Metformin and simvastatin exert additive antitumour effects in glioblastoma via senescence-state: clinical and translational evidence

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Summary

Background Glioblastoma is one of the most devastating and incurable cancers due to its aggressive behaviour and lack of available therapies, being its overall-survival from diagnosis ~14-months. Thus, identification of new therapeutic tools is urgently needed. Interestingly, metabolism-related drugs (e.g., metformin/statins) are emerging as efficient antitumour agents for several cancers. Herein, we evaluated the *in vitro*/*in vivo* effects of metformin and/or statins on key clinical/functional/molecular/signalling parameters in glioblastoma patients/cells.

Methods An exploratory-observational-randomized retrospective glioblastoma patient cohort (n = 85), human glioblastoma/non-tumour brain human cells (cell lines/patient-derived cell cultures), mouse astrocytes progenitor cell cultures, and a preclinical xenograft glioblastoma mouse model were used to measure key functional parameters, signalling-pathways and/or antitumour progression in response to metformin and/or simvastatin.

Findings Metformin and simvastatin exerted strong antitumour actions in glioblastoma cell cultures (i.e., proliferation/migration/tumoursphere/colony-formation/VEGF-secretion inhibition and apoptosis/senescence induction). Notably, their combination additively altered these functional parameters vs. individual treatments. These actions were mediated by the modulation of key oncogenic signalling-pathways (i.e., AKT/JAK-STAT/NF-κB/TGFβ-pathways). Interestingly, an enrichment analysis uncovered a TGFβ-pathway activation, together with AKT inactivation, in response to metformin + simvastatin combination, which might be linked to an induction of the senescence-state, the associated secretory-phenotype, and to the dysregulation of spliceosome components. Remarkably, the antitumour actions of metformin + simvastatin combination were also observed *in vivo* [i.e., association with longer overall-survival in human, and reduction in tumour-progression in a mouse model (reduced tumour-size/weight/mitosis-number, and increased apoptosis)].

Interpretation Altogether, metformin and simvastatin reduce aggressiveness features in glioblastomas, being this effect significantly more effective (*in vitro*/*in vivo*) when both drugs are combined, offering a clinically relevant opportunity that should be tested for their use in humans.

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Research in context

Evidence before this study

Being one of the most devastating cancers worldwide, glioblastoma represents a major clinical challenge for the health systems based on their locally aggressive behaviour and current lack of curative therapies. Indeed, despite significant scientific advances, glioblastoma remains an incurable disease with a survival rate after diagnosis of ~14 months. Effective medical therapeutic tools are severely lacking, and thus, novel approaches are urgently needed.

Added value of this study

The information provided herein, both through clinical observations and experimental evidence, robustly suggests the efficacy of metformin, simvastatin, and particularly, their combination, as a potential and useful therapeutic tool for the management of glioblastomas through the modulation of key oncogenic pathways.

Implications of all the available evidence

Advantageously, because of the clinical safety of these drugs, this new insight offers a clinically relevant opportunity that could be promptly tested for their use in humans with limited unknown/undesired side-effects to improve the current therapies that remain inefficient. The data showed here pave the way for the use of rapidly available drugs that could significantly improve the quality of life of GBM patients.

Introduction

Glioblastoma (GBM) is the most common and lethal primary brain tumour in adults, with <10% of newly diagnosed patients alive at five years.[1–3] Evidence points to a significant increase in GBM incidence in recent years, while the rest of glioma subtypes remained stable.[1,4] The mainstay of treatment for patients with GBM consists of maximal surgical resection followed by a combination of radiation treatment and adjuvant chemotherapy with temozolomide.[5–7] Currently, no truly effective pharmacological therapy exists for GBM patients, and most FDA-approved therapies provide marginal survival benefits.[8] Thus, new therapeutic approaches are urgently needed.

An emerging approach against several cancers is based on the repositioning of drugs already approved to treat other pathologies since they are faster and easier to translate to clinical practice than new drugs.[9] In this scenario, metformin and statins (e.g., simvastatin), commonly used to treat metabolism-related pathologies (i.e., obesity, type-2 diabetes, and hypercholesterolemia) have been tested in different malignant tumours.[10–15] In fact, metformin and statin repurposing is already being tested in a range of clinical trials for a variety of cancers including brain tumours[16,17] (see Table 1). However, some of the antitumour actions of these drugs are still controversial,[18–20] and more importantly, to the best of our knowledge, the potential direct antitumour effects that may exert metformin, statins, and especially, their simultaneous combination, in healthy brain tissue and GBM cells have not been compared side-by-side yet in previous reports. Moreover, the mechanisms by which metformin and statins exert their actions on GBM cells have not been deeply defined.

This study was conceived to pursue a double objective: 1) to explore and compare side-by-side the *in vitro* antitumour effects of metformin and simvastatin alone, as well as the combination of both drugs, in human GBM cells (i.e., primary GBM cell cultures and 2 GBM cell line models), in non-tumour brain human cells, and in primary mouse astrocytes progenitor cell cultures; and 2) to assess the potential clinical benefits that the treatments with metformin, statins, and specially their combination, could exert on overall survival and key pathological parameters using an exploratory, observational, retrospective, randomized cohort of patients with GBM available in our hospital (n = 85), as well as in a preclinical xenograft glioblastoma mouse model. In addition, we analysed the ability of these drugs and their combination to alter the expression of relevant genes and/or activity of multiple key oncogenic signalling pathways and mechanisms that are critical for tumour development, progression, and aggressiveness in human GBM.

Methods

Ethics statement

This study was approved by the Reina Sofia University Hospital Ethics Committee and was conducted in accordance with the principles of the Declaration of Helsinki. The biobank of the public health system of Andalusia (Cordoba Node) coordinated the collection, processing, management, and assignment of all the biological samples used in this study, according to the standard procedures established for this purpose. Written informed consent was obtained from all individuals included in the study. The number of mice used per group/arm was calculated by "resource

Study title	Tumour type	Interventions	Study type	Phase	Study start	Status
Temozolomide, memantine hydrochloride, mefloquine, and metformin hydrochloride in treating patients with glioblastoma multiforme after radiation therapy	Glioblastoma; Gliosarcoma; Supratentorial Glioblastoma	Drug: Mefloquine Drug: Memantine Drug: Metformin Drug: Temozolomide	Interventional	Phase 1	September 14, 2011	Active, not recruiting
Efficacy and safety of atorvastatin in combination with radiotherapy and temozolomide in glioblastoma	Glioblastoma	Drug: Atorvastatin Drug: Temozolomide Radiation: Radiotherapy	Interventional	Phase 2	January 1, 2014	Completed
Treatment of recurrent brain tumors: metabolic manipulation combined with radiotherapy	Brain Neoplasms	Radiation: Partial brain re-irradiation Drug: Metformin Behavioral: low carbohydrate diet	Interventional	Phase 1	June 1, 2014	Unknown†
Metformin, neo-adjuvant temozolomide and Hypo- accelerated radiotherapy followed by adjuvant TMZ in patients with GBM	Glioblastoma	Drug: Metformin	Interventional	Phase 2	March, 2015	Recruiting
Study on low dose temozolomide plus metformin or placebo in patient with recurrent or refractory glioblastoma	Glioblastoma	Drug: Temozolomide + Metformin Drug: Temozolomide + Placebo	Interventional	Phase 2	November 21, 2016	Recruiting
Bioavailability of disulfiram and metformin in glioblastomas	Glioblastoma	Drug: Disulfiram Drug: Metformin	Interventional	Early Phase 1	January 29, 2018	Terminated
A pilot study of ketogenic diet and metformin in glioblastoma: feasibility and metabolic imaging	Glioblastoma	Drug: Metformin Other: Ketogenic Diet	Interventional	Phase 2	December 31, 2020	Recruiting
Study of the safety, tolerability and efficacy of metabolic combination treatments on cancer (METRICS)	Brain Cancer	Drug: Atorvastin Drug: Doxycycline Drug: Metformin Drug: Mebendazole	Interventional	Phase 3	May 23, 2022	Not yet Recruiting
Oxidative Phosphorylation Targeting In Malignant glioma Using Metformin plus radiotherapy temozolomide (OPTIMUM)	Glioblastoma	Drug: Metformin Drug: Temozolomide Radiation: Radiotherapy	Interventional	Phase 2	May, 2022	Not yet Recruiting

†Study has passed its completion date and status has not been verified in more than two years.

Table 1: **Current clinical trials of metformin and statin treatments in adult patients diagnosed with primary brain neoplasms (Data obtained from clinicaltrials.gov; May 2022).**

equation" method based on ANOVA test E = N (number of individuals)-T (number of conditions); E = (5 × 4)–4 = 16; where E is considered adequate in a range between 10 and 20).[21] All *in vivo* experiments were performed according to the European Regulations for Animal-Care under the approval of the university/regional-government research ethics committees.

Reagents

Unless otherwise indicated, reagents and products were purchased from Sigma–Aldrich (St. Louis, MO). Metformin and simvastatin were obtained from Merck KGaA (Darmstadt, Germany).

Patients

We carried out a pilot, observational, and retrospective study with 85 patients designed to assess the putative association between metformin and/or statins treatment with median overall survival. Patients were included and excluded in this study according to compliance with the following criteria. We included all the patients who underwent surgery between 2016 and 2022 as a treatment for brain lesions with an imaging diagnosis compatible with aggressive glioma, and who were also treated with only metformin and/or statins (administered according to the defined daily dosage and not with other metabolism-related drugs) for at least 6 months before and/or after surgery in the post-surgery adjuvant stage. Specifically, a total of 122 patients were identified in the clinical database. Subsequently, we excluded those patients without a genetic study as well as those where the glioma diagnosis had not been confirmed by pathological anatomy. Finally, 85 patients were included in the study who met all these criteria. It should be noted that 86.41% of the patients were treated following the STUPP protocol and none of these patients were being treated with other co-adjuvant or metabolic-related medications. Available demographic and clinical characteristics of the cohort are depicted in Table 2 which supports that our cohort is representative of the wider population of interest within the context of the study.

Primary human GBM/non-tumour cell cultures and cell lines

Fresh human GBM tissues (n = 4; obtained after intracranial surgery) and non-tumour brain tissues [n = 4;

GBM patient cohort	Non-treated patients	Metformin-treated patients	Statin-treated patients	Metformin + Statin-treated patients
Patients (n)	51	8	17	9
Gender (M/F)	33(64.71%)/ 18(35.29%)	6 (75%)/2 (25%)	10 (58.82%)/9 (41.18%)	6 (66.66%)/3 (33.33%)
Age at surgical intervention (mean ± std. dev.)	55.67 ± 15.89	57.00 ± 4.47	55.57 ± 7.08	57.27 ± 10.53
Surgical resection (>95%/95-75%/75-50%/<50%)	21/25/4/1	4/4/0/0	6/9/2/0	4/5/1/0
Relapsed patients	8	0	1	1
%Ki67	24 ± 0.1	25 ± 0.1	26 ± 0.1	23 ± 0.1
$IDH1_{mut}$	2	1	1	1
STUPP protocol (% patient enrolled)	80.39%	100.00%	94.12%	100.00%
Pre-surgery/Post-surgery administration (minimum of 6 months)	–	6/8	13/17	8/9
GBM survival (Median–Months)	12.20	13.00	13.8	15.5
Events per condition (non-censored patients)	49	8	15	8
Survival log-rank test (p value)	–	0.3052	0.6198	0.2316

86.41% of all the patients included were treated following the STUPP protocol. Kaplan–Meier analysis results are showed as log-rank test p value. See the Patients section in Methods for inclusion criteria for GBM patients.

