

## Captopril inhibits Matrix Metalloproteinase-2 and extends survival as a temozolomide adjuvant in an intracranial gliosarcoma model

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### ABSTRACT

**Background:** Captopril is a well-characterized, FDA-approved drug that has demonstrated promise as a repurposed oncology therapeutic. Captopril's known anti-cancer effects include inhibition of Matrix Metalloproteinase-2 (MMP-2), an endopeptidase which selectively breaks down the extracellular matrix to promote cell migration. MMP-2 is a known therapeutic target in gliomas, tumors with significant clinical need. Using an aggressive gliosarcoma model, we assessed captopril's effects on MMP-2 expression in vitro and in vivo as well as its efficacy as an adjuvant in combination therapy regimens *in vivo*.

**Methods:** Following captopril treatment, MMP-2 protein expression and migratory capabilities of 9 L gliosarcoma cells were assessed *in vitro* via western blots and scratch wound assays, respectively. Rats were intracranially implanted with 9 L gliosarcoma tumors, and survival was assessed in the following groups: control; captopril (30 mg/kg/day); temozolomide (TMZ) (50 mg/kg/day), and captopril+TMZ. *In vivo* experiments were accompanied by immunohistochemistry for MMP-2 from brain tissue.

**Results:** *In vitro*, captopril decreased MMP-2 protein expression and reduced migratory capacity in 9 L gliosarcoma cells. In a gliosarcoma animal model, captopril decreased MMP-2 protein expression and extended survival as a TMZ adjuvant relative to untreated controls, captopril monotherapy, and TMZ monotherapy groups (27.5 versus 14 ( $p < 0.001$ ), 16 ( $p < 0.001$ ), and 23 ( $p = 0.018$ ) days, respectively).

**Conclusions:** Captopril decreases gliosarcoma cell migration, which may be mediated by reduction in MMP-2 protein expression. Captopril provided a survival advantage as a TMZ adjuvant in a rat intracranial gliosarcoma model. Captopril may represent a promising potential adjuvant to TMZ therapy in gliosarcoma as a modulator of the MMP-2 pathway.

### 1. Introduction

Gliomas are the most frequent primary tumors of the brain and central nervous system (CNS) in adults, comprising 27% of primary CNS tumors and 81% of all malignant brain tumors in the United States [1]. Of all gliomas, glioblastoma (GBM) is the most prevalent, comprising 47% of malignant CNS tumors with an incidence of 3.20 per 100,000

[1]. First-line treatment for newly diagnosed GBM is maximal tumor resection, followed by adjuvant radiotherapy, concomitant and maintenance temozolomide chemotherapy, potentially with the addition of implanted carmustine wafers [2–4]. Although advances in pharmacotherapy and drug delivery have been made, GBM remains highly treatment-resistant, with correspondingly poor prognosis, due to its infiltrative and heterogeneous nature [2,5–9].

**Abbreviations:** AngII, Angiotensin-II; ARBs, angiotensin-II receptor blockers; AT1R, angiotensin-II type-1 receptor; AT2R, angiotensin-II type-2 receptor; ACE, angiotensin-converting enzyme; ACEI, angiotensin-converting enzyme inhibitor; ACUC, Animal Care and Use Committee; ARRIVE, Animal Research: Reporting of *In Vivo* Experiments; BVZ, bevacizumab; CNS, central nervous system; DPBS, Dulbecco's Phosphate Buffered Saline; ECM, extracellular matrix; GBM, glioblastoma; MMP-2, Matrix Metalloproteinase-2; MMPs, matrix metalloproteinases; TMZ, temozolomide; TIMP-2, tissue inhibitor of metalloproteinases 2; TGF- $\beta$ , transforming growth factor; RAS, renin-angiotensin system; SEM, standard error; OS, overall survival.

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The infiltrative nature and invasive behavior of GBMs contribute to their recurrence, even in the midst of tremendous efforts such as hemispherectomy of the tumor-bearing hemisphere initially reported by Walter Dandy [10]. Interactions between tumor cells and the extracellular matrix (ECM) promote cell migration and play a pivotal role in glioma invasion, particularly with regard to degradation of ECM proteins [11]. Matrix metalloproteinases (MMPs) belong to a family of extracellular zinc-containing endopeptidases, which can selectively break down elements of the ECM. In particular, MMP-2 (also known as 72-kDa gelatinase-A) has been strongly associated with glioma cell migration, given that its activation leads to the disruption of the basement membrane protein collagen IV [12]. MMP-2 is synthesized as an inactive soluble zymogen that requires proteolytic cleavage to be activated. The activated MMP-2 enzyme can degrade the ECM, either directly or via association with integrins, promoting cell migration and invasion [13].

