Inhibition of the angiotensin II type 2 receptor AT2R is a novel therapeutic strategy for GBM

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Introduction and Aims

- Glioblastoma (GBM) is an aggressive primary malignant brain tumour with poor clinical outcomes
- Standard of care consists of surgical debulking followed by radiation and temozolomide, but the tumour invariably recurs, and median survival is only ~14 months[1]
- Repurposing drugs used for the treatment of other diseases is a promising avenue to identify novel treatments for this highly aggressive form of cancer
- One such class of compounds is angiotensin II (AngII) receptor blockers, commonly used to control blood pressure
- Whilst there is a clear oncogenic role for the angiotensin II type 1 receptor (AT1R) in many forms of cancer[2], the role of AT2R is poorly understood, particularly in GBM
- Here, we aimed to characterise the expression of AT2R in primary models of GBM and determine the effects of agonists and antagonists both *in vitro* and *in vivo*

Materials and Methods

- GBM cell lines cultured in DMEM or DMEM/F12 with 10% FBS at 37°C and 5% CO_2
- Expression of AT2R in primary (patient derived) and established GBM cell lines determined by qPCR
- Proliferation assays (SRB and CCK8) to determine the effects of agonists and antagonists of AT2R
- CRISPR/Cas9 mediated knockdown of AT2R to determine specificity of AT2R antagonists
- Spheroid culture using Matrigel to determine the effects of AT2R antagonists in a 3D model, and the effects on cell migration and invasion
- A novel, blood-brain-barrier (BBB) penetrable formulation of the AT2R antagonist EMA401 conjugated to angiopep-2 (A3E) was chemically synthesised for *in vivo* work
- Both subcutaneous and intracranial models of GBM using U87-GFP/luc cells in BALB/C nude mice treated with TMZ, EMA401 and A3E
- Bioluminescent imaging performed using the Lumina III In Vivo Imaging System
- Immunohistochemistry for Ki67, integrin β3 and TUNEL staining to measure proliferation, angiogenesis and apoptosis respectively
- Graphs generated using GraphPad Prism (ver. 9.4.1)