Table 2: **Demographic and clinical characterization of our cohort of patients with GBM which include non-treated patients (n = 51), metformin-treated patients (n = 8), statin-treated patients (n = 17; 13 simvastatin-treated, 3 atorvastatin-treated and 1 rosuvastatin-treated patients), and metformin + statin treated patients (n = 9; 7 metformin + simvastatin and 2 metformin + atorvastatin).**

obtained from lobectomy (epilepsy surgery) and gyrectomy] were collected at the surgery room, placed in sterile cold (4 °C) S-MEM medium (Gibco, Madrid, Spain) complemented with 0.1% BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution and 2.5% HEPES, and immediately transported to the laboratory. Similarly, we also had the opportunity to collect fresh tissues from high-grade (grade III) astrocytomas (n = 4) (Table 3). One piece of the samples collected was included in paraffin to be immediately analysed by expert pathologists, who histologically confirmed all tumour samples and the absence of tumour in the control brain tissues. The remaining pieces were mechanically dispersed and enzymatically digested [using 2% Collagenase IV and 0.15% Trypsin lyophilized powder (Beckton, Dickinson, and Company, Sparks, MD, USA)] to obtain single-cell cultures, as previously reported.[22] The dispersed single cells were cultured onto poly-L-Lysine-coated tissue culture plates in D-Valine DMEM with 10% foetal bovine serum (FBS) complemented with 0.1% BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution, and 2.5% HEPES.

Two human GBM cell line models (U-87 MG and U-118 MG) were obtained from the American Type Culture Collection (ATCC, #HTB-14, RRID:CVCL_0022/ #HTB-15, respectively; Manassas, VA, USA) and cultured according to the supplier's recommendations. Cell lines were systematically checked for mycoplasma contamination by PCR and validated by PCR-STR profiling as previously reported.[22]

Primary mouse astrocytes progenitor cell cultures

These experiments were carried out according to the European Regulations for Animal Care under the approval of the university/regional government research ethics committees. Whole brains of newborn (PND1-3) C57BL/6 (Charles River Laboratories; RRID:IMSR_CRL:027) mice were collected in cold DMEM/F12 medium with L-glutamine (Lonza, #BE12-719F). Mechanical dissociation was done by gentle scraping over a 40 µm mesh. The cell suspension was centrifuged, and the cell pellet was resuspended with 4 mL in growth medium DMEM/F12 with L-glutamine; 1% Gentamicin/Amphotericin (GIBCO, #R01510) and 10% FBS. Cells were plated on uncoated T25 flasks. After 5 days of incubation (37 °C, 5% CO_2), fresh growth medium was added and incubated for 48 h. Then, the flasks were placed at 300 rpm in a shaker at room temperature for 12 h, then 1 h at 37 °C (5% CO_2), and finally for an additional 12 h in the shaker at room temperature to remove oligodendrocytes and microglial cells from culture and obtain isolated primary mouse astrocyte progenitor cell cultures. After shaking, the medium was replaced with growth medium.

Measurements of cell proliferation/viability and migration rates

As described previously,[22] proliferation/viability in response to metformin and/or simvastatin was analysed using AlamarBlue reagent (Biosource International, Camarillo, CA, USA). Briefly, cells were seeded in 96-well plates at a density of 5000 or 10,000 cells/well (for cell lines or primary cell culture, respectively) and serum-starved for 24 h. Then, proliferation was evaluated at 5% FBS every 24 h using the FlexStation-III system (Molecular Devices, Sunnyvale, CA, USA) for up to 72 h. Results were expressed as a percentage referred to vehicle-treated controls. It should be mentioned that dose–response experiments and IC_{50} determination of metformin and simvastatin revealed that the drug

Parameters	Control samples	High-grade (grade III) astrocytomas samples	GBM samples
Fresh samples			
Patients (n)	4	4	4
Gender (M/F)	1(25%)/3(75%)	3(75%)/1(25%)	1(25%)/3(75%)
Age at surgical intervention (mean ± std. dev.)	51.2 ± 5.38	52.25 ± 16.46	59.75 ± 14.59
Grade IV	–	0	4
%Ki67 (mean ± std. dev.)	–	11.7 ± 2.89	26.3 ± 9.46
% of P53 positive	–	75%	100%
% of PAGF positive	–	75%	100%
% of IDH1 positive	–	25%	25%
GBM survival rate (Mean - Months)	–	30.08	11.72

Table 3: **Demographic and clinical characterization of the brain samples [normal-control, glioblastoma (GBM), and high-grade III astrocytomas (AIII)] used to obtain primary human sample-derived cell cultures (n = 12).**

concentration that evoked half maximal inhibitory response was 10 mM and 10 μM, respectively (Fig. 1a–d).

Migration was evaluated by wound-healing assay, as previously reported,[22] only in the U-118 MG and not the U-87 MG cell line due to its incompatibility to obtain migration data with this assay. Briefly, cells were serum-starved for 24 h to achieve cell synchronization, and then, the wound was made using a 200 μL sterile pipette tip. Wells were rinsed and cells were incubated for 6 h and 24 h with supplemented medium without FBS. Wound-healing was compared with the area just after the wound was performed. Images of the scratch were randomly acquired along the wound per well to calculate the area by ImageJ software (RRID:SCR_003070).

Apoptosis analysis

Apoptotic rate in response to metformin and/or simvastatin in GBM cell lines (5000 cells/well onto white-walled multi-well luminometer plates) was performed using Caspase-Glo-3/7 Assay (Promega Corporation, #G8091), as previously reported.[22] Cells were serum-starved for 24 h and subsequently, treated for 24 h before the measurements which were carried out following the manufacturer's protocols.

Tumoursphere formation assay

Tumoursphere formation assay was carried out in GBM cell lines, as previously reported.[22] Briefly, 2000 cells/well were seeded in Corning Costar 24-well ultra-low attachment plates with DMEM/F12 medium supplemented with EGF (20 ng/μL; Merck KGaA). EGF, metformin, and/or simvastatin treatments were added 24 h later and refreshed every 3 days. The number and the area of tumourspheres were determined after 10 days of incubation using an inverted optical microscope and ImageJ software (RRID:SCR_003070).

VEGF secretion

Human VEGF-ELISA Kit (ThermoFisher-Scientific, #RAB0507-1 KT) was used to quantify VEGF secretion in GBM *in vitro* models treated with metformin and/or simvastatin, following the manufacturer's instructions. Briefly, cells were seeded in a 24-well plate (150,000 cells/well) and incubated for 24 h in medium with FBS. Then, cells were starved in medium without FBS, and treatments were added under the same condition for 24 h. The medium was collected, centrifuged, and stored at –20 °C for VEGF determination.

Clonogenic assay

Clonogenic assay was performed in GBM cell lines. Briefly, 300 or 500 cells/well (6-well plate) of U-87 MG and U-118 MG were seeded, respectively. 24 h later, metformin and/or simvastatin were administered, and cells were incubated for 10-12 days. Then, the medium was replaced, cells washed with PBS and crystal violet 0.5% plus glutaraldehyde 6% was added and incubated 45 min at room temperature. Finally, cells were rinsed 3 times with distilled water and left to dry at room temperature. Colonies were measured by ChemiDoc-XRS + System (Bio-Rad, Hercules, CA; SCR_019690) and analysed using ImageJsoftware (RRID:SCR_003070).

Flow cytometry

Flow cytometry was assessed to analysed cell cycle arrest and apoptosis induction. Briefly, U-87/U-118 MG cells were seeded in 6-well plates (~2×10^5 cells/well) and treated with metformin and/or simvastatin for 48 h. Cells were trypsinized, and then washed with PBS. To evaluate the cell cycle arrest cell were also fixed with ice-cold 70% ethanol at 4 °C. FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA; #556547) and 7-aminoactinomycin D (7-AAD) (Invitrogen, Waltham, MA; #A1310) at 10 μg/mL in PBS were used to quantify apoptosis and cell cycle arrest, respectively, according to the manufacturer's instructions. To determine the percentage of apoptotic cells Annexin V+/PI– cells were designated as early apoptotic cells, whereas Annexin V+/PI + cells were identified as late apoptotic cells. Quantification of the