Since the 1990s, *in vivo* studies have described the upregulation of MMP-2 in human glioma. Sawaya et al. reported that MMP-2 enzyme levels in fresh glioma samples were correlated with malignancy phenotype, which increased tumor aggressiveness associated with higher levels of MMP-2 activity and microRNA [12]. MMP-2 expression has additionally been associated with shorter overall survival in patients with grade II-IV astrocytic tumors, and with poorer prognosis in patients with GBM who survived longer than 8.5 months, independent of age and gender [14]. MMP-2 may function in both ECM degradation and tumor angiogenesis, as its overexpression has been observed in both tumor and endothelial cells [15].

Sorbi et al. previously reported that captopril, a common angiotensin-converting enzyme (ACE) inhibitor (ACEI), was able to inhibit MMP-2 in a dose-dependent manner *in vitro* [16]. They demonstrated a reversal of this inhibitory effect by adding zinc, suggesting an underlying mechanism through which captopril inhibits MMP-2, and potentially delays glioma progression [16]. Since then, a few reports have presented further evidence supporting the notion of a possible role of this renin-angiotensin system (RAS) inhibitor in malignant gliomas from the perspective of MMP-2 inhibition [17–19].

In the present study, we evaluated the effects of captopril on MMP-2 expression *in vitro* and *in vivo*, and on survival *in vivo* in an aggressive murine intracranial gliosarcoma model commonly used in GBM research. We hypothesized that captopril treatment can decrease MMP-2 expression, both *in vitro* and *in vivo*, and enhance chemotherapy efficacy with a survival benefit in rats implanted with gliosarcoma tumors.

## 2. Methods

The Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [20] and the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee (ACUC) were followed in the design, analysis, and reporting of the present study.

### 2.1. Cell culture

9 L gliosarcoma cells were obtained from the Brain Tumor Research Center (University of California San Francisco, CA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in 5% CO<sub>2</sub>-humidified incubator, and tested monthly for mycoplasma contamination using Look Out Kit (Sigma-Aldrich, St. Louis, MO, USA). When appropriate, cells were treated with 100, 150, or 200 µM captopril (Sigma-Aldrich, St. Louis, MO, USA), or ultrapure water vehicle (Invitrogen, Carlsbad, CA, USA) for 24, 48, 72, and 96 h.

### 2.2. 9 L tumor xenografts

9 L gliosarcoma cells were implanted to form xenograft tumors and

subsequently passaged in the flank of Fisher 344 (F344) rats (Charles River Laboratories, Cambridge, MA, USA) every 2–3 weeks. For orthotopic xenograft experiments, 9 L gliosarcoma tumors were resected from the carrier animals, cut into 2 mm<sup>3</sup> pieces (approximately 500,000 cells), and placed in sterile 0.9% saline on ice before being surgically implanted intracranially as described below.

### 2.3. Western blot analysis

Cells treated with captopril or ultrapure water (control) were lysed using RIPA buffer, and western blots were performed using rabbit anti-rat MMP-2 [Gelatinase A; 72 kDa Type IV Collagenase] polyclonal antibody (1:1000, AB19015, MilliporeSigma, Burlington, MA, USA) as the primary antibody, and IgG HRP-linked Whole Ab (1:20000, GENA934, GE-Healthcare, Chicago, IL, USA) as the secondary antibody. Data were normalized to respective loading controls using an anti-GAPDH antibody (1:1000, 607902, BioLegend, San Diego, CA, USA), and three biological repeats were quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD, USA).

### 2.4. Migration/*in vitro* wound closure assay

The migration/wound healing assay was performed as described previously [21]. 9 L cells were seeded in 6-well plates and grown until confluent. Cell layers were scraped with a plastic pipette tip, washed with Phosphate Buffered Saline (Invitrogen, Carlsbad, CA, USA), and incubated to allow cells to migrate into the space cleared by the scratch. Using light microscopy, images of identical locations along each wound were acquired at 4-hour intervals. Cell migration was quantified as the average percent wound closure for three biological repeats of each condition (Control and Captopril 200 µM) using ImageJ.

### 2.5. Study animals

Female rats 8–12 weeks old (F344), weighing 125–175 g, were used for all studies. Animals were housed in standard facilities and allowed to eat and drink ad libitum. Their conditions were monitored on a daily basis. All animals were handled following the policies and guidelines of the Johns Hopkins University ACUC. Sample size was calculated using G\*Power version 3.1.9.2 software [22] with the following input parameters: Effect size f = 0.5, α err prob = 0.05, Power (1-β err prob) = 0.8.

### 2.6. Anesthesia

Rats were weighed and anesthesia was induced via intraperitoneal injection of 0.3 ml of a solution containing ketamine hydrochloride (75 mg/kg; 100 mg/ml), xylazine (7.5 mg/kg; 100 mg/ml), and ethanol (14.25%) in sterile saline solution.