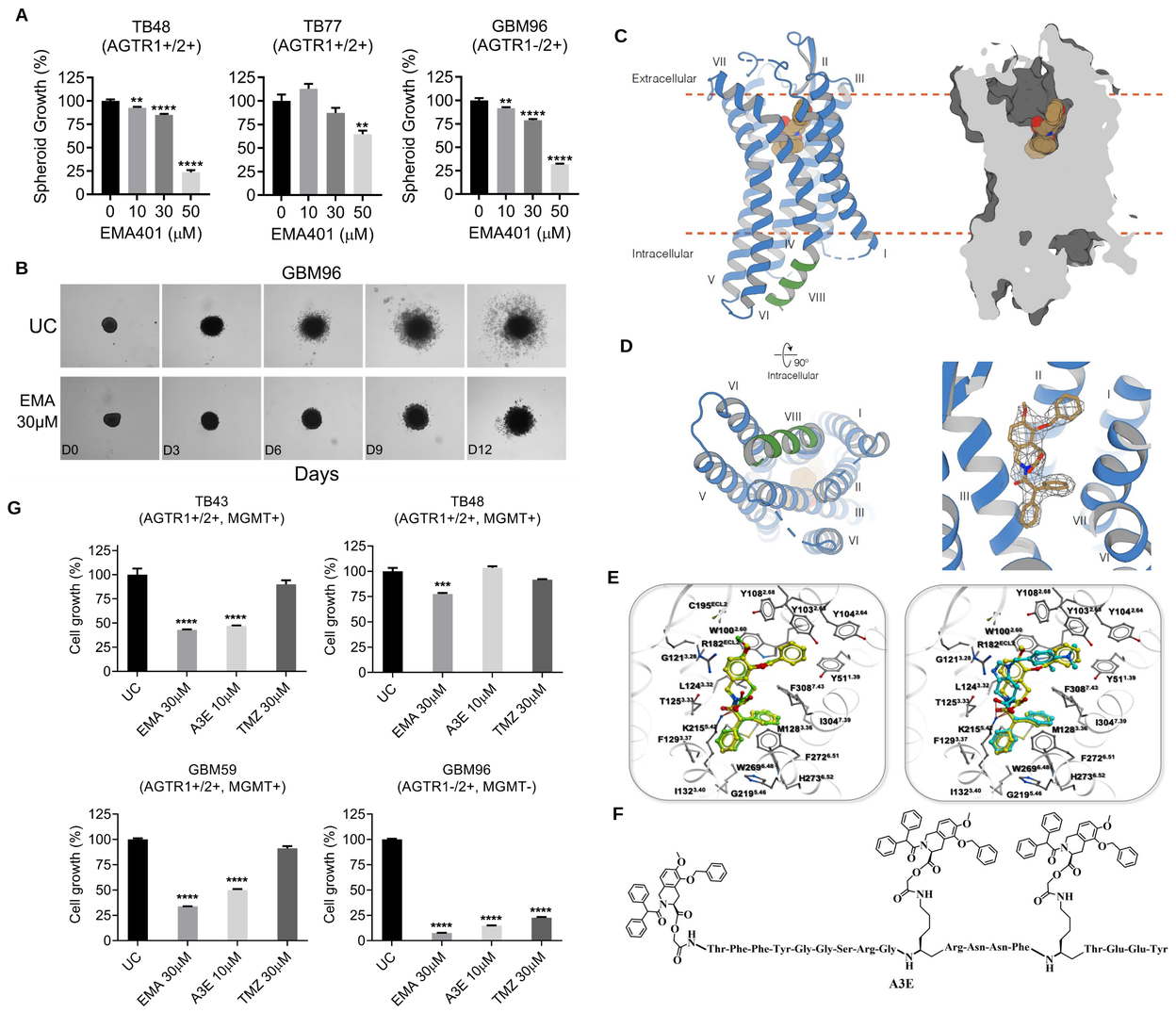

Figure 2. A, CCK8 data from primary GBM cultures grown as spheroids treated with increasing concentrations of EMA401. **B,** images showing the invasion of primary GBM spheroids in to Matrigel treated with EMA401. **C,** overall view of AT2R with the ligand EMA401 in the binding pocket. **D,** intracellular view showing how helix VIII (green) blocks G protein and β-arrestin binding site, and the electron density (grey mesh) of EMA401 in the binding pocket. **E,** predicted docking pose and contacts of EMA401 and PD123,319 in the AT2R binding pocket. **F,** chemical structure of A3E

Results

- In a subcutaneous model of GBM, A3E significantly reduced tumour burden compared to native EMA401, with similar potency to TMZ (**fig. 3A**)
- In an intracranial model of GBM, A3E significantly inhibited tumour growth in a time-dependent manner (**fig. 3B, C**)
- In intracranial tumours, A3E reduced the levels of proliferation and angiogenesis, as shown using Ki67 (**fig. 3D**) and integrin β3 (**fig. 3E**) staining
- A3E also increased the levels of apoptosis, as demonstrated by TUNEL staining (**fig. 3F**)