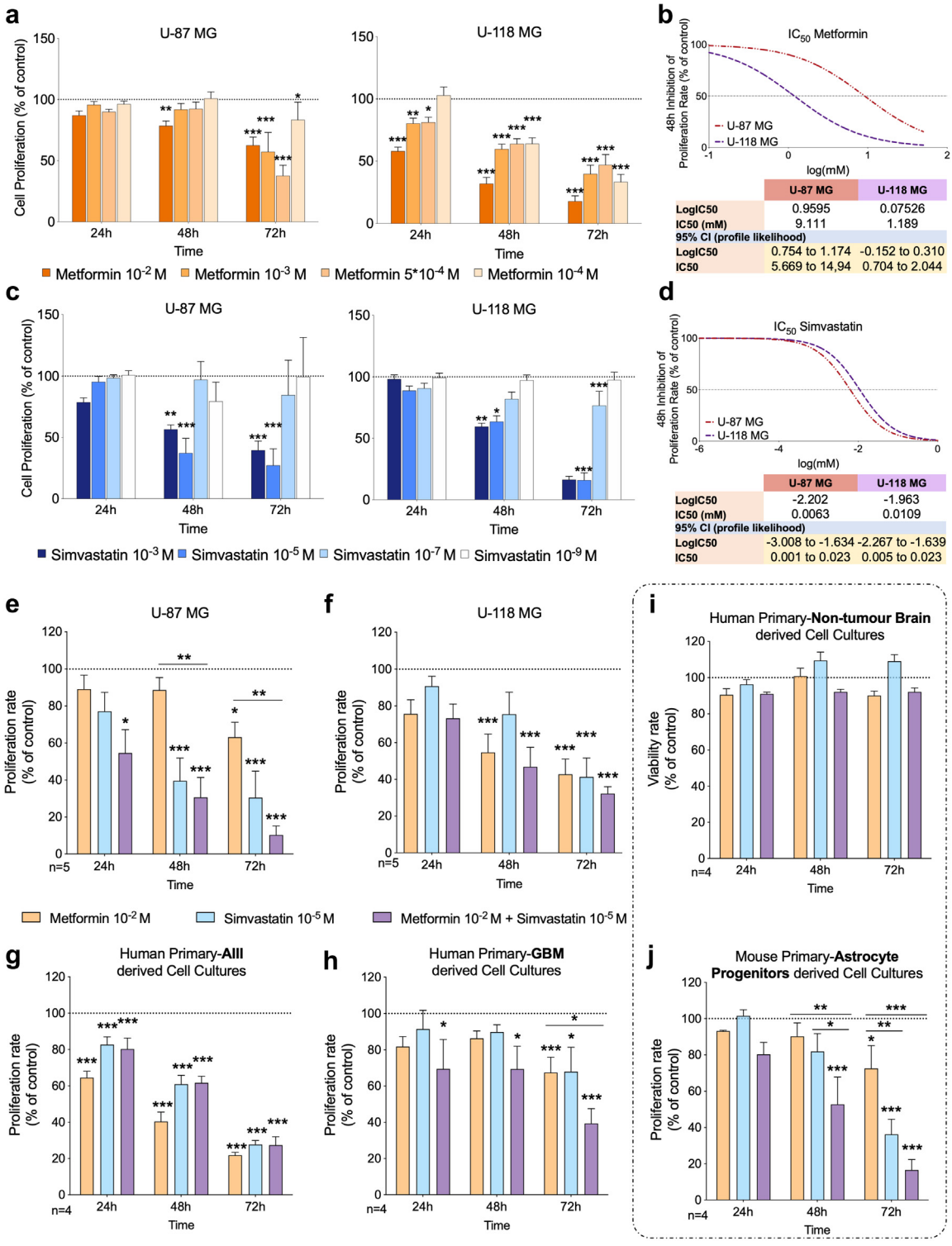

Fig. 1: **Treatments with metformin and/or simvastatin significantly decrease proliferation rate in different glioblastoma (GBM) cell models and high grade (III) astrocytomas, but not the viability in non-tumour brain cells *in vitro*.** **(a)** Metformin dose–response carried out in U-87 MG and U-118 MG cells (n = 4). **(b)** IC_{50} of metformin *in vitro* in U-87 MG and U-118 MG cells. **(c)** Simvastatin dose–response carried out in U-87 MG and U-118 MG cells (n = 4). **(d)** IC_{50} of simvastatin *in vitro* in U-87 MG and U-118 MG cells. Proliferation/viability rates of GBM cell lines [U-87 MG **(e)** and U-118 MG **(f)**; n = 5], primary patient-derived cell cultures from AIII [grade III **(g)**, n = 4] and grade IV-GBM [**(h);**

percentage of cells in G0/G1, S, and G2/M phases of the cell cycle was performed using the Watson Pragmatic model.[23] Cell debris and aggregates were excluded using a sequential gating strategy based on FSC-A versus SSC-A followed by FSC-A versus FSC-H (apoptosis analysis) and FSC-A versus SSC-A followed by 7AAD-A versus 7AAD-W (cell cycle analysis). Cells were analysed using BD LSRFortessa SORP flow cytometer (BD Biosciences San Jose, CA; SCR_018655) and FlowJo v10.8.1 software (BD Biosciences, San Jose, CA; SCR_008520).

Human phosphorylation pathway profiling array

The phosphorylation pathway profiling array was performed using the Human Phosphorylation Pathway Profiling Array C55 kit (Raybiotech, Inc. #AAH-PPP-1-8), following the manufacturer's protocols and as previously reported.[24] Antibody array has been validated by the manufacturer using appropriate cell lysates. Briefly, membranes were incubated with blocking buffer at 25 °C for 30 min and then incubated overnight at 4 °C with 1 mL of U-87 MG cell lysates (1/4 dilution). Then, the membranes were incubated with Detection Antibody Cocktail at room temperature for 2 h and next with horseradish peroxidase (HRP)-labelled anti-rabbit secondary antibody at room temperature for 2 h. The signals were collected after adding ECL-reagent by ImageQuant LAS 4000 (GE Healthcare). Densitometric analysis of the array spots was carried out with ImageJ software using positive control spots as a normalizing factor. In addition, pAMPK, pTP53 and pPTEN were validated by western blot as previously reported (Fig. S1a).[25]

Western blotting

To determine protein levels, cell pellets were resuspended using pre-warmed SDS-DTT sample buffer [62.5 mM Tris–HCl (#10708976001), 2% SDS (#71726), 20% glycerol (#17904), 100 mM DTT (#D0632-5G) and 0.005% bromophenol-blue (#B0126)] followed by sonication for 10s and boiling for 5 min at 95 °C. Proteins were separated by SDS-PAGE electrophoresis with different poly-acrylamide percentage and transferred to nitrocellulose-membranes (Millipore, #1704270). Membranes were blocked with 5% non-fat dry milk (#T145.3) in Tris-buffered saline/0.05%-Tween-20 (#93773) and incubated with the primary-antibody [phosphoTP53 (CST, #9284), phosphoPTEN (CST, #9551), phosphoCDK4 (CST, #12790), phosphoAMPK (CST, #2535),], and their appropriate secondary-antibodies [anti-rabbit (CST, #7074)]. Proteins were developed using an enhanced chemiluminescence-detection system (GE-Healthcare) with dyed molecular-weight markers. A densitometric analysis of the bands was carried out with ImageJ 1.8.0_172 software[26] using total-protein loading (Ponceau-staining, #P3504-10G) as normalizing factor, as previously reported[27] represented using a heatmap.

Preclinical GBM mouse model, and haematoxylin & eosin (H&E) examination

A preclinical xenograft mouse model to test the anti-tumour actions of metformin and/or simvastatin was developed. Specifically, 5-week-old ATHYM-Foxn1nu/nu mice (Charles River Laboratories; RRID:IMSR_CRL:490) were injected subcutaneously with 3×10^6 of U-87 MG cells in both flanks [resuspended in 100 µL of basement membrane extract (Trevigen, #3432-010-01)]. Once the tumour was clearly measurable, each mouse received an intra-tumour injection (8 days after cell-inoculation) with 50 µL of metformin (10^{-2} M), simvastatin (10^{-5} M) or the combination into one flank and vehicle (Dulbecco's Phosphate-Buffered Saline, D1408; used as control) into the other flank (n = 5 mice/treatment condition, with randomly group allocation of each mouse). The treatment was administrated every 2 days for 16 days (total of 8 intra-tumour injections). Tumour growth was monitored every 2 days using a digital calliper. Mice were sacrificed and each tumour was dissected, fixed, and sectioned for histopathologic examination after H&E-staining. Examination of mitosis number and apoptosis was performed by expert pathologists. Additional tumour pieces were placed in liquid nitrogen and then frozen at –80 °C until RNA isolation using Trizol-reagent, as previously reported.[22,25]

RNA isolation and customized quantitative real-time PCR (qPCR) dynamic array based on microfluidic technology

Total RNA from *in vitro*/*in vivo* samples treated with metformin and/or simvastatin was extracted with TRIzol-Reagent (Thermo-Fisher Scientific, Waltham, MA, USA). Total RNA concentration and purity were assessed with the Nanodrop-One Microvolume UV–Vis Spectrophotometer (Thermo-Fisher Scientific). Total RNA was retrotranscribed by using random hexamer primers and the RevertAid Reverse-Transcription Kit (Thermo-Fisher Scientific). A qPCR dynamic array based on microfluidic technology (Fluidigm, #BMK-M-48.48; RRID:SCR_022658) was implemented to determine the expression levels of key genes belonging to the

n = 4], primary non-tumour brain cell cultures [**(i)**; n = 4] and mouse primary-astrocytes progenitor derived cells [**(j)**; n = 4] in response to metformin, simvastatin, and their combination compared to vehicle-treated controls. Four technical replicates (tr) were assessed in each condition. Data represent medians (interquartile range) or means ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001, were set as statistically significant differences vs. control conditions.

Senescence-Associated Secretory Phenotype (SASP) pathway (n = 32). Moreover, qPCR to obtain expression levels of key splicing components and apoptosis-related genes were also performed as previously reported.[22] Specific primers for all human transcripts used in this study (including three housekeeping genes) were specifically designed with the Primer3-software (RRID:SCR_003139; Supplementary Tables S1 and S2). A normalization factor was calculated with the expression levels of *ACTB*, *HPRT*, *GAPDH*, and *Rps11* and the GeNorm 3.3 software, as previously reported.[22]

Conditioned media from metformin, simvastatin, and metformin + simvastatin treated cells

Conditioned media from human GBM cells treated with metformin and/or simvastatin were obtained to uncover the influence of SASP in cell proliferation and telomere length. Specifically, 100,000 cells/well (U-87 MG/U-118 MG) were seeded in 12 well-plates and starved in medium without FBS for 1 h. Then, cells were treated (5% FBS) with vehicle, metformin, and/or simvastatin for 24 h. Subsequently, the media were replaced with a drug-free (5% FBS) medium, and collected after 24 h, centrifuged to remove cells, and stored at −20 °C.

Telomere length determination

The telomere length determination was carried out using Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienceCell, #8918), following the manufacturer's instructions. DNA was obtained from 50,000 cells/well (U-87 MG/U-118 MG) cultured in a 24-well plate and treated with conditioned media taken from cells treated with metformin and/or simvastatin for 72 h. Subsequently, cells were trypsinized, centrifuged, and resuspended in 450 µL of Digestion Buffer (3 mL 5 M NaCl; 30 mL 0.5 M EDTA; 94.5 mL distilled water; 7.5 mL 1 M TRIS; 15 mL 10% SDS) and 25 µL of Proteinase-K. This mix was incubated at 55 °C overnight, then Proteinase K was blocked using 190 µL NaCl 5 M. Finally, DNA was obtained through isopropanol precipitation. Total DNA concentration and purity were assessed by using Nanodrop-One Microvolume UV–Vis Spectrophotometer (Thermo-Fisher Scientific).

β-galactosidase-senescence assay

Senescence Cells Histochemical Staining Kit (#CS0030) was used in U-87 MG/U-118 MG cells, following the manufacturer's protocols. Briefly, 4000 cells/well in a 24-well plate were treated with control, metformin and/or simvastatin for 48 h. β-galactosidase-senescence assay was also measured in cells treated with conditioned media from cells treated with the same treatments for 48 h. Then, an inverted microscope coupled to a digital camera was used to take photographs to visualize and identify blue cells to calculate the number of senescent cells (β-gal positive) in 3 randomly selected fields.