### 2.7. Intracranial tumor model

F344 female rats were anesthetized before intracranial implantation. The head was shaved, and skin prepared with alcohol and Prepodyne solution (Patterson Veterinary, Saint Paul, MN, USA). A midline scalp incision was made, and skin edges were retracted to expose the sagittal and coronal sutures. Anatomical landmarks were identified, and a small burr hole was made in each rat skull centered 3 mm lateral to the sagittal suture and 5 mm caudal to the coronal suture, using an electric drill with a 2-mm cutting bur. A dural opening was made, exposing the cortical surface. A small volume of the cortex and underlying white matter were aspirated, and a single 2 mm<sup>3</sup> tumor fragment was implanted in the resection cavity. The skin incision was then closed with surgical staples, and animals were allowed to recover from anesthesia.

### 2.8. Efficacy study

Fifty rats were intracranially implanted with 9 L gliosarcoma tumor pieces, as described above. Rats were randomly divided into four experimental groups [1]: Control, no treatment ( $n = 17$ ) [2]; Oral captopril, 30 mg/kg/d beginning on day 5 ( $n = 17$ ) [3]; Oral Temozolomide (TMZ) (Merck & Co., Kenilworth, NJ, USA), 50 mg/kg on Days 5–9 ( $n = 8$ ) [4]; Oral TMZ plus Oral captopril, as dosed in the monotherapy groups ( $n = 8$ ). All drugs were dissolved in water, as described previously [23,24]. The maximum non-toxic oral dose of captopril (30 mg/kg/d), determined based on previous experiments, was administered daily via gastric gavage for ten days starting 5 days after tumor implantation [24]. TMZ treatment was given daily via gastric gavage on Days 5–9 after tumor implantation.

### 2.9. Immunohistochemistry

On day 0, 18 additional F344 rats (separate from the efficacy study) were implanted intracranially with 9 L gliosarcoma cells. Rats were divided randomly into two experimental groups [1]: Control ( $n = 9$ ); and [2] Oral captopril, 30 mg/kg/d ( $n = 9$ ). Beginning on day 5, each rat in the latter group received a daily dose of captopril via gastric gavage until they were euthanized. Three rats from each group were sacrificed, perfused with paraformaldehyde, and de-brained at three separate time points: Days 6, 8, and 11. Cryosections (10  $\mu\text{m}$ ) from each tumor site were prepared on paraffin-embedded slides for indirect immunofluorescence. All sections were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, St. Louis, MO, USA), and treated with blocking serum (10% normal goat serum in 0.1% Triton + DPBS). Slides were incubated overnight with a 1:100 dilution of matrix metalloproteinase-2 (MMP-2) mouse anti-rat primary antibody (AB86607, Abcam, Cambridge, MA, USA) in blocking serum. The slides were then incubated with goat anti-mouse secondary antibody. Intracellular MMP-2 was stained with Texas red to contrast with blue DAPI stains on cell nuclei. Images of all stained tumor sites were obtained using an Axio Observer Z1 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) and coded for blind analysis. After staining, 54 sample slides were obtained. Of 54 total slides, 49 were analyzed, excluding five slides due to poor quality. Three photographs at 20x amplification fields per slide were taken for counting. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to establish an MMP-2 expression threshold. All cells at or above that threshold were counted as positive for MMP-2 expression; those below were counted as negative. At least 1400 cells were counted per sample. A separate analyzer, blinded to the treatment groups, then decoded the data.

### 2.10. Statistical analysis

The primary outcome variable measured was survival, which was defined as the time to death from the day of tumor implantation (Day 0). Kaplan-Meier survival curves were generated, and experimental groups were compared using the Mantel-Cox test. An unpaired two-tailed *t*-test was conducted to determine the statistical significance of the MMP-2 positivity analysis, western blot quantification, and migration/scratch wound assay. Continuous data were displayed as mean  $\pm$  standard error (SEM). A *p*-value of  $<0.05$  was considered statistically significant for all comparisons. GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) was utilized for all statistical analyses.

## 3. Results

### 3.1. Captopril reduces MMP-2 protein expression in vitro

Given that captopril treatment can downregulate MMP activity in glioma cells [18], we first sought to assess the protein expression of MMP-2 in 9 L cells following 24, 48, 72, and 96 h of treatment with 100,