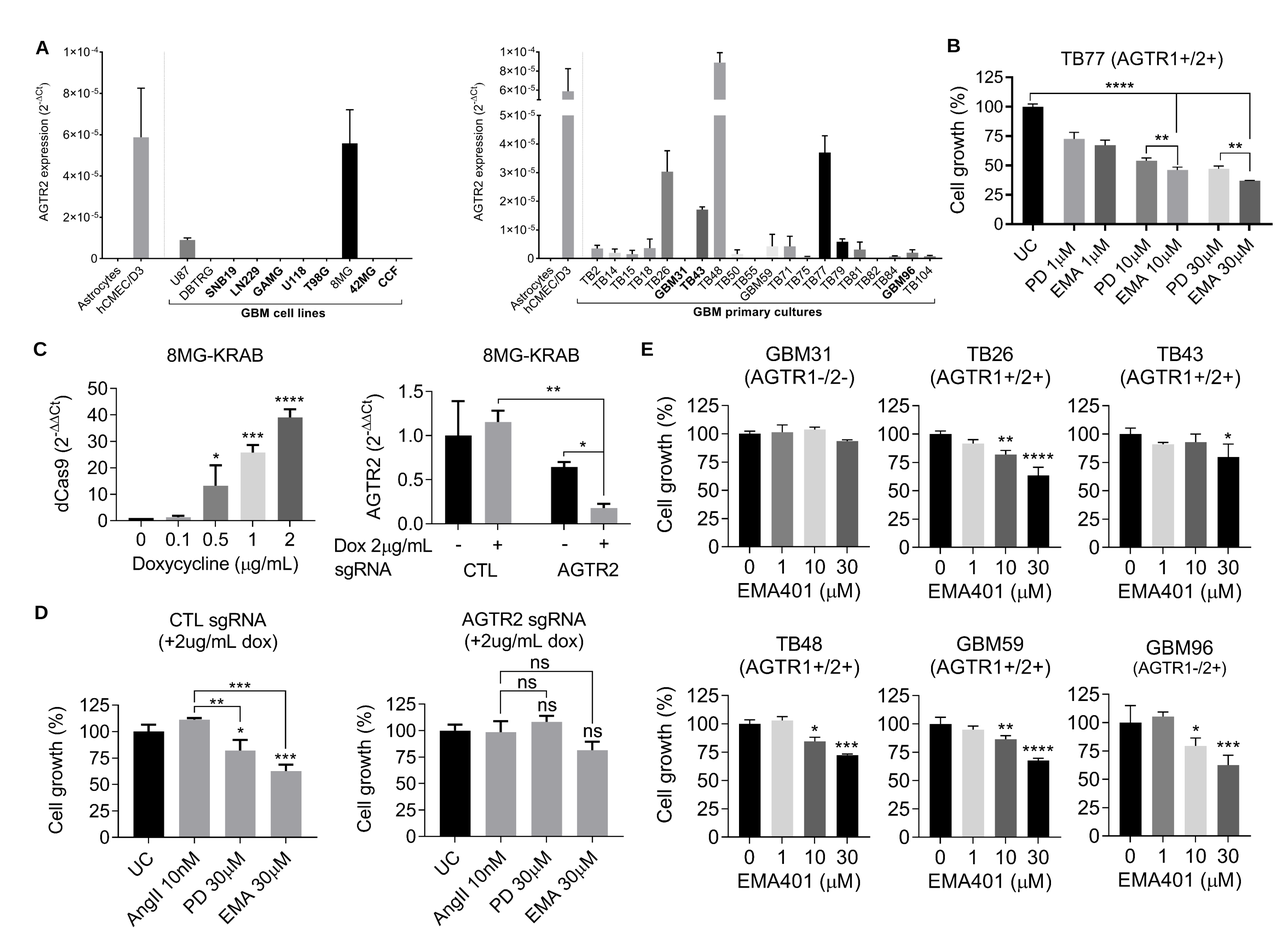

Figure 1. A, qPCR data showing *AGTR2* expression across established and primary GBM cell lines. **B,** proliferation assay showing the effects of two different AT2R antagonists, PD123,319 and EMA401, in a primary GBM cell line. **C,** qPCR data showing the expression of *dCas9* and *AGTR2* in 8MG-dCas9-KRAB lines with and without doxycycline. **D,** proliferation assay showing the effects of AT2R agonists and antagonists in 8MG-dCas9-KRAB lines with either non-targeted control guide RNA's (CTL) or guide RNA's targeting *AGTR2*. **E,** proliferation assay showing the effects of increasing concentrations of EMA401 on primary GBM cell lines.

Results

- *AGTR2* expression is lost in established GBM cell lines, but retained in primary patient derived lines (**fig. 1A**)
- The AT2R antagonists PD123,319 and EMA401 inhibit proliferation in primary GBM cells (**fig. 1B**)
- CRISPR-Cas9 mediated knockdown of *AGTR2* (**fig. 1C**) demonstrates that the effects of PD123,319 and EMA401 are specifically dependent on AT2R (**fig.1D)**
- EMA401 inhibits proliferation in primary GBM cells in both 2D (**fig. 1E**) and 3D (**fig. 2A**) models
- EMA401 also blocks migration and invasion of primary GBM cells into extracellular matrix (**fig. 2B**)
- The structure of EMA401 complexed with AT2R was elucidated (**fig 2C, D, E**) which aided in the design of a BBB penetrable formulation, consisting of three molecules of EMA401 conjugated to angiopep-2 (**fig. 2F**) which has previously been used to deliver drugs across the BBB[3]
- A3E retained its potency against primary GBM cells *in vitro* (**fig. 2G**)

