Authors

Ricardo Gargini[1*†] Berta Segura-Collar[1†], María Garranzo-Asensio[1], Rafael Hortigüela[1,2], Daniel Lobato-Alonso[1], Miguel Moreno-Raja[3], Santiago Esteban[3], Juan M. Sepúlveda-Sánchez[4], Laura Nevola[3*], and Pilar Sánchez-Gómez[1*]

Title

IDP-410: a novel therapeutic peptide that blocks N-MYC over-stability and reduces angiogenesis and tumor progression in glioblastomas

Affiliations

[1]Neurooncology Unit, Instituto de Salud Carlos III-UFIEC, Madrid, Spain.

[2]Current address: Centro de Investigaciones Biológicas Margarita Salas-CSIC, Madrid, Spain

[3]IDP Discovery Pharma S.L., Barcelona, Spain

[4]Instituto de investigaciones Biomédicas I+12, Hospital 12 de Octubre, Madrid, Spain.

† These authors contributed equally

***To whom correspondence should be addressed:**

Ricardo Gargini, rgargini@isciii.es

Pilar Sanchez-Gomez, psanchezg@isciii.es

Laura Nevola, lnevola@idp-pharma.com

Article type: original research

Competing interests: Laura Nevola and Santiago Esteban are co-founders and shareholders at IDP Discovery Pharma. This work was supported in part by IDP Discovery Pharma funds.

Running title: N-MYC inhibition blocks glioma growth

Summary (150-250 words)

Glioblastomas (GBMs) are the most frequent and highly aggressive brain tumors, being resistant to all cytotoxic and molecularly targeted agents tested so far. There is, therefore, an urgent need to find novel therapeutic approaches and/or alternative targets to bring treatment options to patients. Here, we first show that GBMs express high levels of N-MYC protein, a transcription factor involved in normal brain development. A novel stapled peptide designed to specifically target N-MYC protein monomer, IDP-410, is able to impair the formation of N-MYC/MAX complex and reduce the stability of N-MYC itself. As a result, the viability of GBM cells is compromised. Moreover, the efficacy is found dependent on the levels of expression of N-MYC. Finally, we demonstrate that IDP-410 reduces GBM growth in vivo when administered systemically, both in subcutaneous and intracranial xenografts, reducing the vascularization of the tumors, highlighting a potential relationship between the function of N-MYC and the expression of mesenchymal/angiogenic genes. Overall, our results strengthen the view of N-MYC as a therapeutic target in GBM and strongly suggest that IDP-410 could be further developed to become a first-in-class inhibitor of N-MYC protein, affecting not only tumor cell proliferation and survival, but also the interplay between GBM cells and their microenvironment.

Keywords N-MYC, Peptide Inhibitors, protein stability, Glioblastoma, glioma, tumor microenvironment and angiogenesis.

Introduction

Glioblastoma (GBM), classified as a grade IV astrocytoma, is one of the most aggressive forms of cancer, as well as the most frequent malignant primary tumor of the brain [1]. Surgical removal of the tumor followed by radiotherapy and chemotherapy with temozolomide is the common GBM treatment. However, the invasiveness and proliferation rate of tumor cells, as well as their high resistance to conventional therapies favor the recurrence of GBM, leading to the death of the patients in 15 to 20 months after the first diagnosis [2-4]. The development of new types of drugs, especially those capable of reaching the brain, is necessary to enhance the survival of these patients, who have not seen a therapeutic improvement in the last decades. In this sense, the development of small molecules to inhibit the function of transcription factors (TF), as is the case of inhibitors of Brd4 (Bromodomain containing 4), STAT3 (Signal transducer and activator of transcription 3), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) or MYC, is a major focus of interest in different cancers [5].

The MYC family of oncogenes, which includes *c-MYC*, *L-MYC* and *N-MYC*, are dysregulated in many neoplasias, associated with poor patients' prognosis [6, 7]. MYC proteins regulate numerous processes such as cell growth, differentiation and apoptosis, normally through dimerization with MYC-associated protein X (MAX) and formation of a functional transcriptional activator. However, MYC may also bind MYC interacting zinc finger 1, SP1 and other co-factors to repress gene expression [8]. The transcriptional output signature of MYC proteins is highly dependent on the cellular context. L-MYC and N-MYC are distinctly expressed in specific tissues (lung and neuronal tissue, respectively), whereas c-MYC is ubiquitously expressed. *c-MYC* and *N-MYC* can replace each other during normal development [9] or in cancer, where they often show mutually exclusive expression patterns [10] [11].

A two-fold rise in MYC levels suffices to affect cell cycle progression, leading to cancer [12], so the expression of this TF is tightly regulated. Proliferating cells allow for MYC stabilization at a protein level, but quiescent cells degrade the protein through the ubiquitin degradation pathway, a process that depends on the phosphorylation status of MYC proteins [13]. Indeed, overexpression of N-MYC harboring a mutation at threonine 58 that leads to protein stability can originate cerebellar medulloblastomas as well as forebrain gliomas [14]. Direct amplification or overexpression of *MYC* genes have also been found in human brain tumors, including pediatric gliomas, adult GBM and medulloblastomas [13, 15-19]. Moreover, different oncogenic alterations promote the tumorigenicity of glioma stem cells through c-MYC stabilization [20, 21].

MYC proteins are considered undruggable because they belong to the internally disordered proteins (IDPs) group, which lack a stable 3D structure and exist instead as ensembles of rapidly interchanging conformations [22]. On top of that, their intra-nuclear localization supposes an additional challenge. Still, many efforts have been made over the last years to target MYC genes to treat different cancer types, including inhibiting MYC transcription or transcriptional activity, protein stability, dimerization, immune therapy, and synthetic lethality [23-27]. These have allowed establishing the important role of N-MYC in medulloblastomas and neuroblastomas [13]. However, specific downregulation or inhibition of this gene has not been tested yet in GBMs.

In this work, we confirm the strong expression of N-MYC in human GBM samples and mouse models. Notably, no increase is observed in the amount of *MYCN* mRNA. Moreover, we describe the anti-glioma effect of IDP-410, a stapled peptide specifically designed to target N-MYC. By using primary glioma cell lines, we demonstrate that this novel inhibitor reduces the viability of those cells expressing

high levels of this TF. IDP-410 disrupts the N-MYC-MAX complex, reducing the stability of N-MYC protein and the amount of nuclear staining. Systemic treatment with IDP-410 reduces heterotopic and orthotopic glioma growth by attenuating the vascularization of the tumors. Our results show a potential relationship between the function of N-MYC and the expression of mesenchymal/angiogenic genes. Overall, the results presented here suggest that IDP-410 could be a first-in-class inhibitor of N-MYC in GBM, affecting the interplay between the tumor cells and their microenvironment.

