

All data were analyzed by Prism software (GraphPad, San Diego, USA), using one-way ANOVA (with Tukey post-test) or paired *t*-test. Data are presented as mean $\pm$ SD, and statistical differences with $p < 0.05$ were considered significant.

Results

Effects of plasma and cytotoxic concentrations of NSAIDs on C6 cell viability and CD73 expression

We initially determined the effects of treatment with plasma concentrations of NSAIDs. These concentrations were acquired from the information leaflets for the medicines and correspond to the concentrations found in patients' plasma. All plasma concentrations of NSAIDs are presented in Table 1. C6 cells were treated with plasma concentrations of NSAIDs for 48 h and analyzed using the Trypan Blue exclusion dye test or by flow cytometry. As shown in Fig. 1a, the concentrations of NSAIDs employed did not reduce cell viability and did not significantly modulate the expression of the CD73 enzyme in C6 cells (Fig. 1b). Subsequently, C6 cells were treated with cytotoxic concentrations of NSAIDs (Table 1) for 48 h. As shown in Fig. 1c, cell viability was significantly reduced by all of the drugs tested ($p < 0.001$), compared to the DMSO group. In addition, NSAIDs at cytotoxic concentrations did not alter the expression of the CD73 enzyme in GBM cells (Fig. 1d).

Effects of plasma and cytotoxic concentrations of NSAIDs on mononuclear cell viability and CD73 expression

Subsequently, we analyzed cell viability and CD73 expression in mononuclear cells after treatment with NSAIDs for 48 h. In

Fig. 5 C6 cell viability after treatment with DIP, ABT-702, APCP, caffeine, MTX, and AMP for 24 h or 48 h. C6 cells were treated with 1.0 μ M MTX for 24 h (**a**) or 48 h (**b**), with or without exposure to DIP, ABT-702, APCP, or caffeine. Cell viability was evaluated by MTS assay. In addition, the effect of 20 μ M AMP on treatment was evaluated. Data are shown as means $\pm$ SD, $n = 3$ (** $p < 0.001$)

general, we did not observe any changes in cell viability following incubation with plasma concentrations of NSAIDs, in relation to DMSO. The PRX and DCF drugs led to a slight increase in the percentage of viable cells, while NPX and ASA led to a slight decrease in viable mononuclear cells, although these changes were not significant (Fig. 2a). CD73 expression by mononuclear cells was determined by flow cytometry, in the total lymphocyte population and in the following subsets: CD4⁺, CD8⁺, and CD4⁺CD25^{high} (Fig. 2b). None of the concentrations of NSAIDs tested altered CD73 expression by the mononuclear cells, compared to DMSO. Approximately 70% of mononuclear cells (Fig. 2c), 60% of CD4⁺ lymphocytes (Fig. 2d), 50% CD8⁺ lymphocytes (Fig. 2e), and 70% of CD4⁺CD25^{high} lymphocytes (Fig. 2f) expressed CD73 on their surfaces after treatment with DMSO or with plasma concentrations of NSAIDs. Similarly, when median fluorescence

intensity (MFI) of C73 expression was determined, no statistical differences were observed (data not shown).

MTX decreases the viability of glioma cells and upregulates their CD73 expression

After treatment with 1.0 μ M MTX, we observed an approximately 80% reduction in C6 cells viability, relative to DMSO, in glioma cells ($p < 0.001$) (Fig. 3a). In addition, MTX treatment led to either a selection of cells highly expressing CD73 enzyme or a significant increase in CD73 expression on remaining C6 cells (~20%) (about 2-fold greater than DMSO, $p < 0.001$, Fig. 3b–c). In contrast, MTX did not reduce the cell viability of mononuclear cells, (Fig. 3d) or alter their expression of CD73, when compared to DMSO (Fig. 3e–h).