Bioinformatics and statistical analysis

Data were evaluated for heterogeneity of variance using the Kolmogorov–Smirnov test. Statistical differences were assessed by t-test and One-Way/Two-Way ANOVA. All statistical analyses were performed using Prism software 8.0 (GraphPad Software, La Jolla, CA; RRID:SCR_002798). For survival analysis, statistical differences were assessed by Log-rank (Mantel–Cox test). Values of $P < 0.05$ were considered statistically significant. Data represent medians (interquartile range) or means ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001, were set as statistically significant differences vs. control conditions. Multiple linear regression analysis, principal component analysis, and setting control variables were performed in our cohort of patients to minimize the effects of directly and non-directly observable potential confounding variables (i.e., gender, age at surgical intervention, surgical resection, relapsed patients, %Ki67, and IDH1 status; Fig. S1b). Additive-synergic effects of the combination treatment were evaluated with the combination index equation: CI=(D)1/(Dx)1 + (D)2/(Dx)2[28] where (D)1 is the dose of drug 1 to produce 50% cell kill in combination with (D)2; (Dx)1, the dose of drug 1 to produce 50% cell kill alone; (D)2, the dose of drug 2 to produce 50% cell kill in combination with (D)1; (Dx)2, the dose of drug 2 to produce 50% cell kill alone. The CI value determines the drug combination effect, CI > 1.3 indicates antagonism, CI = 1.1–1.3 moderate antagonism, CI = 0.9–1.1 additive effect, CI = 0.8–0.9 slight synergism, CI = 0.6–0.8 moderate synergism, CI = 0.4–0.6 synergism, and CI = 0.2–0.4 strong synergism. In the Human Phosphorylation Pathway Profiling Array, a fold change (log2) of 0.2 was considered as a significant threshold. Clustering, Principal Component Analysis (PCA), and Partial least squares-discriminant analysis (PLS-DA) analyses were performed with MetaboAnalyst Software v.4.0 (McGill University, Quebec, Canada; RRID:SCR_015539). Clustering and enrichment analysis was carried out using STRING v11.0 (https://string-db.org; RRID:SCR_005223), Networkanalyst v3.0 (https://www.networkanalyst.ca; RRID:SCR_016909), and Reactome Database v76 (https://reactome.org; RRID:SCR_003485). Clinical trials information was extracted from https://clinicaltrials.gov (May, 2022; RRID:SCR_002309). Circle plots were implemented in R language 3.5 (RRID:SCR_001905) using the following packages: tidyverse, viridis, and ggplot2.

Role of funding source

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Results

Treatments with metformin or simvastatin, and specially their combination, decreased the proliferation rate of different GBM models but not the viability of non-tumour control cells

Human GBM cell lines (U-87 MG/U-118 MG), primary patient-derived cell cultures [from GBM and high-grade III astrocytomas (AIII)], and primary non-tumour brain cell cultures were initially used to test the direct effects of metformin and/or simvastatin on proliferation/viability rate (Fig. 1). Dose–response experiments of metformin and simvastatin (both able to cross the blood–brain barrier, being simvastatin the unique statin that has been consistently reported to have this capacity[29,30]) in GBM cell lines demonstrated that metformin and simvastatin dose- and time-dependently inhibited the proliferation of both GBM cell models. Specifically, the optimal concentration was 10 mM for metformin and 10 μM for simvastatin (i.e., the lowest doses tested with a maximal effect determined by nonlinear regression; Fig. 1a and b and c and d, respectively), which is in accordance with previous *in vitro* studies in other cancer pathologies.[10–15] Therefore, these doses were used for subsequent experiments.

Metformin and simvastatin treatments alone time-dependently decreased the proliferation rates of both human GBM cell lines (U-87 MG/U-118 MG; Fig. 1e and f), and of primary patient-derived cell cultures from grade III (AIII) and grade IV-GBM (Fig. 1g and h, respectively)]. Remarkably, by and large, the combination metformin + simvastatin additively decreased the proliferation of these cell models compared with the individual treatments, especially in the most aggressive ones [i.e., grade IV-GBM vs. grade III cells (Fig. 1g and h), and in U-87 MG (more aggressive type) vs. U-118 MG cells (Fig. 1e and f)]. Importantly, metformin or simvastatin alone, or in combination, did not statistically alter the viability of non-tumour control cells [$P > 0.1$ at 72 h (t-test); (Fig. 1i)].

Next, the well-characterized and validated mouse primary-astrocyte progenitor derived cell culture (Fig. S1c and d) was used as a model of tumour precursor cells that contribute to the GBM malignancy and therapeutic resistance.[31] Specifically, we found similar results to that previously observed in human GBM cell models since metformin, simvastatin, and especially their combination, significantly decreased the proliferation of these mouse astrocytes progenitor cells (Fig. 1j).

Treatments with metformin, simvastatin, and especially their combination, decreased other key functional parameters *in vitro*

Metformin and simvastatin alone, but especially their combination, significantly reduced GBM cell migration (U-118 MG), being these effects more pronounced at 6 h than at 24 h (Fig. 2a). Likewise, metformin treatment significantly induced apoptosis in U-87 MG (but not in U-118 MG) cells, while simvastatin and metformin + simvastatin significantly increased apoptosis in both GBM cell lines (Fig. 2b), being this induction significantly more elevated in response to metformin + simvastatin treatment compared with the individual treatments in U-87 MG (Fig. 2b). The apoptosis induction was also supported by: 1) flow cytometry analysis which showed that simvastatin alone and metformin + simvastatin treatments were the most efficient inducing apoptosis (Fig. S2a–d), and 2) an upregulation of key apoptosis-related genes in all our *in vitro* models after metformin or simvastatin alone, and especially after their combination (Fig. S2e and f). Tumoursphere formation assays used to quantify the proliferation capacity of cancer stem/progenitor cells revealed that metformin alone decreased tumoursphere number and area, while simvastatin alone and the combination of metformin + simvastatin totally impaired the tumoursphere formation (Fig. 2c) in GBM cells. We also observed in our *in vitro* models a clear decrease in VEGF secretion in response to metformin and simvastatin alone (being simvastatin treatment less effective on cell lines; Fig. 2d). Nevertheless, metformin + simvastatin combination showed a clear anti-tumour effect in terms of VEGF secretion (Fig. 2d).

The potential of these drugs to induce senescence phenotype was also tested by a β-galactosidase assay. Our results demonstrate that all the treatments induced a senescence state in GBM cells, being the effect of metformin + simvastatin combination stronger than metformin and simvastatin alone (Fig. 2e). Additionally, the induction of senescence state after the treatment with metformin and simvastatin was corroborated with an increment of cell arresting in G0/G1 cell cycle phase and a decrease of CDK4 protein expression in both cell lines, but especially when metformin and simvastatin were combined (Fig. 2f, Fig. S1a).

Moreover, the analysis of the colony formation assays unveiled that metformin alone, but especially simvastatin alone and the combination of both drugs significantly decreased the number of colonies in GBM cells (U-87 MG/U-118 MG; Fig. 2g). Therefore, our results revealed that metformin and simvastatin treatments impair the progression, development, and aggressiveness of GBM cells inducing a senescence phenotype *in vitro*, and that these effects are, in general, significantly stronger when both drugs are combined (Fig. 2h).

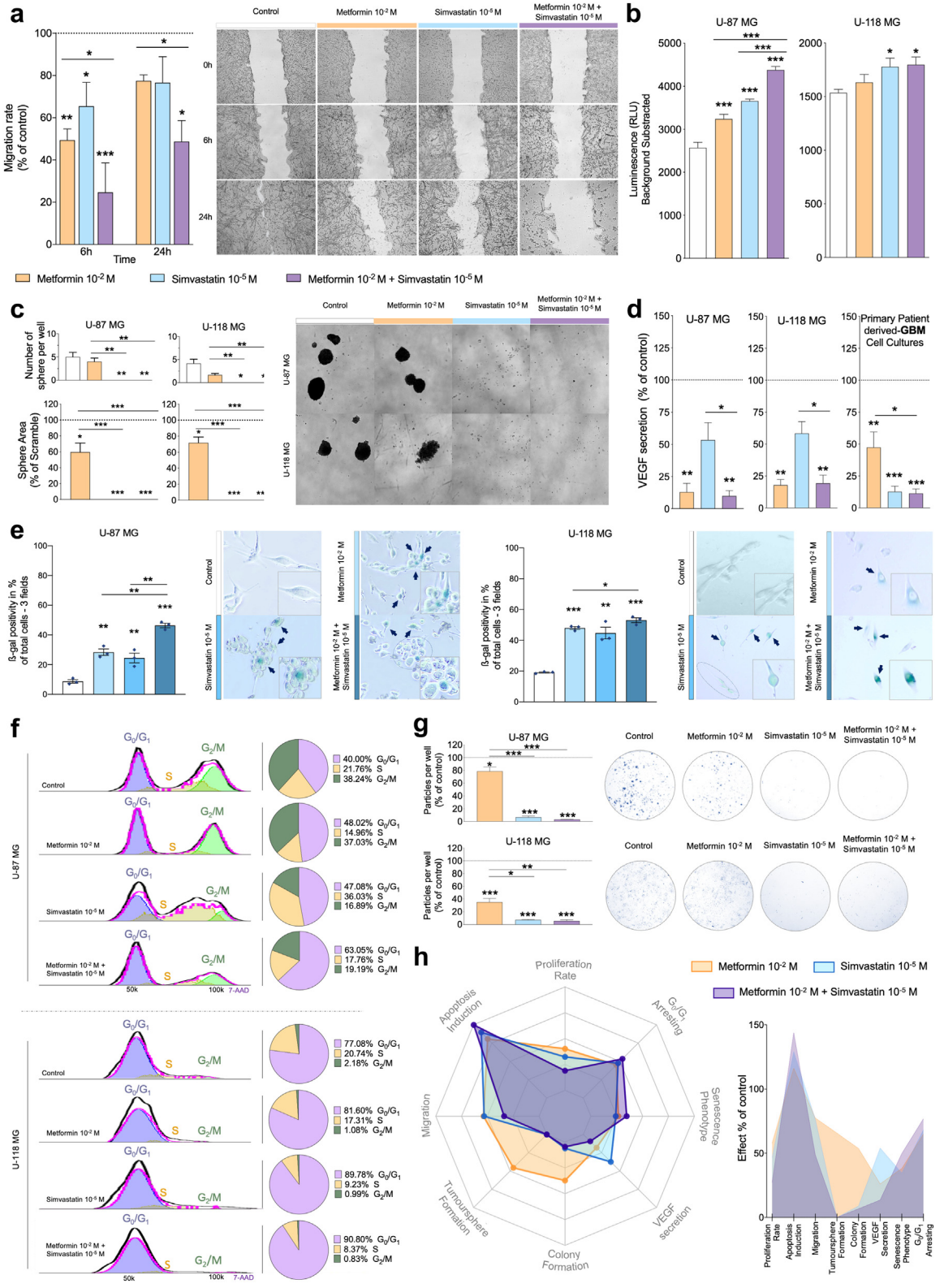


***Fig. 2*: Treatments with metformin, simvastatin, and specially their combination, decreased key functional parameters in glioblastoma (GBM) cells *in vitro*.** Effect of the treatment with metformin, simvastatin, and their combination compared to vehicle-treated control cells in:

Treatments with metformin and/or simvastatin modulated the phosphorylation levels of different proteins associated with key oncogenic pathways in GBM cells

Phosphoprotein analysis in GBM U-87 MG cells uncovered different molecular pathways that could be involved in the observed antitumour actions of metformin and/or simvastatin in GBM cells (Fig. 3a and Fig. S3). As expected, metformin exerted its actions through the activation of AMPKa (PRKAA1), which possibly induced the subsequent downregulation of several key downstream elements of the AKT pathway [including RPS6KB1 (p70S6K), PRAS40 (AKT1S1), PTEN, RPS6] and the increase in TP53, MAP14, JNK, and ERK1/ERK2 (MAPK1/MAPK3) levels (Fig. 3a,b and Fig. S4). Moreover, metformin treatment tended to induce an overall increase in the phosphorylation levels of various TGFβ proteins (i.e., ATF2, FOS, SMAD1, SMAD2, SMAD4, SMAD5), but these differences did not reach the significant threshold (Fig. 3a,b and Fig. S4).