Materials and Methods

Human samples

Normal tissue (NT) was obtained postmortem from non-pathological brain samples. NT and glioma samples (fresh frozen or embedded in paraffin) were obtained after patient´s written consent and with the approval of the Ethical Committee at Hospital 12 de Octubre (Madrid, Spain) (CEI 14/023 and CEI 18/024). The primary cell lines (Supplementary Table S1) were generated as previously described [28]. They belong to the Biobank of Hospital 12 de Octubre. They were maintained in stem cell medium (Neurobasal (Invitrogen) supplemented with B27 (1:50) (Invitrogen); GlutaMAX (1:100) (Invitrogen); penicillin-streptomycin (1:100) (Lonza); 0.4% heparin (Sigma-Aldrich); and 40 ng/ml EGF and 20 ng/ml bFGF2 (Peprotech)) and passaged after enzymatic disaggregation using Accumax (Millipore).

IDP-410 synthesis and characterization.

The linear peptide was synthesized with automatic synthesizer using 9-fluorenylmethoxycarbonyl/tertbutyl solid phase peptide chemistry. Ring-closing metathesis reaction was performed in solution with a first-generation Grubbs catalyst

after cleaving the linear peptide from the resin, as previously described (Scott J.M. et al., "Application of Ring-Closing Metathesis to the Synthesis of Rigidified Amino Acids and Peptides",1996, J. Am. Chem. Soc., 1996, 118 (40), pp 9606–9614). The compound was purified by HPLC-RP (C-18 column; Pump A: H_2O with 0,1% TFA; Pump B Acetonitrile with 0,1% TFA) using a linear gradient 44%-54% of B in 15 minutes (purity grade 95% by HPLC) and identified by ESI-MS.

In vitro treatments

The stock solution of IDP-410 (IDP Discovery pharma) was prepared at 10mM in PBS. For viability assays, 5000 cells were seeded in triplicate wells of a 96-well microplate coated with matrigel (Bekton-Dickinson, 15mg/ml stock solution diluted 1:100 in DMEM medium (Lonza)). 24h later, cells were treated with IDP-410 (at the indicated concentrations in each assay) and viability was measured after 72h of treatment. For that, cells were incubated with Hoechst 33342 (1:200, Sigma-Aldrich) and propidium iodide (1:1000, Merck) and fluorescence was measured in a Cytell Cell Imaging System (GE Heatlhcare Life Sciences). Wells containing non-treated cells were considered as 100% viability for each tested cell line.

For the N-MYC degradation experiment, GBM6 and GBM7 cells were grown in presence of DMSO or MG132 (Millipore) 10 µM for 1h and then the cells were incubated with IDP-410 (10 µM) for 6h. Then, cells were collected and lysed and subsequently analyzed by western blot, as described below.

Wound healing assay

HBMEC cells were seeded at a density of 5×10^5 cells per well into 24-well flat-bottom microplates and incubated in 10% FBS-supplemented DMEM until sealed. Then, the wound was performed by scratching across the bottom of the well using a pipette tip. Medium was removed and cells were washed with PBS prior to adding the conditioned medium (CM). Glioma CM was obtained by incubating GBM6 cells

for 48h in presence of 5μM IDP-410 or PBS. After 48 h, the medium was removed and replaced with fresh starving medium for 6h. Then, CM was collected, briefly spun and the supernatant was used for the wound-healing assays. To calculate wound-closing rate, pictures were taken at different times and wound area was measured as square pixels using ImageJ.

In vivo assays

Animal experiments were reviewed and approved by the Research Ethics and Animal Welfare Committee at our institution (Instituto de Salud Carlos III, Madrid) (PROEX 244/14 and 02/16), in agreement with the European Union and national directives. For subcutaneous transplantations, cells (3×10^6) were resuspended 1:1 in culture media and Matrigel (BD) and then subcutaneously injected into athymic nude Foxn1nu mice (Harlan Iberica). When the tumors reached a visible size, we started measuring them with a caliper. At that point, animals started receiving IDP-410 at 15 mg/kg through intravenous (i.v) injections (twice a week). Control animals were treated with the PBS solvent. 2h before sacrifize, the animals received intraperitoneal injections of 5-Bromo-2′-deoxyuridine (BrdU) (Sigma Aldrich) (50mg/Kg) in saline solution.

To establish the intracranial tumors, we injected 100.000 GBM6 cells (resuspended in 2 μl of culture stem cell medium) with a Hamilton syringe into Nude mice. The injections were made into the striatum (coordinates: A–P, −0.5 mm; M–L, +2 mm, D–V, −3 mm; related to Bregma) using a Stoelting Stereotaxic device. Two weeks after the tumor implantation, we started treating the animals. For that, IDP-410 was dissolved in PBS+1% Polysorbate and injected subcutaneously (15/mg/Kg/day). Control animals were treated with this solvent. Tumor growth was monitored in an IVIS equipment (Perkin Elmer) after intraperitoneal injection of D-luciferin (75 mg/kg;

PerkinElmer). Animals were sacrificed when they showed signs of disease and the brains were processed for cellular and molecular analysis.

Quantitative reverse-transcriptase PCR (qRT-PCR)

RNA was extracted from the tissue or the cell pellets using RNA isolation Kit (Roche) and it was digested with DNase I (Roche) according to the manufacturer's instructions. cDNA was synthesized with SuperScript II Reverse Transcriptase (Takara). Quantitative real time PCR (qRT-PCR) reactions were performed using the Light Cycler 1.5 (Roche) with the SYBR Premix Ex Taq (Takara). The primers used for each reaction are indicated in Supplementary Table S2. Gene expression was quantified by the double delta Ct method.

Inmunofluorescent (IF) and Inmunohistochemical (IHC) staining and quantification

Tumor tissues were embedded in paraffin and cut with a microtome. The slides were heated at 60ºC for 1 hour followed by deparaffinization and hydration, washed with water, placed into antigen retrieval solution (pressure-cooking) in 10 mM sodium citrate pH 6.0. Paraffin sections were permeabilized with 1% Triton X-100 (Sigma-Aldrich) in PBS and blocked for 1 hour in PBS with 5% BSA (Sigma), 10% FBS (Sigma) and 0,1% Triton X-100 (Sigma). For the detection of ki67 or BrdU, sections were incubated with pre-heated 2N HCl for 15 min, followed by 10 min incubation with 0,1 M sodium borate pH 8,5, and then continued with the blocking solution as described above. The following primary antibodies (Supplementary Table S3) were incubated O/N at 4ºC. The second day, sections were washed with PBS three times prior to incubation with the appropriate secondary antibody (Supplementary Table S3) for 2h at room temperature. Prior to coverslip application, nuclei were counterstained with DAPI. Imaging was done with Leica SP-5 confocal microscope.

Otherwise, IHC sections were incubated with biotinylated secondary antibodies (1:200 dilution). Target proteins were detected with the ABC Kit and the DAB kit (Vector Laboratories).