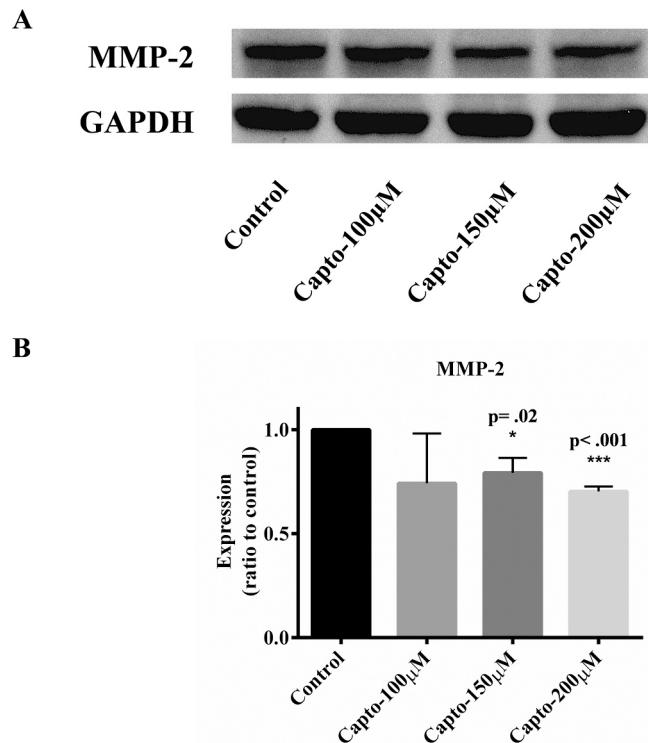
150, or 200  $\mu\text{M}$  of captopril. No change in MMP-2 protein expression was observed at any concentration at 24, 48, or 72 h (data not shown). However, at 96 h, MMP-2 expression was significantly decreased at both 150 and 200  $\mu\text{M}$  of captopril ( $p = 0.02$  and  $p < 0.001$ , respectively). Captopril therefore reduced MMP-2 protein expression in a dose-dependent manner (Fig. 1).

### 3.2. Captopril decreases 9 L gliosarcoma cell migration in vitro

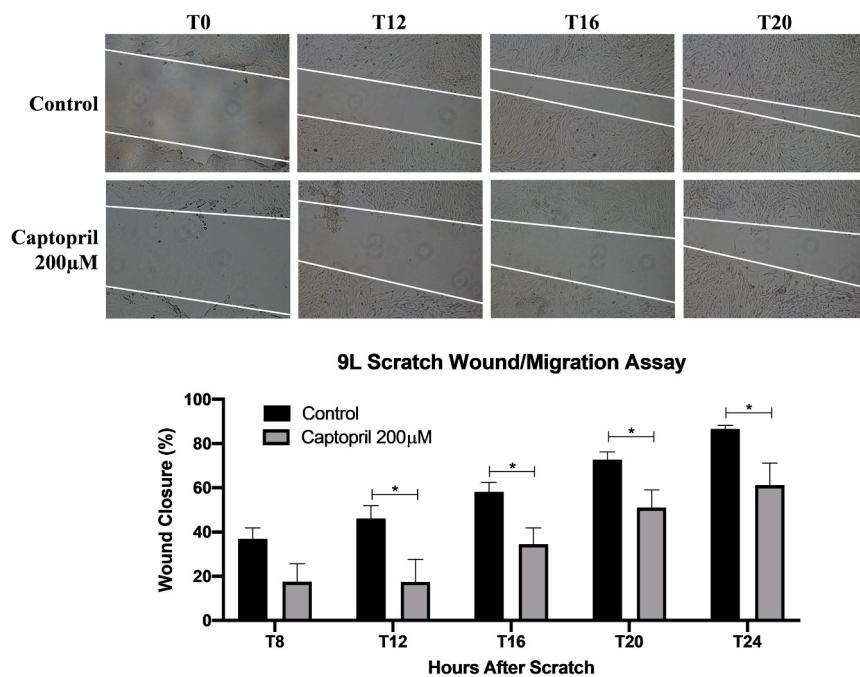
We next investigated the effects of Captopril on 9 L gliosarcoma cell migration. Scratch wound assays revealed that pre-treatment with 200  $\mu\text{M}$  of Captopril reduced the capability of 9 L gliosarcoma cells to migrate and close the wound created by a plastic P200 pipette. This reduction was statistically significant at all time points tested beyond 12 h post-scratch ( $p < 0.05$ ) (Fig. 2).

### 3.3. Captopril extends survival in a gliosarcoma model in vivo

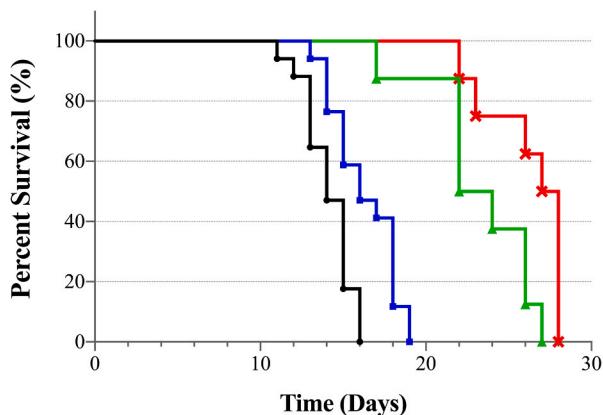
None of the animals in the efficacy study showed any signs of toxicity. The control group had a median survival of 14 days (range: 11–16 days), while the captopril group had a median survival of 16 days (range: 13–19 days,  $p = 0.001$ ). The TMZ group displayed a significant increase in median survival of 23 days (range: 17–27 days) compared to both the control group and the captopril group (both  $p < 0.001$ ). The TMZ plus captopril group demonstrated a significant survival benefit with a median survival of 27.5 days (range: 22–28 days) compared to the control, captopril-only, and TMZ-only groups ( $p < 0.001$ ,  $p < 0.001$ , and  $p = 0.018$ , respectively) (Fig. 3).