Fig. 6 Cell viability of co-cultured C6 cells after treatment with DIP, ABT-702, APCP, caffeine, MTX, and AMP for 24 h or 48 h. C6 cells were co-cultured with mononuclear cells and treated with MTX for 24 h (**a**) or 48 h (**b**), with or without previous exposure to DIP, ABT-702, APCP, or caffeine. Cell viability was evaluated by MTT assay, and the effect of 20 μ M AMP on treatment was evaluated. Data are shown as means $\pm$ SD, $n = 3$ and $n = 4$, respectively (** $p < 0.001$)

Inhibition of adenosine uptake or phosphorylation does not influence MTX-mediated cytotoxic effects

Given that MTX incubation decreased the viability of C6 cells (Fig. 3a) and led to an associated increase in CD73 enzyme expression (Fig. 3c–b), we hypothesized that MTX may exert its effects via a mechanism involving the adenosinergic system. To test this hypothesis, C6 glioma cells were treated with MTX alone or in combination with dipyridamole (DIP, an adenosine uptake inhibitor) and ABT-702 (an adenosine kinase inhibitor), as described in Table 2, for 24 h (Fig. 4a) or 48 h (Fig. 4b). Neither ABT-702 nor DIP alone modulated C6 cell viability, compared to DMSO, following 24 h and 48 h of incubation. In contrast, MTX alone significantly reduced cell viability at both time points, while DIP and ABT-702 were

unable to alter the cytotoxic effect mediated by MTX in GBM cells (Fig. 4a–b).

The cytotoxic effect of MTX on glioma cells is not altered by the adenosinergic modulation, even in the presence of high levels of AMP

To evaluate the intracellular and extracellular influence of adenosine in MTX-mediated effects, we exposed glioma cells to adenosinergic modulators, such as DIP, ABT-702, APCP (inhibitor of CD73), and caffeine (CAF, non-selective antagonist of P1 adenosine receptors), as described in Table 2, with or without MTX. In addition, given the upregulation of CD73 in glioma cells following MTX treatment, we looked at the effect of mimicking the high levels of ADO in the tumor

microenvironment [10] by evaluating the influence of exogenous AMP (20 μ M) on the above-mentioned treatments. Our hypothesis was that AMP in excess, combined with CD73 upregulation, would generate elevated adenosine, exacerbating the effects of MTX. MTX significantly reduced C6 cell viability, compared to DMSO, following 24 h (Fig. 5a) and 48 h incubation (Fig. 5b) to the same extent, both in the presence or absence of AMP. In addition, purinergic inhibitors did not modulate the cytotoxic effect of MTX (Fig. 5a–b).

The cytotoxic effect of MTX on glioma cells is not altered by adenosinergic modulation, even in the presence of mononuclear cells and exogenous AMP

In addition, to create a tumor-like microenvironment, we evaluated the influence of the co-culture of C6 with mononuclear cells on cell viability, after treatment with MTX, DIP, ABT-702, and APCP inhibitors and AMP, as described in Table 2, for 24 h and 48 h. Of note, after 24 h of treatment, we did not observe the inhibitory effect of MTX on glioma cell viability as shown in Fig. 5a, and we did not find a significant difference in cell viability, including in cells exposed to MTX (Fig. 6a). This result suggests that co-culture with mononuclear cells can decrease the cytotoxic effect of MTX, since in the presence of the same concentration and time of incubation, MTX was no longer cytotoxic (Fig. 6a) in C6 cells. However, after 48 h of treatment, MTX decreased the percentage of viable cells by approximately 70%, when compared to DMSO ($p < 0.001$) (Fig. 6b). Performance of co-culture in the presence of inhibitors did not alter the cytotoxic effect of MTX, and exogenous AMP did not alter cell viability, even in the presence of mononuclear cells (Fig. 6a–b).