Simvastatin treatment also induced a decrease in the phosphorylation levels of different elements of the AKT pathway, wherein some of these changes were similar to that previously found in metformin-treated cells (i.e., RPS6KB1, AKT1S1, and RPS6), but also unique in some cases (i.e., RAF1, Fig. 3a,c and Fig. S4). Moreover, as previously observed with metformin, simvastatin treatment induced a significant increase in ERK1/ERK2 phosphorylation levels and an overall, but significant, activation in the phosphorylation levels of various TGFβ proteins (Fig. 3a,c and Fig. S4). Furthermore, simvastatin treatment induced upregulation in the phosphorylation levels of some elements of the JAK/STAT pathway [i.e., TYK2 and PTPN11 (SHP2), Fig. 3a,c and Fig. S4].

Remarkably, while treatment with metformin or simvastatin alone did not significantly alter the activity of many of the elements belonging to these signalling pathways, the combination of both drugs significantly altered the activity of these elements [i.e., JAK/STAT-pathway (EGFR, JAK1, JAK2, SCR, STAT1, STAT5, STAT6), NF-κB-pathway (EIF2A, HDAC2, NF-KBIA, MSK1, TAK1), and TGFβ-pathway (ATF2, FOS, SMAD1, SMAD2, SMAD4, SMAD5)] and/or drastically increased their phosphorylation levels as compared with the individual treatments [i.e., MAPK-pathway (ERK1/ERK2, TP53, and CREB), JAK/STAT-pathway (particularly, PTPN11-SHP2)] (Fig. 3a,d and Fig. S4). These results showing the alteration in the phosphorylation levels of 22 proteins belonging to these key oncogenic signalling pathways (Fig. 3e), especially the drastic (i.e., additive) activation of ERK1/ERK2 and TGFβ-pathway together with the previous *in vitro* results (Fig. 2), suggest a well-defined induction of a senescence state[32–35] in GBM cells co-treated with metformin and simvastatin.

Co-treatment with metformin and simvastatin altered the expression levels of key elements involved in SASP and the spliceosome in GBM cells

We next interrogated whether the treatments with metformin and/or simvastatin were able to induce an alteration in the expression levels of key genes involved in the SASP, and spliceosome in GBM cells [cell lines (U-87 MG/U-118 MG) and in primary-derived GBM cell cultures]. First, we measured a battery of 32 key SASP-related genes[36] by microfluidic qPCR in cells treated with metformin and/or simvastatin. A non-supervised clustering bioinformatic approach (random-forest algorithm; Fig. S5a) and the Principal Component Analysis (PCA; Fig. S5b) with the expression levels of the 32 SASP genes did not reveal a clear clustering or discrimination pattern between the different treatments. However, when all the GBM experimental cell models treated with metformin and/or simvastatin were grouped (mean values of U-87 MG + U-118 MG + primary-derived cell cultures), a clear differential alteration was found across each treatment, especially in the combination treatment (Fig. 4a). In line with this, the molecular fingerprint comprised by the combination of the 20 genes, whose expression was most altered, revealed a differential and unique expression pattern of these SASP-related genes in response to the co-treatment with metformin + simvastatin compared with the individual treatments and vehicle-treated controls [random-forest algorithm (Fig. 4b) and Partial Least Squares-Discriminant Analysis (PLS-DA); Fig. 4c]. Moreover, the Variable Importance in Projection (VIP)

(a) Migration rate of GBM U-118 MG cells ([n = 5; 3 technical replicates (tr)] representative images of the migration capacity are included); **(b)** Apoptosis induction in GBM U-87 MG and U-118 MG cells (n = 3); **(c)** Sphere number and area in GBM U-87 MG and U-118 MG cells (n = 3; tr = 4; representative images of formation of tumourspheres are shown); **(d)** VEGF secretion in U-87 MG, U-118 MG, and primary patient-derived GBM cells (n = 4; tr = 2); and **(e)** Percentage of β-galactosidase positive cells (cells positive in blue) in GBM U-87 MG and U-118 MG cells (n = 3; tr = 2) in response to metformin, simvastatin, and their combination compared to control condition and their corresponding representative images. **(f)** Percentage of cells in each cell cycle phase (right panel) determined by the Watson Pragmatic model (left panel) in U-87 MG and U-118 MG cells (n = 3; tr = 1) after 72 h of treatment with metformin, simvastatin, and their combination compared to control condition. **(g)** Colony formation in GBM U-87 MG and U-118 MG cells (n = 3; tr = 3). **(h)** Summary of the effects of metformin and/or simvastatin treatments (vs. control) on different functional parameters associated with the progression, development, and aggressiveness of GBM cells. Data represent medians (interquartile range) or means ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001, were set as statistically significant differences vs. control conditions.

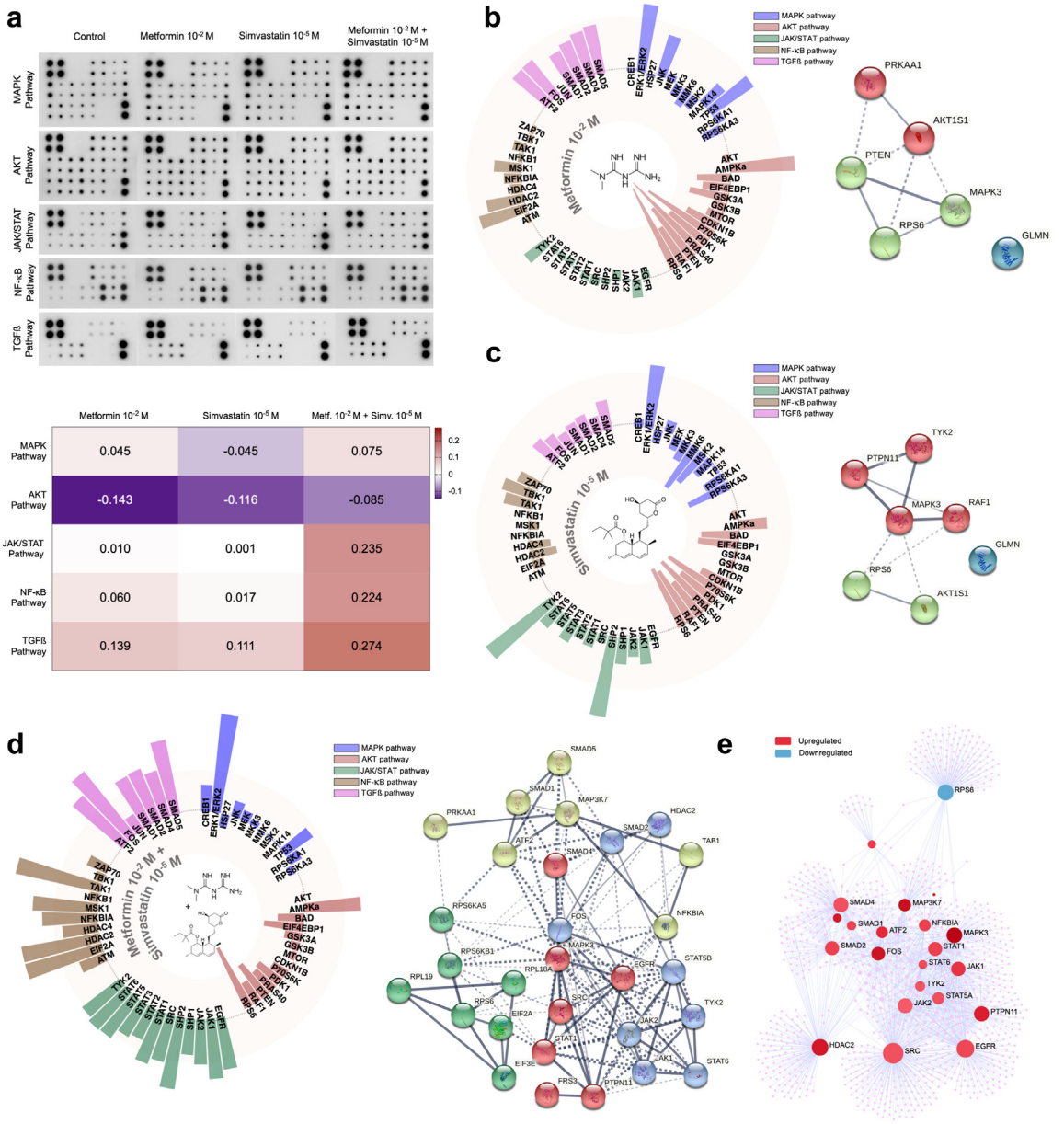


***Fig. 3*: Treatment with metformin, simvastatin, and their combination altered the phosphorylation levels of different proteins associated with different key oncogenic pathways in GBM cells. (a)** *Upper panel*: Membranes of the phosphoprotein array showing the spots (phosphorylation levels) quantified of 5 oncogenic signalling pathways (MAPK, AKT, JAK/STAT, NF-kB, and TGFβ pathways; 55 phosphorylated proteins) in response to the treatments with metformin, simvastatin, and their combination (24 h, n = 3; tr = 1, pooled) in GBM U-87 MG cells. *Lower panel*: Heatmap showing the fold-change mean corresponding to each pathway in response to metformin, simvastatin, and their combination. **(b-d)** Log2 (Fold Change) of individual phosphorylation protein levels in response to metformin **(b)**, simvastatin **(c)**, and their combination **(d)** compared with vehicle-treated controls cells [threshold: log2(FC) = 0.2]. The significantly altered phosphorylated proteins were analysed using the STRING database. **(e)** Functional association network of the significantly dysregulated proteins (upregulated in red and downregulated in blue) in response to the combination of metformin and simvastatin using the Network Analyst software.

score (Fig. 4d) of PLS-DA (Fig. 4c) revealed that *IL6*, *CXCL3*, *CCR3*, and *CDKN2D* were the highest-score components that were differentially expressed across the 4 treatment conditions in GBM cells (Fig. 4d).