For quantification, slides were scanned at 63X or 40X magnification. The number of BrdU-positive cells per field was counted with Fiji-ImageJ software and normalized with the total number of cells. For the quantification of the vasculature, we counted the number of dilated vessels per field, substrated from endomucin IHC.

Western Blot (WB) analysis

Protein extracts were prepared by re-suspending cell pellets or tumor tissue samples in lysis buffer (50 mM Tris (pH 7.5), 300 mM NaCl, 0.5% SDS, and 1% Triton X-100) and incubating the cells for 15 min at 100º. The lysed extracts were centrifuged at 13,000 g for 10 min at room temperature and the protein concentration was determined using a commercially available colorimetric assay (BCA Protein Assay Kit). Approximately 30 µg of protein were resolved by 10% or 12% SDS-PAGE and they were then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). The membranes were blocked for 1 h at room temperature in TBS-T (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20) with 5% skimmed milk, and then incubated overnight at 4ºC with the corresponding primary antibody (Supplementary Table S3) diluted in TBS-T. After washing 3 times with TBS-T, the membranes were incubated for 2 h at room temperature with their corresponding secondary antibody (Supplementary Table S3) diluted in TBS-T. Proteins were visible by enhanced chemiluminiscence with ECL (Pierce) using Amersham imager 680 and the signal was quantified by Fiji-ImageJ software.

Immunoprecipitation

GBM6 cells were incubated in the presence of DMSO or IDP-410 (5µM) for 6h. Then, the cells were collected, washed in PBS and the protein extracts were generated in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin) during 30 min. After that, the extracts were pre-cleared for 1 hr with Protein A/G-Sepharose (Santa Cruz Biotechnology, Inc.) and immunoprecipitated (IP) using anti-N-MYC antibodies (Cell signaling) or IgG as a control. The IP fractions were analyzed by WB with N-MYC (Santa Cruz Biotech) and MAX (Cell signaling) antibodies.

In sillico analysis

The Cancer Genome Atlas (TCGA) GBM dataset was accessed via UCSC xena-browser (https://xenabrowser.net) for extraction of N-MYC gene´s expression level.

Statistical Analysis

GraphPad Prism 5 software was used for data presentation and statistical analysis. For bar graphs, the level of significance was determined by a two-tailed un-paired Student´s t-test. The difference between experimental groups was assessed by Paired t-Test and one-way ANOVA. To analyze the survival of nude mice, we used the Kaplan–Meier method and evaluated with a two-sided log-rank test. For correlation analysis between each gene, expression data were tested by Pearson' s correlation coefficient and Spearman' s correlation coefficient. P values < 0.05 were considered significant (*p < 0.05; **p < 0.01; *** p< 0.001; **** p< 0.0001; n.s., non-significant). All quantitative data presented are the mean ± SEM.

Results

N-MYC is highly expressed in gliomas

To evaluate whether N-MYC is dysregulated in glioblastoma cell lines, we first studied its presence by quantifying its RNA levels. We did not find an increase in *N-MYC* transcription in GBM samples compared to normal brain, both in our cohort (Fig. 1a) or in the analysis of TCGA samples (RNAseq or microarray data) (Supplementary Fig. 1a-b). By contrast, using protein extracts from patients' samples we observed a clear upregulation of N-MYC protein in GBMs compared to non-tumor (NT) tissues (Fig. 1b and c). Some of the patient derived xenografts (PDX) also express high levels of N-MYC protein (Fig. 1d), with frequent nuclear staining in the mouse tumors (Fig. 1e) and a strong consistency between the WB and the IF quantification (Fig. 1f). These results confirm the increased levels of N-MYC protein in GBM and suggest that the main mechanisms for this upregulation must be related to increased translation and/or stabilization of the protein.

Effect of IDP-410 N-MYC inhibition in glioma growth in vitro

We studied IDP-410, a novel stapled peptide designed to specifically target and inhibit the function of N-MYC, as therapeutic agent in GBM, its mechanism of action and the biological consecuences of inhibiting N-MYC in vitro. For that, we first analyzed the levels of expression of N-MYC in a battery of primary GBM cell lines (Fig. 2a). The exposure of GBM6 (high N-MYC expression) to increasing concentrations of IDP-410 clearly reduced the viability of the cells (Fig. 2b). Moreover, 10μM of IDP-410 triggered the cell death of all the primary cell lines expressing high levels of N-MYC (Fig. 2c). By contrast, there was very little effect of this compound in GBM8, GBM10 or fibroblasts, which express reduced amounts of

N-MYC (Fig. 2c). A direct comparison of the effect of IDP-410 in GBM6 (high N-MYC), GBM2 (low N-MYC) and fibroblasts (no N-MYC) (Fig. 2d) showed the strong correlation between the expression of the target and the sensitivity to the drug (Fig. 2e), reinforcing its specificity.

IDP-410 induces N-MYC protein degradation in vitro and disrupts N-MYC/MAX complex

The incubation of GBM6 cells with increasing amounts of IDP-410 for 6 hours reduced the amount of N-MYC protein in the cells (Fig. 3a-b). Notably, the enhanced degradation induced by IDP-410 was blocked in the presence of the proteasome inhibitor MG132, both in GBM6 and GBM7 cells (Fig. 3c-e). We also observed an impairment of N-MYC and MAX interaction in the presence of IDP-410 (Fig. 3f). Taking together, these results suggest that the IDP-410 inhibitory effect is associated with the dissociation of the N-MYC/MAX complex and the degradation of N-MYC.

IDP-410 suppresses glioma growth in vivo

To evaluate whether the effect of IDP-410 observed in vitro translates to an in vivo setting, we injected GBM6 cells subcutaneously in immunodeficient mice and treated the animals intravenously (i.v.) with IDP-410. The growth curve of the tumors shows that systemic treatment with IDP-410 reduced significantly the tumor size, in comparison to vehicle (PBS) (Fig. 4a-b). Immunofluorescence staining of tissues from both sets of mice demonstrated a reduced nuclear N-MYC expression after IDP-410 treatment (Fig. 4c). Furthermore, BrdU incorporation was reduced in IDP-410 treated mice in comparison with control tumors (Fig. 4d-e), whereas the percentage of apoptotic cells (measured through activated Caspase 3 staining) was not different

between the two groups of mice (Supplementary Fig. 2). These results suggest that N-MYC inhibition impairs the growth of GBM tumors but does not promote cell death in immune-supresed, tumor bearing mice xenografts.