Discussion

GBM is the most malignant and common brain tumor, and, despite intense efforts, there is still no cure for this neoplasia [4, 5, 31]. Several factors contribute to this condition, including tumor location, a high degree of invasiveness to adjacent tissues, presence of the blood-brain barrier, and high histological complexity. In addition, the lack of understanding of the pathophysiology of this disease is one of the limiting factors for its treatment [4, 32, 33].

The presence of the inflammatory infiltrate in the microenvironment of this neoplasm is directly related to the degree of malignancy [34]. It is well known that inflammation provides a favorable microenvironment for cancer progression. As such, the role of anti-inflammatory drugs, in this context, has been widely investigated. Silveira and collaborators [35], for example, showed that ketoprofen, administered in nanocapsules, reduced tumor growth and decreased the malignant characteristics of GBM when implanted in rat brains.

In addition, it has been demonstrated that this drug, loaded in nanocapsules or not, increases the activity of E-NTPDases and decreases the activity of adenosine deaminase in rat mononuclear cells. It has been suggested that the modulation of these enzymes by ketoprofen contributes to increase adenosine levels and, thus, may reduce inflammation associated with cancer and thereby reduce tumor growth [35].

Our research group has also studied the effect of anti-inflammatory drugs on GBM growth and the relationship of these molecules with the adenosinergic system. For example, Bernardi and collaborators studied the effect of indomethacin and showed an antiproliferative effect on GBM cells, in association with increased expression of the CD73 enzyme [17], the major enzyme responsible for producing adenosine in the extracellular milieu [36, 37]. Thus, it was hypothesized that indomethacin augments CD73 expression, in turn generating more adenosine in the extracellular environment, which then exerts its effects via the A3 receptor [17]. Different from the NSAIDs tested, indomethacin is an indole-aceto derivative and its structural difference may positively influence CD73 expression. Moreover, dexamethasone also reduced GBM cell growth and increased the expression of CD73 [22]. However, in this work, the mechanism mediating the effects of adenosine on cell growth was not explored [22]. In the present study, we show that different NSAIDs, at cytotoxic concentrations, reduced C6 cell viability, without any change in CD73 expression. Furthermore, when we tested NSAIDs at pharmacologically relevant concentrations, they did not alter CD73 expression in either mononuclear cells or T lymphocyte subsets (CD4⁺, CD8⁺, and CD4⁺CD25^{high}), indicating no association of CD73 expression with the potential anti-inflammatory mechanism of the drugs.

In this context, our aim was to first evaluate CD73 expression on C6 glioma cells and mononuclear cells after treatment with non-mechanism-related NSAIDs: naproxen, piroxicam, meloxicam, ibuprofen, diclofenac, acetylsalicylic acid, nimesulide, and ketoprofen. Our results did not indicate any changes in the expression of the CD73 enzyme at the concentrations of NSAIDs drugs and conditions tested.

In a study previously published by our group, we reported that methotrexate (MTX)-loaded lipid nanocapsules reduced GBM growth and increased the expression of CD73 enzyme in vivo [38]. It was hypothesized that the upregulation of CD73 and adenosine production, provided by MTX, induce greater immune suppression in the tumor microenvironment, reducing regulatory T lymphocytes, which play an important role in GBM progression, leading effector immune cells to be recruited [29, 38]. As the plasma and cytotoxic NSAIDs concentrations did not change CD73 expression, we then sought to investigate a role for adenosinergic mechanisms in MTX-induced CD73 upregulation and GBM cytotoxicity. In the

present work, we demonstrated that MTX treatment reduced cell viability by approximately 80%. In addition, MTX led to a significant increase in CD73 expression, corroborating previously published data [38]. In mononuclear cells, MTX did not modulate cells viability, possibly because MTX acts mainly on cells that are growing exponentially, such as cancer cells [28]. Previous studies have shown that MTX increases CD73 expression on mononuclear cells from tumor microenvironments [29, 38]. However, the results obtained in the present *in vitro* study showed that MTX did not directly affect CD73 expression on the surface of peripheral mononuclear cells, suggesting the need for multiple factors from the tumor microenvironment (such as high levels of TGF- β) for the regulation of CD73 expression on lymphocytes [39].