Based on previous data indicating the important role of the splicing in senescence,[37] together with the fact that metformin and simvastatin treatments have been linked to the modulation of spliceosome component,[38–44]

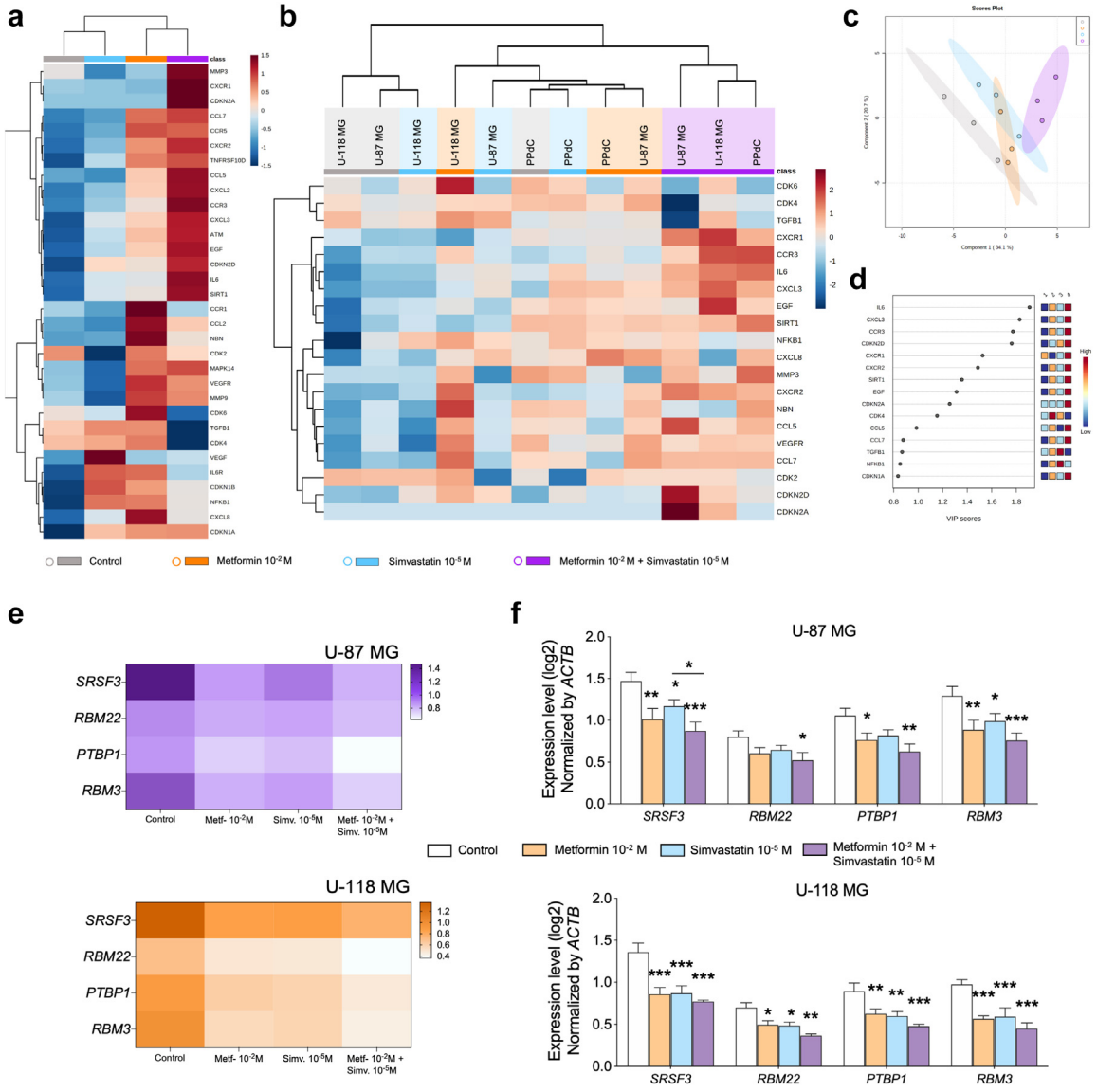


Fig. 4: **The combination of metformin and simvastatin altered the expression levels of key elements involved in the Senescence-Associated Secretory Phenotype (SASP), and in the splicing process in GBM cells. (a)** Hierarchical heatmap generated using the expression levels of 32 SASP genes in all GBM experimental cell models treated with metformin, simvastatin, and the combination of both drugs (n = 3; tr = 2; mean value U-87 MG + U-118 MG + primary-derived cell cultures). Hierarchical heatmap showing the expression levels of 20 top altered genes **(b)** and Partial Least Squares Discriminant Analysis (PLS-DA) **(c)** of the expression of SASP genes in response to metformin, simvastatin, and their combination in GBM cells [cell lines (U-87 MG and U-118 MG) and primary patient-derived GBM cells (PPdC); data represent the mean value of 3 experiments (4 treatment conditions/experiment) in each GBM cell model]. **d)** Variable Importance in Projection (VIP) score of the expression levels of SASP key genes in GBM cells. **e)** Heatmap generated using the expression levels of 4 spliceosome components and **(f)** individual expression levels of these components in response to metformin, simvastatin, and their combination in GBM cells (n = 3; tr = 2; data represent mean value of U-87 MG + U-118 MG). Data represent medians (interquartile range) or means ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001, were set as statistically significant differences vs. control conditions.

we next sought to determine whether the expression levels of the 4 spliceosome components that we have recently reported to be the most important in GBM pathophysiology (*SRSF3*, *RBM22*, *PTBP1*, and *RBM3*)[22] were altered in response to metformin and/or simvastatin in GBM cells (Fig. 4e and f). Our data revealed an overall downregulation in *SRSF3*, *RBM22*, *PTBP1*, and *RBM3* levels in response to metformin and simvastatin

alone, but especially to the co-treatment of metformin + simvastatin, in GBM cells (Fig. 4e and f).

Conditioned media from metformin and/or simvastatin treated cells induced a senescence state and telomere elongation in GBM cells

We interrogated whether the conditioned media derived from metformin and/or simvastatin treated cells were able to induce a senescence state or senescence-linked features such as an alteration in the elongation of telomeres in GBM cells using various experimental approaches (Fig. 5a). Our result indicated that the medium derived from cells co-treated with metformin + simvastatin completely blunted the proliferation capacity of GBM cells (U-87 MG/U-118 MG) compared to media derived from control- and metformin- or simvastatin-treated cells (Fig. 5b and c). Furthermore, the administration of conditioned media derived from cells treated with metformin and simvastatin alone or with metformin + simvastatin significantly increased the telomere length in GBM cells (Fig. 5d and e). In line with this, we found that the number of senescent cells (using the ß-galactosidase-senescence assay) were significantly higher in response to the conditioned media of metformin- and simvastatin-treated cells, but especially in response to the combination of both drugs (additive effect; Fig. 5f). In fact, the morphology of the cells treated with all these conditioned media (metformin, simvastatin, or metformin + simvastatin) was clearly altered (Fig. S5c), showing a senescence-morphology phenotype, and further suggesting a senescence state transition.

Beneficial clinical outcome of metformin and/or statins treatments on overall survival of patients with aggressive glioma and on tumour progression of a preclinical mouse model

Treatment of aggressive glioma patients (grade IV-GBM and grade III astrocytomas; n = 85) with metformin, statins, and their combination was associated with an overall longer median survival compared with patients who had not been treated with these drugs [metformin vs. non-treated patients, P = 0.3052; simvastatin vs. non-treated patients P = 0.6198; combination vs. non-treated patients P = 0.2316; Log-rank (Mantel–Cox test)]. Particularly, our data indicated that patients with aggressive gliomas not treated with these drugs had a worst median survival rate (i.e., 12.2 months) compared with patients treated with metformin (i.e., 13.0 months), statins (i.e., 13.8 months), and their combination (i.e., 15.5 months) (Table 2, and Fig. 6a).

The *in vivo* intra-tumour administration of metformin and simvastatin alone, but especially their combination, reduced tumour volume and weight compared with control-treated tumours in a preclinical-xenograft U-87 MG GBM model (Fig. 6b–d). Specifically, tumour volume clearly showed that GBM progression *in vivo* was stopped in tumours treated with metformin and simvastatin alone vs. control-tumours (that continued their progression; Fig. 6b). Notably, the combination treatment significantly reduced tumour volume and weight vs. control-tumours and vs. metformin or simvastatin treated tumours (Fig. 6b–d). Furthermore, mitosis number was decreased, while apoptosis was increased, in metformin, simvastatin, and especially their combination, treated tumours vs. control-tumours (Fig. 6e–g). This apoptosis induction was also corroborated by the upregulation of key apoptosis-related genes (Fig. S6). As previously observed *in vitro*, administration of metformin + simvastatin-treated tumours *in vivo* significantly altered the expression pattern of key genes involved in the SASP vs. control-treated tumours and vs. metformin- or simvastatin-treated tumours (Fig. 6h). In fact, the molecular fingerprint comprised by the expression of SASP-related genes revealed a differential and unique pattern in response to the co-treatment with metformin + simvastatin compared with the individual treatments and vehicle-treated controls (PLS-DA; Fig. 6i). Notably, the VIP score of PLS-DA revealed that several elements of the SASP-related genes were similarly altered *in vitro* (Fig. 4d) and *in vivo* (Fig. 6j) across the 4 treatment conditions (i.e., *CXCR1*, *CXCR2*, *IL6*, *CDKN2A*, *SIRT1*, *CDKN2D*, *CCR3* and *EGF*).

Finally, we also confirmed a similar alteration in the expression levels of the 4 key spliceosome components (*SRSF3*, *RBM22*, *PTBP1*, and *RBM3*) *in vivo* (Fig. 6k and l) than the previously observed *in vitro* on GBM cells (Fig. 6k and l) in response to the co-treatment with metformin + simvastatin compared with vehicle-treated controls.

Discussion

GBM is the most common and lethal primary malignant brain tumour in adults. Despite many efforts, gold-standard therapy has not significantly evolved since the introduction of adjuvant temozolomide in 2005 and the prognosis remains poor. Indeed, the average overall survival of GBM patients is under 18 months,[1] thereby reinforcing the necessity to find novel therapeutic approaches. An emerging approach against several cancer types is based on repositioning drugs already approved for other pathologies since they are faster and easier to translate to clinical practice than new drugs are. In this scenario, recent studies from our group have demonstrated that metformin and statins, 2 types of drugs commonly used to treat metabolism-related pathologies,[45–47] and specially their combination, exert strong antitumour actions in different cancer types.[10–14] Actually, the clinical relevance of the repurposing of these drugs in patients with GBM and brain neoplasms is supported by the number of clinical trials that are being currently implemented with metformin or statins