To determine if IDP-410 is able to cross the blood brain barrier and block the growth of orthotopic gliomas, we injected GBM6 cells into the brain of Nude mice. Two weeks later, we started treating the animals with vehicle (Polisorbate 1% in PBS) or IDP-410, injected subcutaneously. As the cells express the luciferase reporter, we were able to follow up tumor progression in situ and we determined that IDP-410 reduced the growth of GBM6 cells (Fig. 5a). Moreover, it increases the survival or the tumor-bearing animals (Fig. 5b). The analysis of the excised tumors showed a strong downregulation of nuclear N-MYC in IDP-410 treated samples (Fig. 5c) and a decrease in the amount of N-MYC protein, measured by WB (Fig. 5d). In agreement with the results obtained in the heterotopic tumor setting, the amount of proliferative cells in the intracranial tumors was also diminished after systemic IDP-410 treatment (Fig. 5e). Altogether, these results suggest that the IDP-410 reaches the brain, decreases the stability of N-MYC protein and inhibits the growth of GBM cells.

IDP-410 affects tumor proliferation and vascularization in treated mice

MYC proteins regulate the crosstalk between the tumor and the host and have been linked to processes of proliferation and angiogenesis [29-31]. To explore the possible anti-tumor mechanisms of N-MYC inhibition by IDP-410, we selected a series of 27 genes know to be regulated by N-MYC and mainly associated with these two processes (Fig. 6a). We observed that the mRNA expression of some of these genes was reduced when GBM6 and GBM3 cells were cultured in the presence of IDP-410 (Fig. 6b and c). Notably, a very similar inhibition pattern was observed in

the intracranial GBM6 tumors after systemic IDP-410 treatment (Fig. 6d). Furthermore, we confirmed that the expression of important angiogenesis- (*VEGFA*, VEGF) and proliferation- (*MIK67*, Ki67) related genes correlated positively with N-MYC protein levels in a panel of glioma PDXs (Fig. 6e and f; Supplementary Fig. 3).

It is well known that glioma cells, particularly in high-grade tumors, express a variety of genes that promote different steps of the angiogenesis process [32]. To study if N-MYC inhibition by IDP-410 could affect this pro-angiogenic function of gliomas, we first performed an in vitro assay using GBM6 cells. We examined the effect of conditioned media (CM) from these glioma cells (treated or not with IDP-410) on the wound healing capacity of HBMEC cells. Fig. 7 a-b shows that treatment with IDP-410 impaired the effect of glioma CM on the endothelial migration of HBMEC cells.

We have recently shown that mesenchymal transformation is key to induce angiogenesis in GBM, even for the generation of tumor blood vessels [33]. When we characterized the tumor tissue from IDP-410 treated animals, we observed a clear reduction on the amount of TAZ and αSMA, which are mesenchymal-related proteins [34], in comparison with control samples (Fig. 7c). Moreover, we observed that IDP-410 treated tumors contained reduced levels of the angiogenic factor VEGF (Fig. 7d). In addition, the IHC staining with endomucin antibodies showed a strong reduction in the number of dilated blood vessels in the presence of IDP-410 (Fig. 7e). Taken together, our results suggest that the anti-tumor effect of N-MYC inhibition by IDP-410 is mediated, at least in part, by the decrease in tumor-cell proliferation and by the reduction of neo-vascularization processes.

Discussion

GBM is one of the deadliest tumor types with a very short life expectancy upon diagnosis. Its treatment does not succeed mainly because of its highly heterogeneous and plastic nature [35]. Thus, there is an urgent need to search for new therapeutic strategies. MYC proteins are one of the most wanted targets due to their notorious oncogenic features [5, 6, 23, 26]. However, a long standing consideration for the clinical development of MYC inhibitors is the potential side effects due to its capacity to regulate proliferation in normal tissues. Indeed, systemic inhibition of Myc in transgenic mice models for continuous long periods (4 weeks) was shown to affect high proliferating tissues, such as skin and gastrointestinal tract, but found to be well tolerated, not to alter the homeostasis of the tissues, and the effect readily reversed when Myc was reactivated [36]. In that sense, targeting specifically N-MYC, with a more neural-restricted pattern of expression, has become of interest for many pediatric cancers but also for certain adult neoplasia, including GBM [24].

Here, we have confirmed the overexpression of *N-MYC* gene compared to normal tissue in a percentage of human GBM samples and PDXs. Interestingly, we found no correlation between the levels of protein and mRNA. This suggest that protein stabilization is an important regulatory event and that *N-MYC* transcriptional data cannot be used to evaluate the overexpression of N-MYC protein in gliomas [19]. More importantly, we show here compelling evidence supporting the anti-tumor effect of a novel stapled peptide IDP-410 specifically designed to target N-MYC in those GBM expressing high levels of N-MYC, both in vitro and in vivo. These results demonstrate the therapeutic potential of IDP-410, which shows capacity to reach subcutaneous and intracranial tumors when administered systemically, with high

tollerability. Moreover, the data suggest that N-MYC (protein, not mRNA) should be used as a biomarker of response to IDP-410.

The nuclear localization of MYC proteins and their intrinsically disordered structure hampers the development of classical small molecule inhibitors against these TFs. Strategies to impair MYC protein function via its transcription and protein or mRNA stability have been developed and some of them have reached the clinic [5, 23, 26, 27]. The most recent example is Omomyc, a miniprotein analog to cMYC currently in Phase I for treatment of solid tumors (NCT04808362). It was shown that Omomyc, when conditionally expressed in transgenic models, antagonizes the function of MYC family members and reduce the growth of different types of cancer including GBMs [26]. When administered systemically and intranasally, Omomyc was shown to inhibit tumor growth in lung cancer animal models [37]. However, its potential to reach the brain and act against gliomas has not been evaluated [38].

Our results show that the IDP-410 peptide acts by reducing the interaction of N-MYC with its partner MAX and favoring the degradation of the protein. Systemic IDP-410 treatment induced a decrease in cell proliferation and a clear inhibition of glioma vascularization. In agreement with that, IDP-410 reduce the pro-angiogenic properties of glioma cells in vitro. As a possible mechanism to explain this effect, we observed a strong downregulation of *VEGFA* expression in tumors treated with IDP-410. Moreover, we show a significant correlation between *VEGFA* transcription and the amount of N-MYC protein in GBM xenografts. VEGFA, which is normally secreted by tumor cells, is a key player in angiogenesis as it binds to the endothelial VEGFR2 and promotes proliferation and migration, leading to new vessel-like structures [39-41]. Therefore, our results suggest for the first time a direct regulation of GBM angiogenesis by N-MYC inhibition through the modulation of the expression of *VEGFA* and possibly other vascular-related molecules. This is in agreement with the

widely accepted role of c-MYC as a modulator of different aspects of the tumor microenvironment, including the vasculature [29, 42] and with the correlation of *N-MYC* amplification with angiogenesis in neuroblastoma [43].