**Fig. 1.** Captopril treatment results in decreased MMP-2 expression. (A) Western blot analyses of MMP-2 expression in 9 L cells 96hrs after captopril treatment. A reduction in MMP-2 expression was observed at higher captopril concentrations while GAPDH (loading control) remained without changes at any captopril concentration. (B) Quantifications of Western blot analyses using Image J software. The ratio to control showed a significant reduction of MMP-2 expression at both 150 and 200  $\mu\text{M}$  of captopril (\* $p < 0.05$ , \*\*\* $p < 0.001$ , respectively,  $n = 3$ ).



**Fig. 2.** 9 L Scratch Wound/Migration Assay. Pre-treatment with Captopril (200  $\mu$ M) decreased the ability of 9 L gliosarcoma cells to close the wound created by a P200 pipette in the scratch wound/migration assay compared to control; the difference was significant from 12 to 24 h after the scratch (\* $p < 0.05$ ). White lines represent the cells' migrating front, and the space between the white lines represents the area cleared by the scratch wound and the space into which cells are migrating.



— Captopril  
— Control  
— TMZ  
\* Captopril and TMZ

**Fig. 3.** Increased survival of captopril treated rats implanted with 9 L gliosarcoma. Kaplan-Meier survival curves following intracranial implantation of 9 L tumors in rats and treatment with vehicle control (water, black curve,  $n = 17$ ), captopril (30 mg/kg, blue curve), TMZ (50 mg/kg, green curve,  $n = 8$ ), or TMZ and captopril (TMZ 50 mg/kg, captopril 30 mg/kg, red curve,  $n = 8$ ). Captopril led to a significant increase in median survival (16 days) compared with controls (14 days) ( $p = 0.001$ ). The combination of captopril and TMZ produced a significant increase in median survival (27.5 days) compared to TMZ alone (23 days) ( $p = 0.018$ ) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

### 3.4. Captopril reduces MMP-2 protein expression *in vivo*

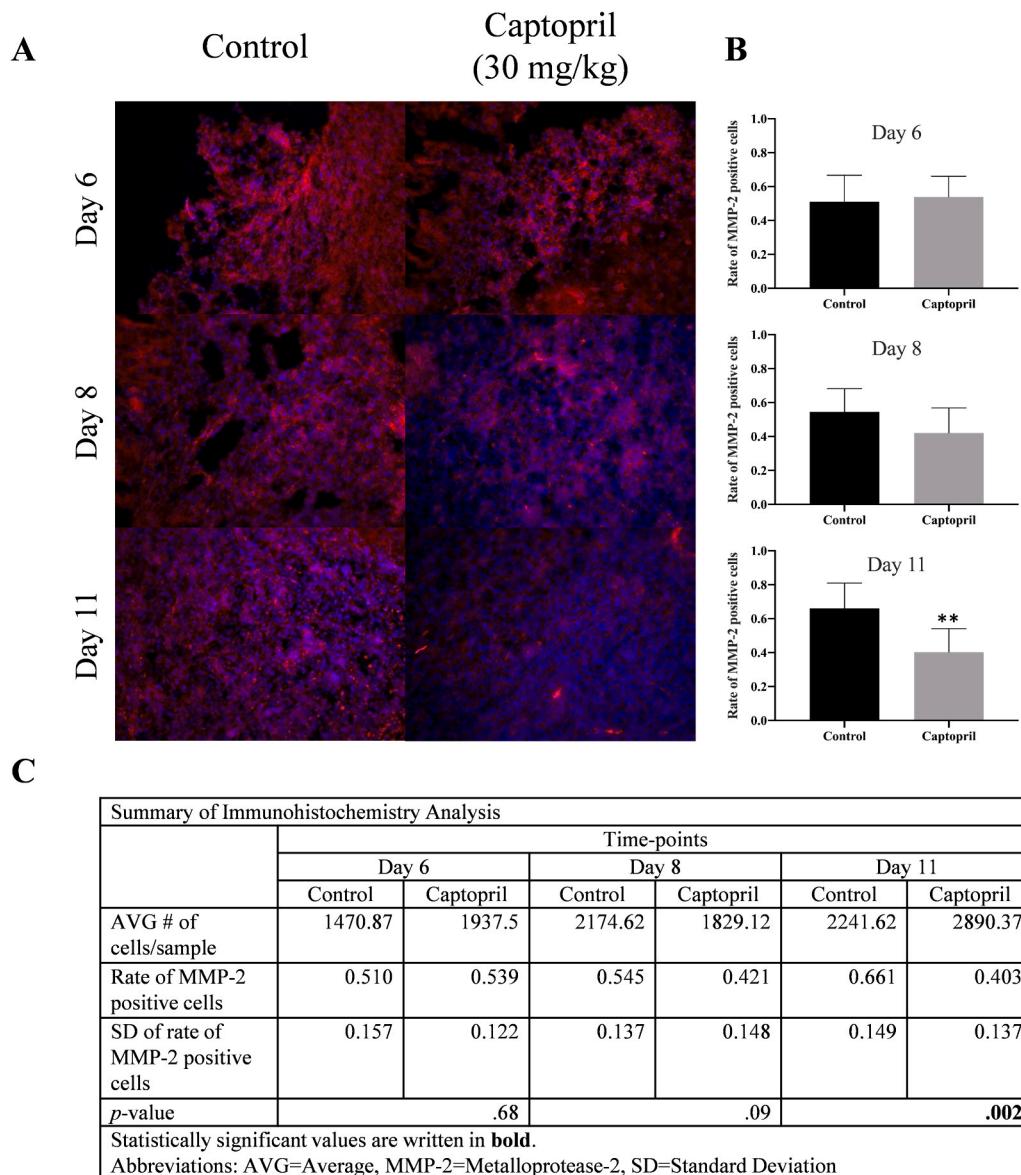
A total of 49 slides were analyzed, each rendering at least three random images. The rate of MMP-2-expressing cells was calculated by dividing the number of MMP-2 positive cells by the total of cells counted in each of the samples of the microscopic analysis. The average positivity was calculated for the control and captopril groups at the indicated days. A summary of the cell counting results is shown in Fig. 4.