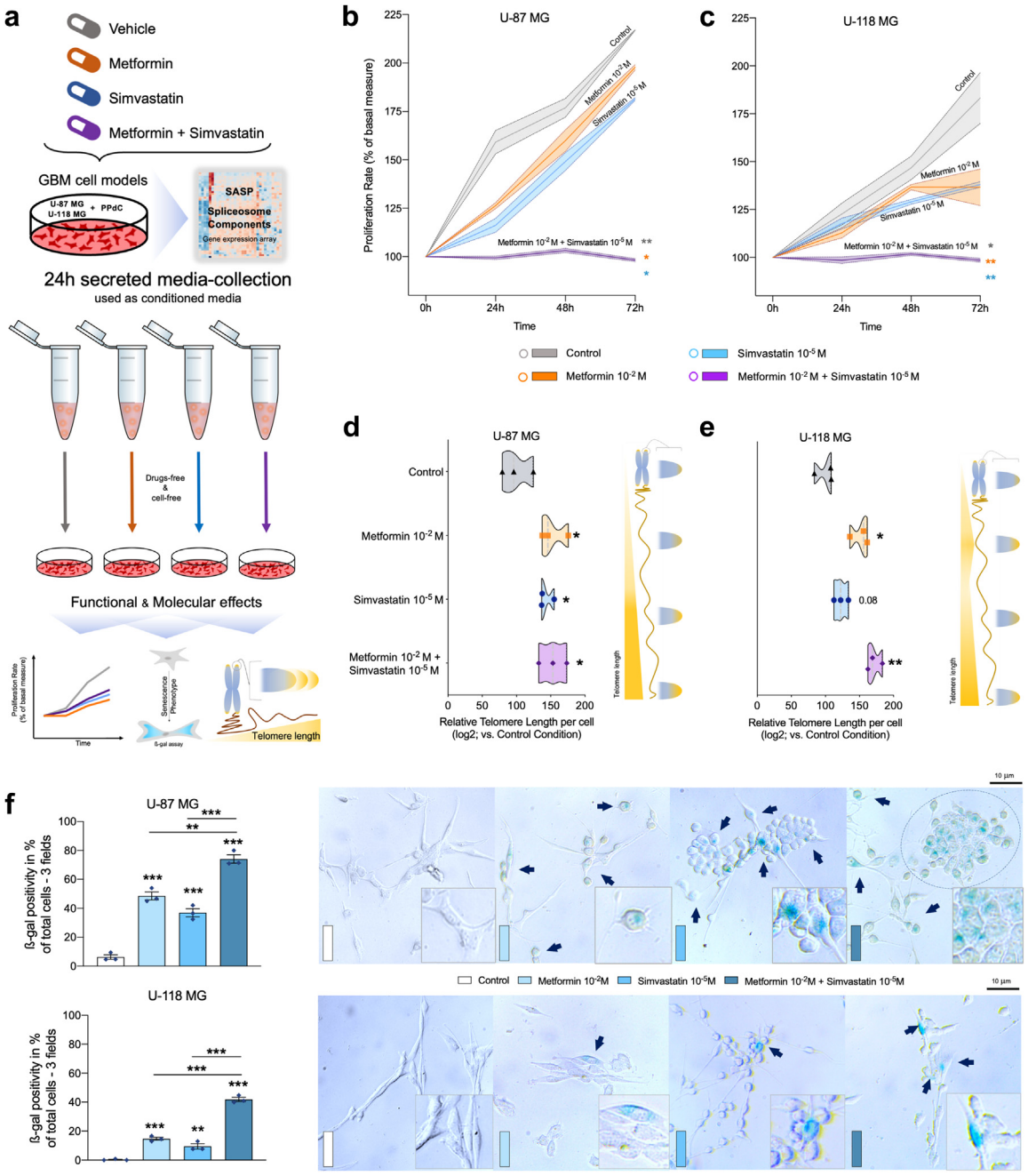
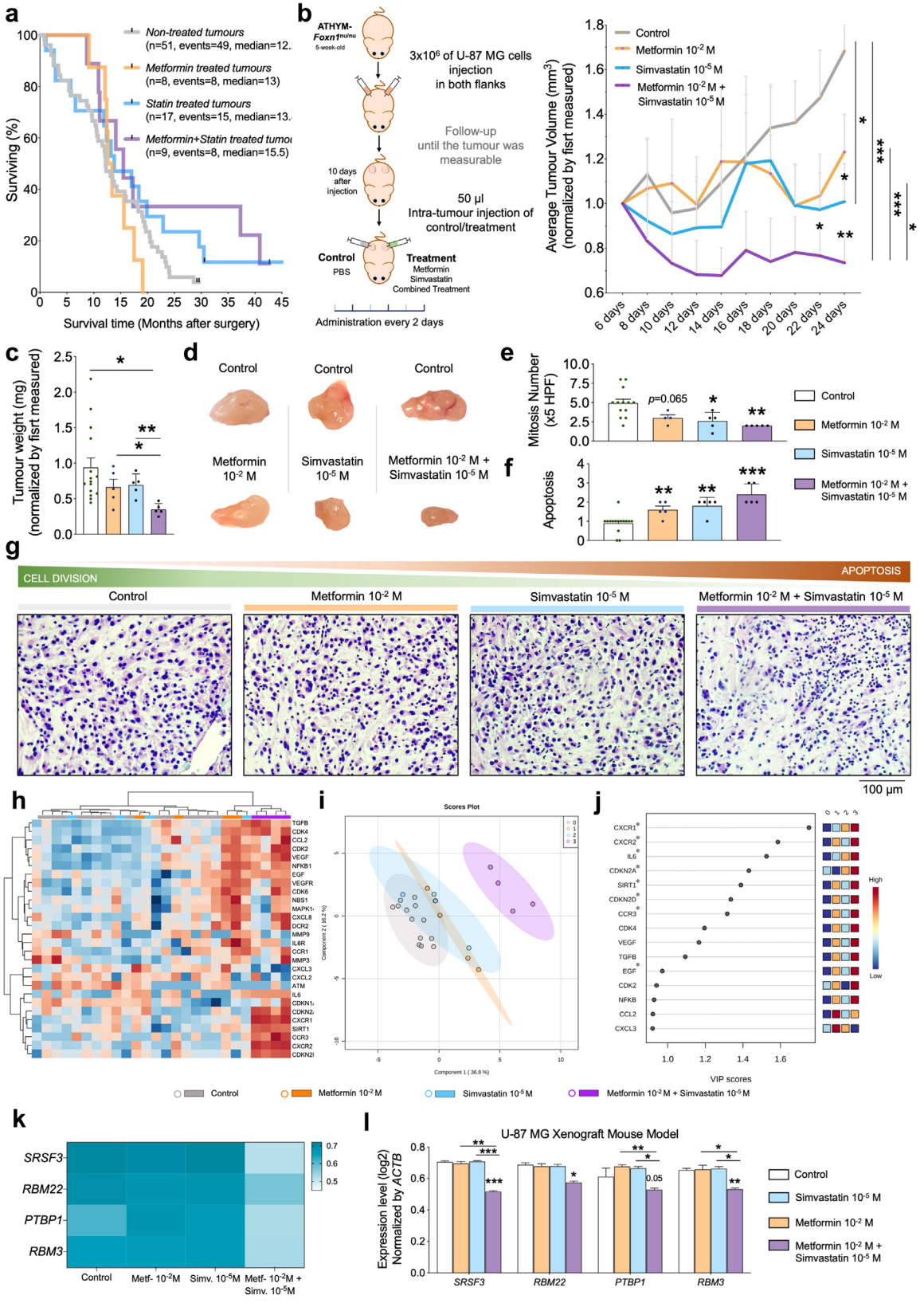


Fig. 5: **Conditioned media of metformin, simvastatin, and metformin + simvastatin treated cells induced a senescence state and an elongation in the telomere of GBM cells. (a)** Diagram showing the workflow carried out to determine whether the treatments with metformin and/or simvastatin are able to induce a senescence state or alteration in the elongation of telomeres in GBM cells. **(b)** Proliferation rates in U-87 MG and **(c)** U-118 MG cell lines (U-87 MG/U-118 MG) in response to the conditioned media of cells treated metformin, simvastatin, and their combination compared to control condition (n = 3; tr = 4). **(d)** Telomere length determination in U-87 MG and **(e)** U-118 MG cells in response to the conditioned media of cells treated metformin, simvastatin, and their combination compared to control condition (n = 3; tr = 2). **(f)** Percentage of β-galactosidase positive cells (cells positive in blue) in response to the conditioned media of cells treated metformin, simvastatin, and their combination compared to control condition and their corresponding representative images (n = 3; tr = 2). Data represent medians (interquartile range) or means ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001, were set as statistically significant differences vs. control conditions.


a
Non-treated tumours (n=51, events=49, median=12.
Metformin treated tumours (n=8, events=8, median=13)
Statin treated tumours (n=17, events=15, median=13.
Metformin+Statin treated tumo (n=9, events=8, median=15.5)
Surviving (%)
Survival time (Months after surgery)
b
ATHYM-Foxn1nu/nu 5-week-old
3x10⁶ of U-87 MG cells injection in both flanks
Follow-up until the tumour was measurable
10 days after injection
50 µl Intra-tumour injection of control/treatment
Control PBS
Treatment Metformin Simvastatin Combined Treatment
Administration every 2 days
Average Tumour Volume (mm³) (normalized by fisrt measured)
Control
Metformin 10⁻² M
Simvastatin 10⁻⁵ M
Metformin 10⁻² M + Simvastatin 10⁻⁵ M
6 days 8 days 10 days 12 days 14 days 16 days 18 days 20 days 22 days 24 days
c
Tumour weight (mg) (normalized by fisrt measured)
d
Control Control Control
Metformin 10⁻² M
Simvastatin 10⁻⁵ M
Metformin 10⁻² M + Simvastatin 10⁻⁵ M
e
Mitosis Number (x5 HPF)
p=0.065
f
Apoptosis
g
CELL DIVISION
APOPTOSIS
Control
Metformin 10⁻² M
Simvastatin 10⁻⁵ M
Metformin 10⁻² M + Simvastatin 10⁻⁵ M
100 µm
h
TGFB CDK4 CCL2 CDK2 VEGF NFKB1 EGF VEGFR CDK6 NBS1 MAPK1 CXCL8 DCR2 MMP9 IL6R CCR1 MMP3 CXCL3 CXCL2 ATM IL6 CDKN1A CDKN2A CXCR1 SIRT1 CCR3 CXCR2 CDKN2D
i
Scores Plot
Component 1 (36.8 %)
Component 2 (16.2 %)
j
CXCR1 CXCR2 IL6 CDKN2A SIRT1 CDKN2D CCR3 CDK4 VEGF TGFB EGF CDK2 NFKB CCL2 CXCL3
High
Low
VIP scores
Control
Metformin 10⁻² M
Simvastatin 10⁻⁵ M
Metformin 10⁻² M + Simvastatin 10⁻⁵ M
k
SRSF3
RBM22
PTBP1
RBM3
Control
Metf- 10⁻²M
Simv. 10⁻⁵M
Metf- 10⁻²M + Simv. 10⁻⁵M
l
U-87 MG Xenograft Mouse Model
Expression level (log2) Normalized by ACTB
SRSF3 RBM22 PTBP1 RBM3
Control
Simvastatin 10⁻⁵ M
Metformin 10⁻² M
Metformin 10⁻² M + Simvastatin 10⁻⁵ M

individually (Table 1). However, the data published to date on the potential therapeutic effects of metformin, statins, and specially their combination, in GBM *in vivo* and *in vitro* is quite limited and fragmentary.[16,17,48] Therefore, this study was aimed to evaluate whether the treatment with metformin and statins, alone or in combination, could translate into a beneficial overall survival in GBM patients and/or might exert direct antitumour actions in GBM cells.

Specifically, a pilot, observational retrospective study of the potential clinical context of the use of metformin or any type of statin, alone or in combination, revealed that aggressive glioma patients not treated with these drugs had a trend towards a worst median survival rate (i.e., 12.2 months) compared with patients treated with metformin (i.e., 13.0 months), statins (i.e., 13.8 months), and their combination (i.e., 15.5 months). These data are in accordance with our recent report using a cross-sectional retrospective cohort of patients with prostate cancer, which indicated that the co-treatment of metformin + statins might be associated with better prognosis (e.g., longer biochemical recurrence-free survival) than patients not treated with these drugs.[14] Obviously, we should underline that the main limitation of this work might be the limited number of patients treated with these drugs and the retrospective observational nature of the analysis. However, our data add compelling evidence that the treatment with metformin, statins, and specially their combination, seems to translate into a beneficial overall survival in GBM patients, an observation that certainly warrants further investigation through a well-designed clinical trial with a controlled follow-up and a complete characterization of the patients (e.g., MGMT status), and by applying the accurate doses and likely changing the administration route of the drugs.