High-grade gliomas are extremely dependent on their vascular microenvironment, which allows the tumor to gather needed nutrients and oxygen for its growth and promotes the progression of the disease. Florid angiogenesis is associated with the appearance of mesenchymal features, which is linked to the aggressiveness of gliomas [33]. Furthermore, tumor cells that have become mesenchymal invade the adjacent parenchyma or may even interact with endothelial cells, a property that allows them to govern the brain vasculature [44]. Our recent results indicate that these processes are mediated by increases in TAZ and α-SMA expression in glioma cells [33, 45]. Moreover, tumor TAZ activation is often correlated with increased levels of angiogenesis while endothelial TAZ activation is needed for the formation of blood and lymphatic vessels [46]. In addition, this transcription factor controls the mesenchymal transformation of gliomas, which is associated with changes in the tumor microenvironment [47]. The expression analysis in tumors treated with IDP-410 suggest a reduction in the mesenchymal features of gliomas after N-MYC inhibition. We propose here that this change in tumor phenotype, added to the *VEGFA* decrease, might reduce the vascularization and the growth of the tumors.

Overall, our data highlights the relevance of N-MYC in glioma pathology, and in particular its role via the over-stabilization of the protein. Moreover, we have demonstrated the efficacy and mechanism of action of IDP-410, a newly designed peptide that targets directly N-MYC protein. Although the results obtained shed some hope for a possible GBM stratified treatment, more insight into IDP-410's effects need to be gathered. Especially interesting would be to study the effect of IDP-410

in the immune component of the microenvironment and its combination with immune checkpoint inhibitors in GBM tumors, as MYC inhibitors can enhance the anti-tumoral immune response [48]. Moreover, combinatorial approaches with anti-angiogenic molecules, which have failed to block GBM growth [49], would be desirable. In any case, the results presented here shows the great therapeutic potential of IDP-410 in GBM as it disrupts the supportive tumor microenvironment, which is probably less susceptible to the appearance of resistant subclones with different genetic alterations.

Acknowledgements

This work was supported by Ministerio de Economía y Competitividad: (Acción Estratégica en Salud) and FEDER funds: PI16/01550 to JMS, by “Asociación Española contra el Cancer grants: INVES192GARG to RG, GCTRA16015SEDA to JMS and IDEAS20095SÁNC to PSG, and by Ministerio de Ciencia, Innovación y Universidades and FEDER funds: RTI2018-093596 to PSG. MGA was supported by the Young Employment Initiative (Comunidad de Madrid).

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Figure 1

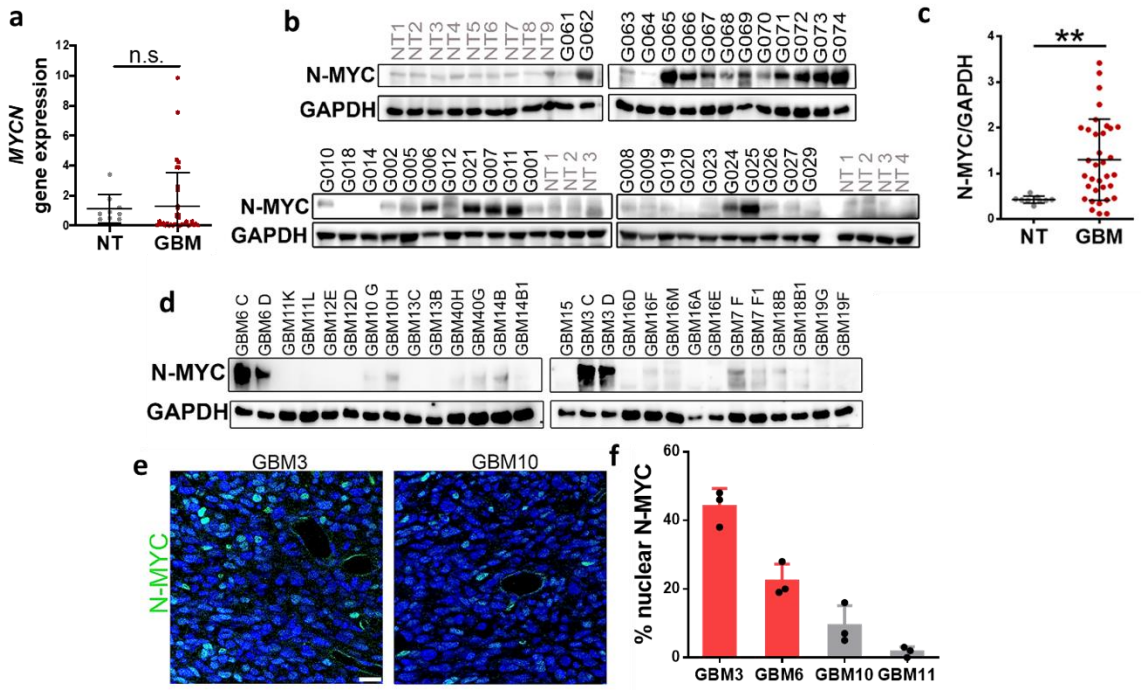

Fig 1. N-MYC expression in gliomas. a Quantification of *N-MYC* mRNA levels by quantitative reverse-transcriptase PCR (qRT-PCR) in a cohort of human glioblastomas (GBM) and normal brain tissues (NT) (n = 35 and n = 9). *HPRT* was used for normalization. **b-c** Western blot (WB) analysis of N-MYC levels in the cohort of GBM (n = 35) and NT (n = 9) and quantification in c. GAPDH was used as a loading control. **d** WB analysis of N-MYC levels in tumor tissue from a panel of PDXs (patient-derived xenografts). GAPDH was used as a loading control. **e** Representative images of the immunofluorescence staining of N-MYC in the PDX´s tumor tissue. **f** Quantification of cells with nuclear N-MYC in (e). **p < 0.01; n.s. non-significant.