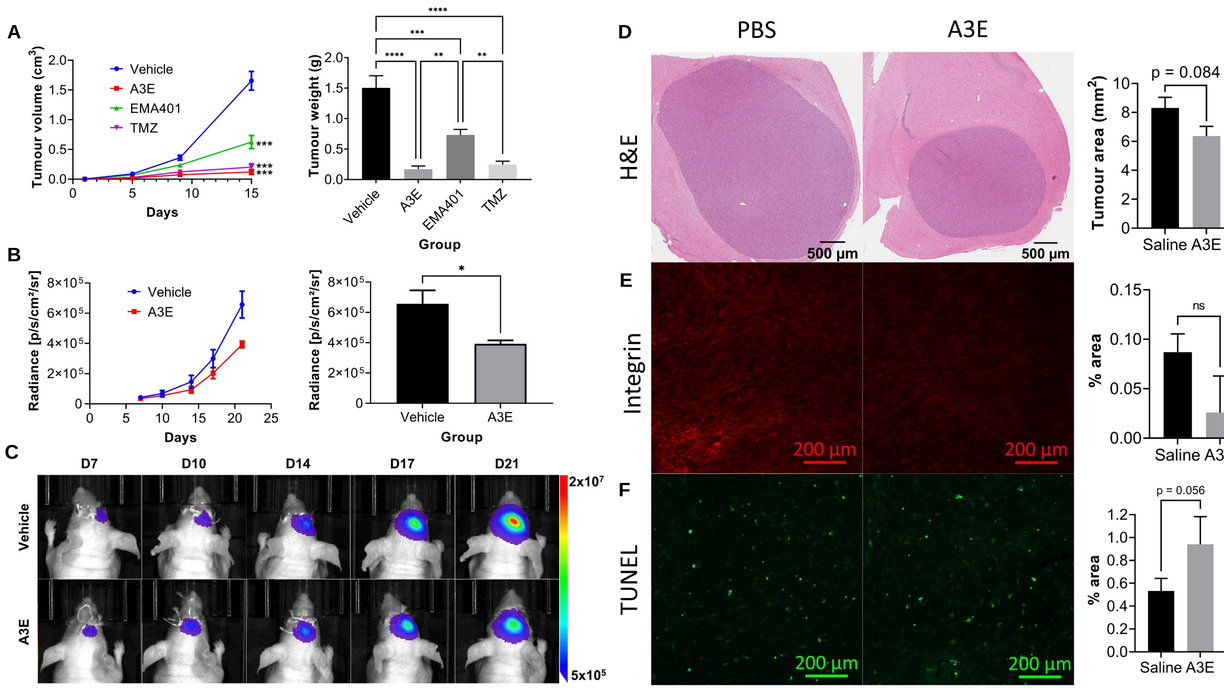

Figure 3. A, tumour volume and weight from U87 tumours treated with EMA401, A3E and TMZ. **B,** bioluminescent signal from intracranial U87-GFP/luc tumours treated with A3E. **C,** representative images of the bioluminescent signal of mice treated with A3E or vehicle. **D,** H&E, **E,** integrin β3 and **F,** TUNEL staining in sections from intracranial U87-GFP/luc tumours treated with A3E or vehicle

Conclusions

- Inhibition of AT2R using EMA401 in primary GBM leads to a decrease in proliferation in both 2D and 3D models
- EMA401 was conjugated to angiopep-2 to create a novel, targeted, BBB-penetrable formulation of EMA401, which we dubbed A3E
- In an intracranial model of GBM, inhibition of AT2R with A3E lead to decreases in tumour burden, proliferation and angiogenesis, as well as an increase in apoptosis

References

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2. Perdomo-Pantoja, A., et al., Renin angiotensin system and its role in biomarkers and treatment in gliomas. *J Neurooncol*, 2018. 138(1): p. 1-15.
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