The beneficial clinical observation previously described in patients was further supported by different *in vitro* and *in vivo* models demonstrating a marked reduction in the progression and aggressiveness features of GBM cell cultures (i.e., proliferation/migration rates, tumoursphere/colony formation, VEGF-secretion, together with apoptosis and senescence induction using different cell lines and patient-derived cell cultures), and of a preclinical xenograft GBM mouse model (reduction in tumour size/weight, number of mitosis, and increase in apoptosis) in response to metformin or simvastatin alone, but specially to their combination (i.e., overall additive effects), which is consistent with previous observations found in other cancer types.[10–14] Therefore, the results of this and previous studies suggest that the utility of metformin or simvastatin alone, but specially their combination, might be an effective therapeutic tool for patients with different tumour pathologies, including GBM. Remarkably, the additive anti-proliferative effect of the co-treatment with metformin + simvastatin was significantly more pronounced in the most aggressive GBM cell models tested (U-87 MG vs. U-118 MG, and grade IV-GBM vs. grade III astrocytomas),[49] while treatment with metformin or simvastatin alone, or their combination, did not alter the viability of non-tumour control brain cells. Therefore, based on these observations, it is tempting to suggest that the anti-proliferative actions of metformin and/or simvastatin might be specific for tumour cells (with high metabolic rates) and not for healthy brain cells (with low metabolic rates), which could represent an important benefit for patients harbouring brain pathologies. Moreover, our data also demonstrate that treatment with metformin or simvastatin alone, and specially their combination, significantly decreased the proliferation of mouse astrocytes stem/progenitor cells, a model of tumour precursor cell population that contribute to the GBM malignancy and therapeutic resistance.[23,31] Clearly, further work and additional models will be required to complete our understanding of this complex process. However, when viewed together, these data might be clinically important and hold a potential

***Fig. 6: In vivo* pharmacological administration of metformin, simvastatin, and their combination disrupts GBM progression and mimic the molecular drug effects observed *in vitro*.** **(a)** Kaplan–Meier survival curves discerning between patients with aggressive gliomas treated with metformin, statins, or their combination, and patients not treated with these drugs obtained from our pilot, observational, and retrospective study including 85 patients. Doses were administered following the prescribed daily dose: metformin (between 500 and 1000 mg/daily), and statins (between 20 and 40 mg/daily for simvastatin and 10–20 mg/daily for atorvastatin). **(b)** Diagram showing the generation of a preclinical-xenograft GBM model by inoculation of U-87 MG cells (n = 15; left panel), average tumour volume (right panel) and weight **(c)** of intra-tumour injection of metformin, simvastatin, and their combination vs. control-treated tumours. **(d)** Image of a representative tumour from each treatment condition at sacrifice day is shown. **(e)** Histopathological evaluation of mitosis number (x5 high-power field; HPF) and **(f)** apoptosis of all the U-87 MG xenograft tumours. **(g)** Representative images of H&E staining comparing tumours treated with metformin, simvastatin, and their combination vs. control-treated tumour samples. All these evaluations were determined by experienced pathologists. **(h)** Hierarchical heatmap generated using the expression levels of relevant SASP genes in all the U-87 MG xenograft tumours treated with control (PBS), metformin, simvastatin, and the combination of both drugs **(i)** and the Partial Least Squares Discriminant Analysis (PLS-DA) of the SASP genes in each condition. **(j)** Variable Importance in Projection (VIP) score of the expression levels of SASP key genes in the same samples. **(k)** Heatmap generated using the expression levels of 4 spliceosome components and **(l)** individual expression levels of these components in response to metformin, simvastatin, and their combination intra-tumours administration in U-87 MG xenograft tumours. Data represent medians (interquartile range) or means ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001, were set as statistically significant differences vs. control conditions.

translational implication since the current therapeutic strategies for GBM are not efficient at reducing tumour volume/growth or augment survival rate, which is likely due, in part, to the resistance acquired by tumours, particularly by neural progenitor cells, to different current drugs.[31,38,50,51]

We next explored the capacity of metformin and/or simvastatin to modulate the activity of key oncogenic signalling pathways in GBM cells. As expected, we found metformin, but not simvastatin, actions were mediated through the activation of AMPK-signalling, considered the central mediator of metformin actions[52] and a key sensor of cellular metabolism through its downstream effectors.[53] Besides, we found that metformin and simvastatin alone modulated the levels of several key molecular elements of the AKT-pathway [i.e., RPS6KB1, AKT1S1 and RPS6 (both drugs), PTEN (only metformin) and RAF1 (only simvastatin)], while simvastatin, but not metformin, also induced an activation of some elements of the JAK/STAT-pathway (i.e., up-regulation of PTPN11 and TYK2 phosphorylation levels), which in general support and extend previous data in other cancer cells.[14,52,54–59] Remarkably, the co-treatment of metformin + simvastatin significantly potentiated (i.e., additively) the modulation of a wide variety of molecular elements of these signalling pathways (i.e., MAPK, AKT, and JAK-STAT), and also significantly activated various elements of additional key oncogenic routes (i.e., NF-κB and TGFβ pathways) in GBM cells (i.e., total of 22 significantly modulated proteins in metformin + simvastatin vs. 5 in metformin vs. 7 in simvastatin treated cells). To the best of our knowledge, the present report provides a comparison, side-by-side, of the different intracellular signalling pathways that underlie the direct antitumour effects evoked by metformin, simvastatin, and their combination in cancer cells. In fact, these data demonstrate that the combination of both drugs exerts strong, additive, antitumour actions in GBM cells compared with the individual treatments through the robust activation and/or amplification of multiple oncogenic signalling pathways which could have significant implications in the cellular metabolism.[53,60–64]

In this context, one of the most relevant observations of our study is that the combination of metformin + simvastatin drastically enhanced the phosphorylation levels of ERK1/ERK2, a molecular event that might be associated with a beneficial senescence-state in cancer cells.[32] We also found elevated phosphorylation levels of TP53 that could be linked to a previous activation of ATM/ATR-CHK2/CHK1 pathway which is able to induce a DNA damage response together with a cell cycle arrest.[65] These ideas would be further supported by the fact that the combination of metformin + simvastatin also induced an overall significant activation of NF-κB and TGFβ pathways (that was not found in response to the individual treatments), as well as an inactivation of AKT pathway (i.e., increase in AMPK phosphorylation levels and decrease in downstream effectors) in cancer cells.[32–35] Indeed, we found that metformin- and simvastatin-treated cells alone, but specially their combination, induced an alteration in the expression levels of key genes involved in the SASP in GBM *in vitro* and *in vivo* (e.g., *IL6*, *CCR3*, *CDKN2D*, etc.). In this sense, some products of these genes have been reported to be secreted by cancer cells to induce senescence microenvironment.[36,66–68] Actually, we also demonstrated that the treatment with the conditioned media derived from metformin or simvastatin, but especially from metformin + simvastatin, treated cells were able to induce a senescence state (i.e., by increasing the number of senescence induced cells, and by blunting or completely blocking the proliferation capacity of GBM cells). Additionally, these treatments evoked a significant elongation of telomeres (likely as an outcome of senescence state), which are in accordance with previous results indicating that metformin or simvastatin has been associated with a telomere lengthening in non-tumour diseases.[69,70] A telomere lengthening is a proof of lifespan extension and tumour survival,[71] but it could be also therapeutically beneficial because it is able to revert the 'telomere shortening state' in the tumour cells producing a return to the mortality stage 1 (M1) or 2 (M2) which could undergo growth arrest signals.[72] Although this beneficial effect of telomere lengthening in response to metformin and simvastatin should be further confirmed, we demonstrate herein that the treatment with these drugs clearly exerts antitumour effects on brain tumour cells. Hence, based on all these results reported herein, it seems reasonable to suggest that metformin and simvastatin alone, but specially the co-administration of both drugs, might also exert antitumour actions in GBM cells through the induction of a senescence-state linked to a SASP *in vitro* and *in vivo*.

Our report also provides sufficient information demonstrating that treatments with metformin and simvastatin (*in vitro*), but specially their combination (*in vitro* and *in vivo*), induced an alteration in the expression levels of critical genes belonging to the splicing machinery (i.e., *SRSF3*, *RBM22*, *PTBP1*, and *RBM3*), an essential cellular machinery involved in the tumour behaviour of cancer cells and associated with cancer development, progression, and aggressiveness.[22] In this context, it is important to mention that the senescent phenotype of cancer cells has been associated with the alteration of gene expression and alternative splicing;[37] therefore, based on our overall data, we might suggest that the beneficial antitumour actions, and the associated significant induction to a senescence state,[37] in response to metformin and simvastatin, but specially their combination, might be linked to a downregulation, both *in vitro* and *in vivo*, of the

expression levels of the most relevant oncogenic elements of the splicing machinery (i.e., *SRSF3*, *RBM22*, *PTBP1*, and *RBM3*[22]) in GBM cells.

Taken together, our results support and extend previous data indicating that the antitumour actions of metformin, simvastatin, and specially their combination, in cancer cells are not only confined to the alteration of aggressiveness features (i.e., proliferation/migration rates, tumoursphere/colony formation, cell cycle arrest, VEGF-secretion, and apoptosis induction), and also included the induction of senescence together with the regulation of the expression of key genes involved in the SASP and in the splicing machinery, which, in conjunction, might promote a senescence-state, a telomere lengthening, and an attenuation in the aggressiveness behaviour of GBM cells. Obviously, further work will be required to complete our understanding about this complex process and to fully elucidate the translational potential behind these interesting and potentially relevant observations. Nonetheless, these findings suggest a putative window of opportunity for these drugs, especially their combination, as a potential new therapeutic tool for the management of human GBM.

Contributors

A.C.F.F. and R.M.L conceived and designed the project and interpreted results. A.C.F.F. M.E.G.G, J.M.P.G., J.M.C., C.D.R., C.B.A., A.T.D., R.S.S, J.S., and R.M.L. coordinated GBM samples collection. J.M.C., C.D.R., C.B.A., A.T.D., and J.S.. Identified and consented patients for the study. A.C.F.F performed the main experimental assays and bioinformatic analyses. M.E.G.G., J.M.P.G., A.J.M.H., J.M.C., C.D.R., C.B.A., E.T., A.T.D., R.S.S, E.P.S., R.M.O.S., J.M.J.V., M.T.S., M.L., M.D.G., J.P.C., and J.S. provided important experimental and analytical support and/or helped to discuss the results. A.C.F.F. and R.M.L wrote the manuscript. M.E.G.G., J.M.P.G., A.J.M.H., J.M.C., C.D.R., C.B.A., E.T., A.T.D., R.S.S, R.M.O.S., J.M.J.V., M.T.S., M.L., M.D.G., J.P.C., and J.S. revised the manuscript for important intellectual content. R.M.L supervised the work. A.C.F.F, M.E.G.G and R.M.L verified the underlying data. All authors read, approved, and verified the final version of the manuscript.

Data sharing statement

The datasets used and/or analysed during the current study available from the corresponding authors on reasonable request.

Declaration of interests

The authors declare that no-competing financial and/or non-financial interests concerning the work exist.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104484.

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