Figure 2

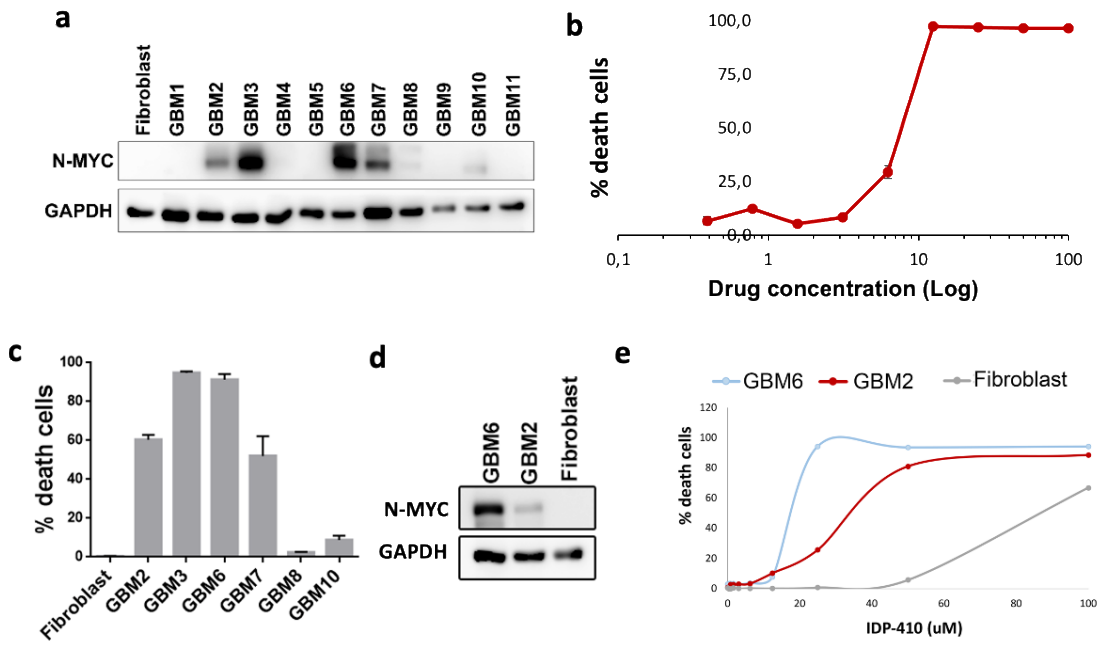

Fig 2. IDP-410 inhibits gliomas growth in vitro. a Western blot (WB) analysis of the levels of N-MYC in a panel of human GBM cells. Diploid human fibroblasts were used as a non-tumor control. GAPDH was used as a loading control. **b** Determination of the percentage of cell death in GBM6 cells exposed to increasing concentrations of IDP-410. **c** Analysis of the percentage of dead cells after IDP-410 treatment of a panel of human GBM cells. **d** WB showing N-MYC levels in human GBM6, GBM2 and fibroblasts. GAPDH was used as a loading control. **e** Analysis of cell death in the presence of increasing concentrations of IDP-410 in glioma cells with high (GBM6), low (GBM2) or no (fibroblasts) N-MYC expression.

Figure 3

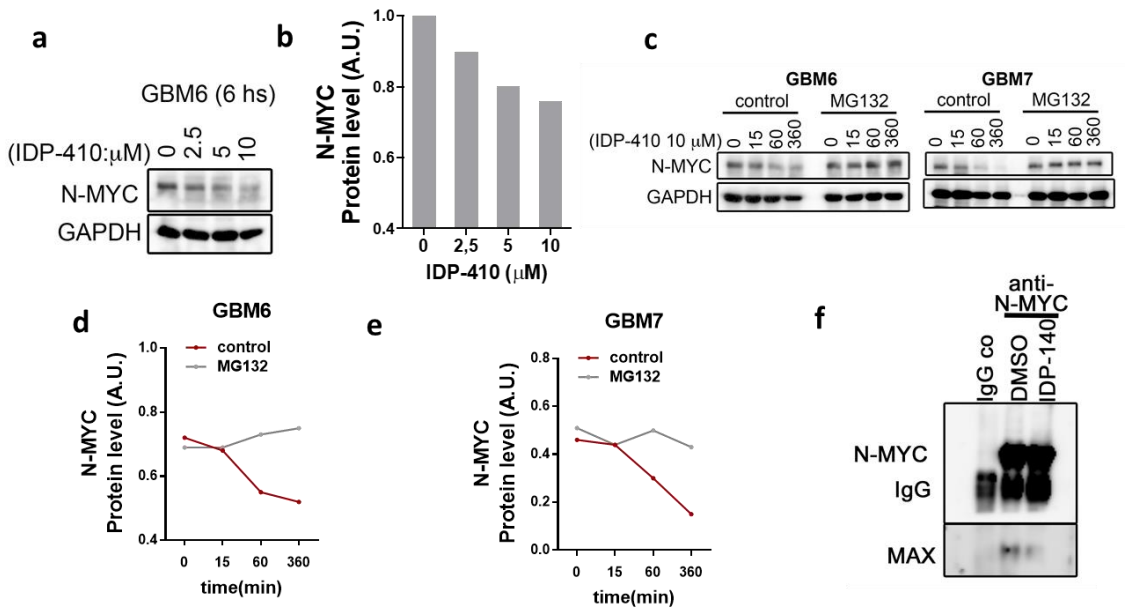

Fig 3. IDP-410 induces N-MYC degradation. a-b Western blot (WB) (a) and quantification (b) of N-MYC levels in GBM6 cells treated for 6h with increasing IDP-410 concentrations. GAPDH was used as a loading control. **c** WB analysis of N-MYC expression in GBM6 and GBM7 cells, incubated for different times in the presence of IDP-410 (10 μM), with or without the proteasome inhibitor MG132 (20 μM). GAPDH was used as a loading control. **d-e** Quantification of the WB in (c) for GBM6 (d) and GBM7 (e) cells. **f** Extracts from GBM6 cells, incubated in the presence of DMSO or IDP-410, were immunoprecipitated (IP) using anti-N-MYC antibodies. The IP fractions were analyzed by WB with N-MYC and MAX antibodies. Control IP with IgG is shown on the left lane.