Initially, on day 6 of captopril treatment, the rate of MMP-2 positive cells was similar between the control and the captopril groups (0.51 versus 0.53, respectively,  $p > 0.05$ ). However, the rate of MMP-2 positive cells progressively decreased in the captopril group following additional days of treatment (Days 8 and 11). In contrast, an increase in the cell positivity rate was observed in the control group, leading to significant differences between both groups. On Day 8, the rate of MMP-

2 positive cells was 0.54 for the control group and 0.42 for the captopril group ( $p = 0.09$ ). The difference further increased on Day 11, with a rate of 0.66 positive cells for the control group and 0.40 for captopril group ( $p = 0.002$ ) (Fig. 4).

### 4. Discussion

GBM is the most common primary malignant brain tumor in adults and represents a significant unmet clinical need in neuro-oncology. These tumors are recurrent, leading to poor prognosis, due in large part to their infiltrative and invasive nature. MMPs have been implicated in migratory, invasive, and angiogenic properties in GBM and represent a potential therapeutic target in this cancer. Recent evidence has demonstrated potential benefits of adjuvant cancer therapy with RAS inhibitors, including captopril, in part due to their MMP-



modulation capability [25]. Based on this rationale, we undertook the present study evaluating the effects of captopril on MMP-2 expression *in vitro* and *in vivo* as well as on survival *in vivo* in an aggressive murine intracranial gliosarcoma model commonly used in GBM research.

We demonstrate that captopril treatment significantly decreases the migratory capability of gliosarcoma cells *in vitro*. Most importantly, we demonstrate that captopril treatment *in vivo* resulted in a survival benefit as monotherapy while adjuvant captopril produced a robust survival benefit compared to control, captopril monotherapy, and TMZ monotherapy groups (27.5 days versus 14, 16, and 23 days,  $p < 0.001$ ,  $p < 0.001$ , and  $p = 0.018$ , respectively).

#### 4.1. Extended survival observed in the captopril monotherapy group suggests an underlying mechanism of the ACEIs independent of the captopril's TMZ potentiation

Our data implies that the captopril's ability to inhibit MMP-2 expression may be responsible in part for the observed survival benefit. The captopril treatment reduced MMP-2 expression *in vitro* and *in vivo*, validated through western blots (of 9 L cells) and immunohistochemistry (of brain tumor tissue from an intracranial rat gliosarcoma

**Fig. 4.** Decreased MMP-2 expression in tumor tissue after captopril treatment. (A) Representative photographs (20x amplification) of sections of rat brain stained 6, 8, or 11 days after 9 L tumor implantation and captopril treatment (30 mg/kg). Intracellular MMP-2 was stained using Texas red to contrast with blue DAPI nuclear stains. (B) There was a significant decrease in the percentage of MMP-2 positive cells in the captopril compared to controls following 11 days of treatment ( $**p < 0.01$ ). (C) Table demonstrating the average number and rate of MMP-2 positive cells in each treatment group on day 6, 8, and 11 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

model), respectively. MMP-2 is a key endopeptidase, which has been closely correlated with glioma invasiveness due to its activation and disruption of the basement membrane (specifically collagen IV). Der-yugina et al. [13] previously noted that the MMP-2 system promotes cell migration, and Ramachandran et al. [14] described that higher MMP-2 expression was directly related to a more aggressive histological grade and, therefore, a poorer prognosis in 89 astrocytoma patients, confirming a previous study with similar results by Jäälinöjä et al. [26].