Subsequently, to investigate the role of the adenosinergic system in the cytotoxic effects of MTX on C6 cells, we evaluated the role of intracellular adenosine in the antiproliferative effect of MTX. For this, we treated C6 cells with dipyrindamole (DIP) and ABT-702, which block adenosine uptake [40] and inhibit adenosine kinase [41], respectively. The aim of associating DIP or ABT-702 with MTX treatment was to assess whether the inhibitors would reverse, at least in part, the cytotoxic effect of MTX. The treatment of C6 cells with DIP or ABT-702 did not significantly alter the cytotoxic effect of MTX. These results suggest that the cytotoxic effect of MTX on C6 cells is not related to an imbalance in intracellular nucleosides/nucleotides, under the aforementioned experimental conditions.

To verify the results obtained in relation to the intracellular role of adenosine and to assess the extracellular influence of adenosine on the cytotoxic effect of MTX, C6 cells were treated with DIP, ABT-702, APCP, or caffeine (CAF), in the presence or absence of exogenous AMP. Our results suggest that neither the CD73 inhibitor nor the adenosine antagonist was able to alter cell viability in the presence or absence of MTX at both times tested (24 h and 48 h). Moreover, treatment with AMP did not significantly alter the viability of the cells, showing that high levels of AMP, and consequently adenosine via upregulated CD73, do not alter the cytotoxic effect of MTX.

The GBM microenvironment is composed of great cellular variability, and immune cells have considerable importance in chemotherapy-related mechanisms [42, 43]. Based on this, we tried to reproduce a tumor microenvironment, to some extent, in our study model by coculturing C6 cells with mononuclear cells. Following coculture of the cells, and after treatment with DIP, APCP, ABT-702, AMP or CAF, and MTX, we found (after 24 h of treatment) that neither MTX nor the inhibitors significantly reduced the viability of C6 cells when cultured together with mononuclear cells. It is well known that mononuclear cells release growth factors, cytokines, and extracellular vesicles, among other factors, which can interact with cancer cells and may, therefore, decrease the

cytotoxic activity of MTX and/or modulate cancer cell growth for 24 h. In fact, the great cellular heterogeneity in the GBM microenvironment is one of the factors that has been associated with therapeutic failure [44, 45]. However, after a longer treatment period (48 h), MTX was able to significantly reduce the viability of C6 cells; furthermore, the addition of exogenous AMP and inhibitors/antagonists did not alter the viability of C6 cells either.

Conclusions

In conclusion, of the drugs tested in this study, only MTX was able to upregulate CD73 expression on the surface of GBM cells. However, the adenosinergic interventions with APCP, DIP, ABT-702, and CAF did not cause significant differences in the antiproliferative effect of MTX, suggesting that the adenosinergic system does not have any direct role in the cytotoxic effect induced by MTX in C6 glioma cells. Further studies are necessary to understand the involvement of the adenosinergic system in the mechanism by which MTX exerts its effects on GBM progression.

Authors' contributions The investigation was planned by D.V.L. and F.F. Cell culture experiments were performed by D.V.L., A.F.D., J.N.S., and L.F.L.S. with guidance from F.F. and A.M.O.B. The anti-rat CD73 antibody was donated by J.S. Data processing, and the writing of this manuscript was performed by A.F.D. and L.F.L.S. with input from all co-authors. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Daniela Vasconcelos Lopes declares no competing interests.

Amanda de Fraga Dias declares no competing interests.

Luiz Fernando Lopes Silva declares no competing interests.

Juliete Nathali Scholl declares no competing interests.

Jean Sévigny declares no competing interests.

Ana Maria Oliveira Battastini declares no competing interests.

Fabício Figueiró declares no competing interests.

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