Figure 4

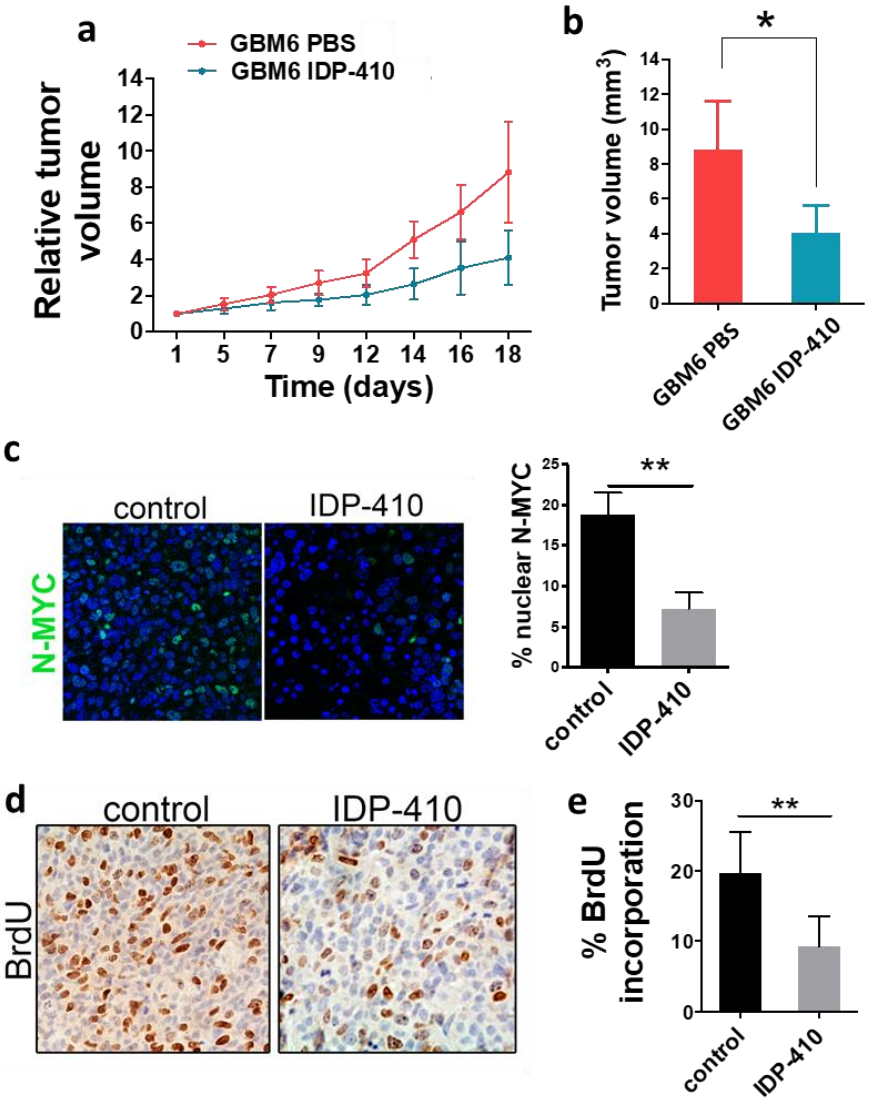

Fig 4. Systemic IDP-410 impairs the subcutaneous growth of gliomas. a Growth curve of GBM6 cells implanted in the flank of nude mice, which were treated by bolus intravenous route (i.v.) with vehicle or IDP-410 twice a week (dose 15 mg/kg). **b** Determination of the tumor volume at the final end point in (a). **c** Representative images of the immunofluorescence analysis of nuclear N-MYC in tumors treated with vehicle (control) or IDP-410. Quantification is shown on the right. **d-e** Determination of tumor cell proliferation measured by incorporation of BrdU and quantified using immunohistochemistry in tumors treated with vehicle (control) or IDP-410. *$p < 0.05$; **$p < 0.01$.

Figure 5

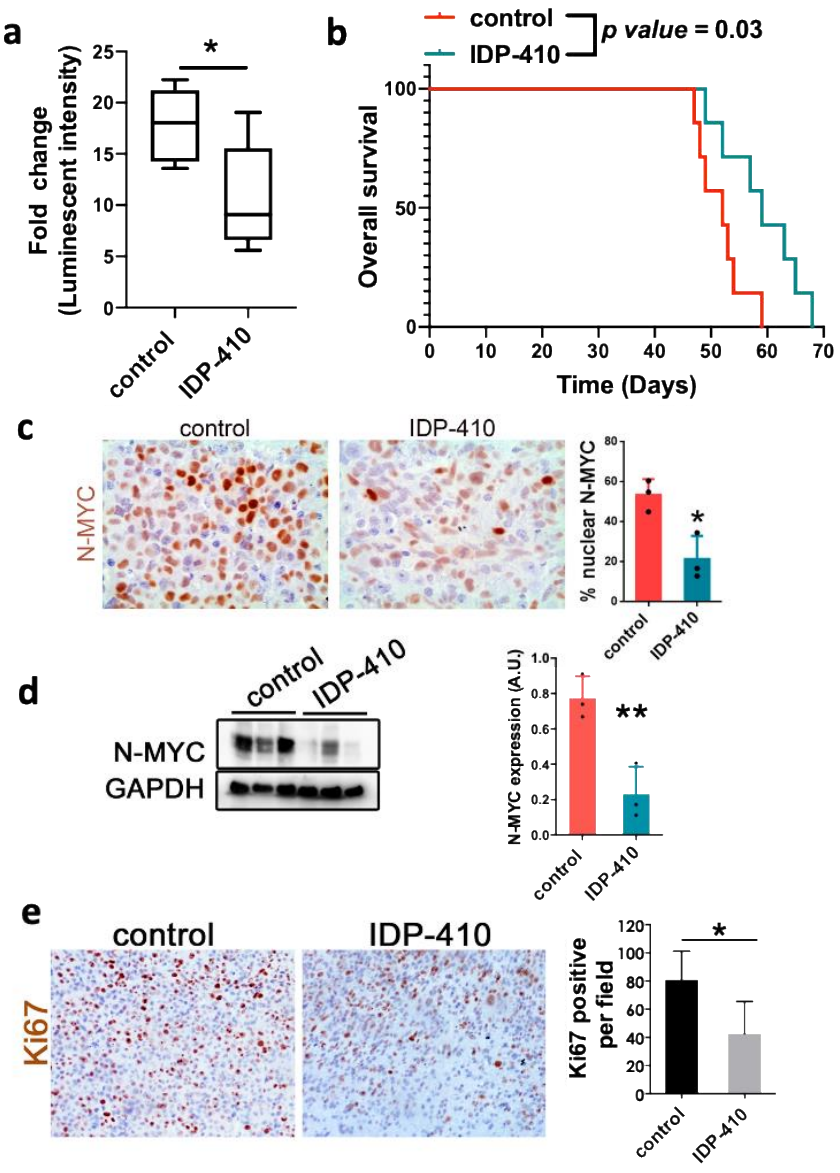

Fig 5. Systemic IDP-410 impairs the intracraneal growth of gliomas. **a** GBM6 cells were intracranially injected in Nude mice and the animals were treated with vehicle (control) or IDP-410 (15mg/Kg; 5 days a week). The graph shows the fold increase in luminescence (between day 40 and day 52 after cell injection) in control and IPD-410 treated mice. **b** Kaplan-Meier survival curve of GBM6-bearing mice treated with vehicle (control) or IDP-410. **c** Representative pictures of the immunohistochemical (IHC) staining of N-MYC in tumors from (b). Quantification of the nuclear N-MYC staining is shown on the right (n=3/condition). **d** Western blot of N-MYC levels in GBM6 intracraneal tumors from (b). GAPDH was used as a loading

control. Quantification is shown on the right (n=3/condition). **e** Representative pictures of IHC staining of Ki67 in tumors from (b). Quantification is shown on the right (n=3/condition). *p < 0.05, **p < 0.01.