Captopril's inhibition of the ACE/AngII/AT1R axis (ACE-mediated RAS inhibition) may also contribute to the therapeutic effect observed in our model. A few retrospective clinical studies showed an improvement in the overall survival (OS) of patients with GBM who were taking RAS inhibitors for non-cancer indications [27,28]. Januel et al. [27] found that functional independence, progression-free survival, and OS were significantly higher in patients on RAS inhibitors compared to controls. Levin et al. [28] reported similar effects of RAS inhibitors on OS in GBM patients who received cytotoxic therapy with bevacizumab (BVZ). Additionally, an OS advantage was identified in a subset of recurrent GBM patients taking low-dosage BVZ who were exposed to RAS inhibitors compared to controls. Although we hypothesize that the observed survival benefit of captopril monotherapy may be attributed to

a similar mechanism of action from the aforementioned studies, we note that those studies share a mutual limitation, which is that patients treated with ACEIs and angiotensin-II receptor blockers (ARBs) were pooled together in the survival analyses, since ACEIs and ARBs have different mechanisms underlying their antineoplastic effects [25]. A relevant difference in the mechanisms of action of ARBs versus ACEIs is the site of the ACE/AngII/AT1R axis where each drug acts. While ARBs selectively inhibit the angiotensin-II type-1 receptor (AT1R), ACEIs act upstream of this pathway and, consequently, inhibit both AT1R and the angiotensin-II type-2 receptor (AT2R). In pathologic situations, AT1R has been generally related to proliferative and angiogenic attributes while AT2R is associated with antiproliferative properties [25,29]. Therefore, we can infer that the net effect of the ACEIs, such as captopril, might be, at least in part, the sum of the inhibition of these angiotensin-II receptors.

#### 4.2. Prolonged survival in rats receiving combination of TMZ and captopril suggests that there exist additional pathways through which captopril augments therapeutic effects of TMZ

As an ACEI, captopril may enhance the therapeutic effect of TMZ by increasing tumor perfusion and decreasing tumor-related desmoplasia. These are particularly important to consider given our combination therapy data *in vivo* demonstrating that captopril enhanced the effects of TMZ, a central component of glioblastoma therapy (Fig. 5). Through ACE inhibition, captopril not only decreases the conversion of angiotensin-I to angiotensin-II, a potent vasoconstrictor, but also enhances bradykinin, an inflammatory mediator and potent vasodilator [30]. Under this premise, Zhang et al. [31] previously reported that captopril increased the expression of bradykinin, and consequently, increased tumor perfusion and the size of the endothelial gaps in the tumor vasculature of a U87 glioma model. Additionally, they found that captopril pretreatment provided an increase in the concentration of paclitaxel-loaded nanoparticles reaching tumor tissues, thus, promoting considerable tumor regression.

Apart from tissue permeability and blood vessel caliber, tumor perfusion can be obstructed by desmoplasia exerting compression on the tumor vasculature. This desmoplastic formation is mediated in part by the transforming growth factor (TGF)- $\beta$ , a key protein that has been associated with tumor growth, angiogenesis, and suppression of immune surveillance. TGF- $\beta$  also appears to modulate MMP-2 activity in malignant gliomas. As described by Wick et al. [32] TGF- $\beta$  simultaneously upregulates cell surface  $\alpha_v\beta_3$  integrin (resulting in greater activation and

expression of MMP-2) and downregulates TIMP-2 expression (a partial inhibitor of MMP-2), leading to enhancement of invasiveness of glioma cells. Captopril may decrease production of TGF- $\beta$  via upstream AT1R blockade and therefore provide an additional contributing factor to the decrease in MMP-2 expression that we observed in our results. Several studies [33–35] support this notion and also provide insight into mechanisms underlying captopril's ability to potentiate the effects of other therapies. Chauhan et al. [33] previously reported that losartan (an ARB) and, to a lesser extent, lisinopril (an ACEI, similar to captopril) reduce the production of collagen-I and hyaluronan in an orthotopic AK4.4 pancreatic tumor mouse model and consequently decreased tumor-related desmoplasia. They suggested that angiotensin-II inhibitors, acting via AT1R, might diminish the tumor interstitial matrix by reducing the expression of profibrotic signals (such as TGF- $\beta$ 1), thereby decompressing tumor vessels and increasing perfusion – and therefore chemotherapeutic drug delivery [33]. This captopril-mediated perfusion enhancement leading to better therapeutic effect has also been observed in radiotherapy, an oxygen-dependent treatment. An increase in intratumoral oxygen delivery following captopril treatment enhanced tumor radiosensitivity [34]. Moreover, given that TGF- $\beta$  is a contributor to radiation-induced fibrosis, captopril might additionally prevent radiation-induced brain damage by inhibiting TGF- $\beta$  production [35].

#### 4.3. Limitations

Although our study contributes to the recent literature exploring RAS inhibitors in gliomas, it is not without limitations. Further investigations are needed for comprehensive understanding whether captopril inhibits cell migration and extends survival in various GBM models in addition to the 9 L. Additionally, the mechanism of action of captopril in GBM should be further elucidated, with and without adjuvant TMZ. Future studies should further investigate the mechanisms underlying these beneficial effects in order to provide additional insight into GBM cancer biology and inform future preclinical and clinical research efforts.

#### 5. Conclusions

In the present study, we report increased survival using captopril as adjuvant therapy to TMZ in a rat intracranial gliosarcoma model. These effects may have been mediated in part by a reduction in MMP-2 expression and migratory capability of gliosarcoma cells. Additional studies are needed to elucidate the mechanism underpinning the adjuvant effect of captopril (or other RAS inhibitors) in combination with

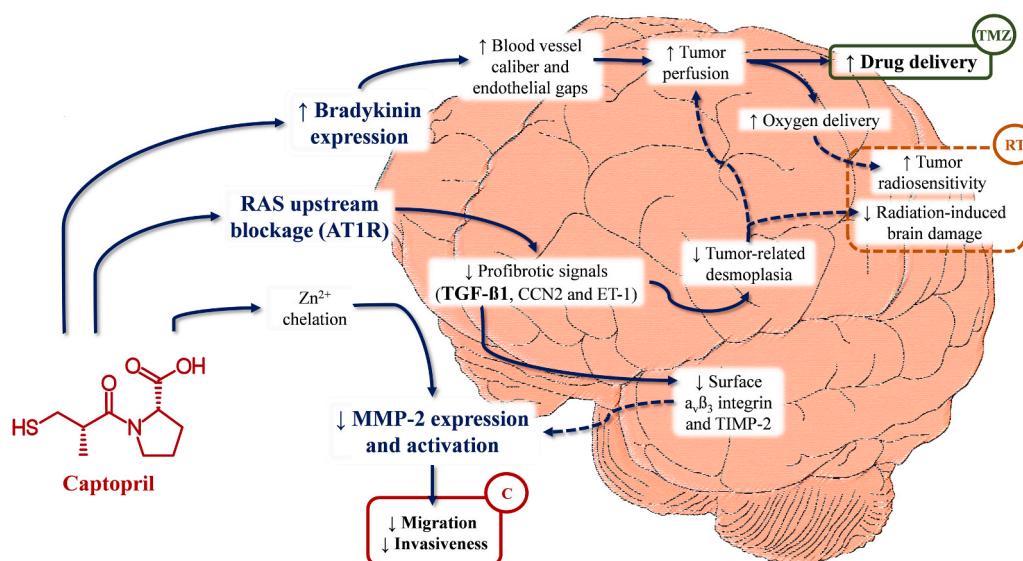


Fig. 5. Potential mechanisms of action of captopril in GBM. Schematic describing possible mechanisms of action of captopril in GBM.

TMZ for the treatment of GBM. Our findings represent a critical first step towards better understanding the potential beneficial effects of RAS inhibitors as GBM therapeutics.

## CRediT authorship contribution statement

- Leon Pinheiro:** Conceptualization, Methodology, Analysis, Writing.  
**Alexander Perdomo-Pantoja:** Conceptualization, Methodology, Analysis, Writing.  
**Joshua Casas:** Conceptualization, Methodology, Analysis, Review.  
**Sakibul Huq:** Methodology, Analysis, Writing, Review.  
**Iddo Paldor:** Methodology, Analysis, Review.  
**Veronica Vigilar:** Methodology, Review.  
**Antonella Mangraviti:** Methodology, Analysis, Review.  
**Yuan Wang:** Methodology, Review.  
**Timothy F. Witham:** Resources, Supervision, Funding Acquisition, Review.  
**Henry Brem:** Resources, Supervision, Funding acquisition, Review.  
**Betty Tyler:** Resources, Supervision, Funding acquisition, Project administration, Review.

## Declaration of interest

The authors declare no competing interests with respect to this work. Dr. Witham receives research support from the Gordon & Marylin Macklin Foundation and is a consultant for Depuy/Synthes Spine. He is also on the Medical Advisory Board and stock holder for Augmedics. Dr Henry Brem is a paid consultant to Insightec and chairman of the company's Medical Advisory Board. Insightec is developing focused ultrasound treatments for brain tumors. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict-of-interest policies. He also received research funding from NIH, Johns Hopkins University, and Acuity Bio Corp\* and philanthropy and he is a consultant for AsclepiX Therapeutics, StemGen, InSightec\*, Accelerating Combination Therapies\*, Catalio Nexus Fund II, LLC\*, LikeMinds, Inc\*, Galen Robotics, Inc.\* and Nurami Medical\*. Betty Tyler has research funding from NIH and is a co-owner for Accelerating Combination Therapies\*. Ashvattha Therapeutics Inc. has also licensed one of her patents (\*includes equity or options).

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