Figure 6

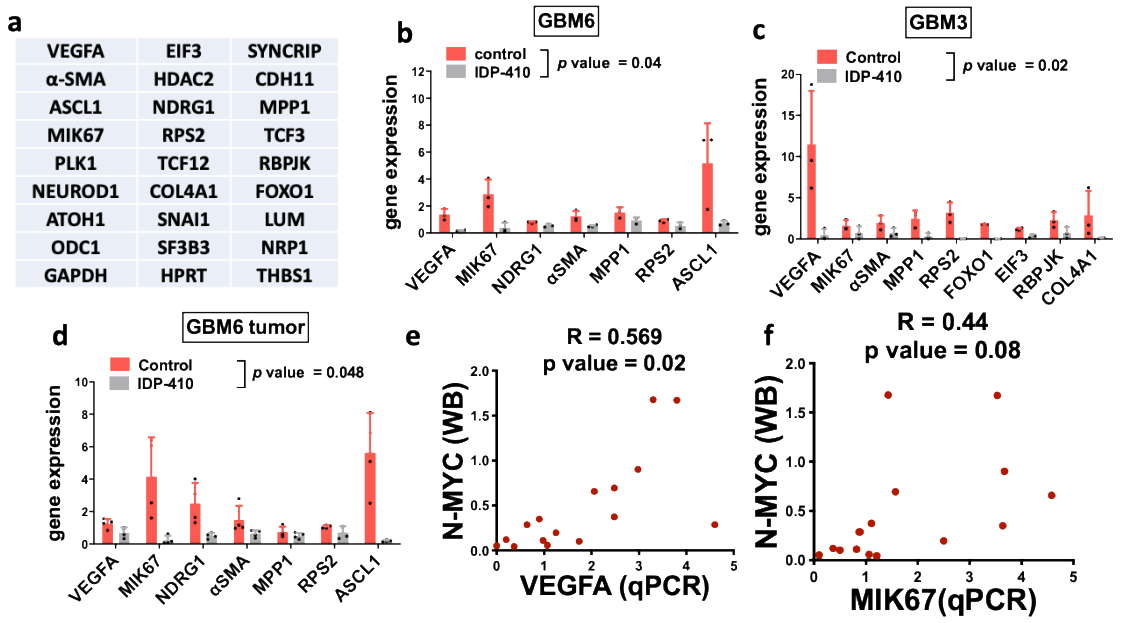

Fig 6. Effect of IDP-410 in proliferation and angiogenesis-related genes regulated by N-MYC in gliomas. a N-MYC upregulated gene signature. **b-c** Quantitative reverse-transcriptase PCR (qRT-PCR) analysis of several *N-MYC*-related genes (from a) in GBM6 (b) and GBM3 (c) cells treated with vehicle (control) or IDP-410. *HPRT* was used for normalization. **d** qRT-PCR analysis of a signature of N-MYC-related genes in GBM6 intracranial tumors from Fig 5. **e-f** Pearson´s correlation analysis between the expression of *VEGFA* (d) and *MKI67* (e) and the amount of N-MYC protein in a panel of PDXs (Supplementary Fig. 3).

Figure 7

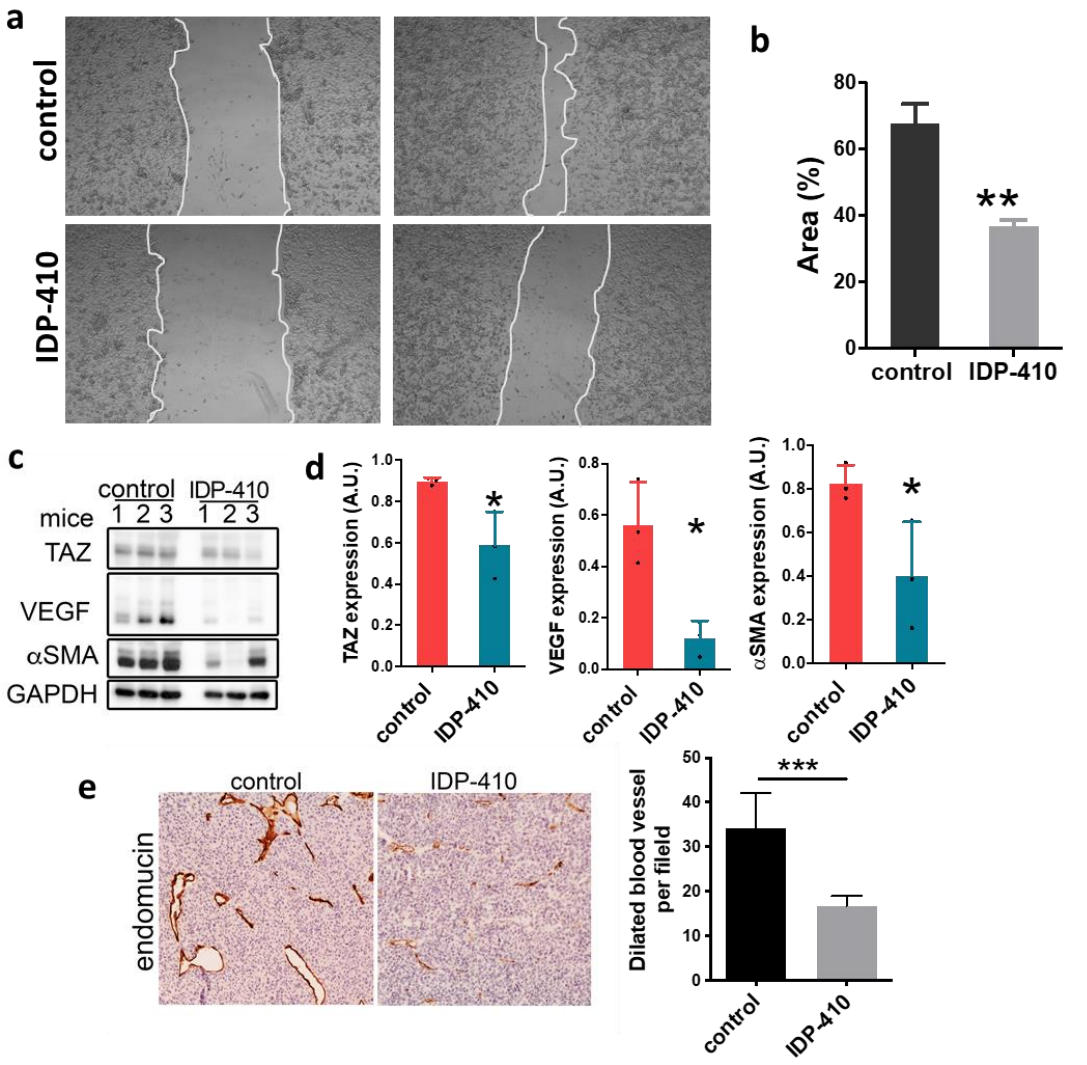

Fig 7. IDP-410 impairs vascularization of gliomas. a Representative images from a wound healing test of HBMEC cells incubated in the presence of conditioned media from control or IDP-410 treated GBM6 cells. **b** Quantification of the percentage of the scratch area reduction in (a). **c** Western blot analysis of TAZ, VEGF and αSMA in GBM6 tumors treated with vehicle (control) and IDP-410. GAPDH was used as a loading control. **d** Representative pictures of the immunohistochemical staining of endomucin in GBM6 control and IDP-410 tumors. Quantification is shown on the right (n=3/condition). **e** Quantification of the number of dilated blood vessels in (d) (n = 3). **p < 0.01; ***p < 0.001.

CoI Gargini

CoI Moreno

CoI Nevola

CoI Lobato

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Supplementary Figures and Tables