Targeted therapy based on p53 reactivation reduces both glioblastoma cell growth and resistance to temozolomide

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Abstract. Glioblastoma (GB) is the most common and aggressive malignant tumor of the central nervous system. Despite current intensive treatment regimens, consisting of surgical resection followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) chemotherapy, the prognosis of patients with GB remains extremely poor. Considering that alterations of the p53 tumor suppressor pathway have a key role in both GB development and resistance to TMZ treatment, the re-activation of p53 could be an effective therapeutic approach against GB. In this study, we challenged p53 wild-type and mutant GB cell lines with RITA, a molecule originally identified for its ability to restore p53 functions, although it was subsequently shown to act also through p53-independent mechanisms. We examined the effects of RITA on GB cell viability, through MTS and clonogenic assays, and analyzed cell death through cytoflourimetric analyses. In all the tested GB cell lines, RITA significantly reduced the cell proliferative and clonogenic potential and induced cell accumulation in the S and/or G2/M cell cycle phases and massive p53-dependent apoptosis. Moreover, RITA was more effective than the well-known p53 re-activating molecule, nutlin-3, and did not affect the viability of normal astrocytes. In addition, RITA decreased survivin expression and induced DNA damage, two mechanisms that likely contribute to its anti-tumor effects. Furthermore, RITA synergized with TMZ and was able to decrease the expression of MGMT, which is a crucial player in TMZ resistance. Thus, although further studies are warranted to clarify the exact mechanisms of action of RITA, the data of this study suggest the potential of such an approach for GB therapy, which may also help to overcome resistance to TMZ.

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Abbreviations: CDKN2A, cyclin-dependent kinase inhibitor 2A; CI, combination index; DDR, DNA damage response; γ-H2AX, phospho-histone H2AX; GB, glioblastoma; GEM, gemcitabine; GOF, gain-of-function; IC_{50}, half maximal inhibitory concentration; JNK, c-Jun N-terminal kinase; MGMT, O(6)-methylguanine-DNA methyltransferase; NHAs, normal human astrocytes; PFTα, pifithrin-α; PI, propidium iodide; PTEN, phosphatase and tensin homolog; RITA, reactivation of p53 and induction of tumor cell apoptosis; ROS, reactive oxygen species; shRNA, short hairpin RNA; TMZ, temozolomide; MDM, murine double minute

Key words: RITA, p53, glioblastoma, apoptosis, survivin, DNA damage, temozolomide, MGMT

Introduction

Glioblastoma (GB) is the most common and aggressive malignant tumor of the central nervous system, characterized by a high degree of proliferation, angiogenesis, necrosis and invasiveness (1). According to the most recent World Health Organization guidelines, GB is classified as a grade IV diffuse astrocytic tumor (2), which can develop either *de novo* (primary GB) or through the malignant progression of lower-grade astrocytomas (secondary GB).

The current standard treatment for GB consists of surgical resection, followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) chemotherapy (3,4). However, despite this intensive approach, almost all patients experience recurrence (1) and the prognosis of patients with this

malignancy remains extremely poor, with the median survival ranging between 12 and 15 months from diagnosis (5). Therefore, more effective therapies are urgently required.

The inactivation of the p53 tumor suppressor is one of the most common molecular alterations occurring in GB. Indeed, the p53 pathway has been found to be altered in 87% of GB cases (6), by either mutations/deletions of the *TP53* gene itself (6,7) or defects affecting other members of the pathway, such as the amplification of the two main p53 negative regulators, murine double minute (MDM)2 and MDM4, and mutations/deletions of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus, encoding the MDM2 inhibitor, p14ARF (6). Similar high frequencies of p53 mutations have been observed among lower-grade astrocytomas and secondary GBs, suggesting an important role of p53 alterations in the early stages of GB development (8). Individuals carrying p53 germline mutations are predisposed to the development of astrocytomas, further supporting a crucial role of p53 mutations in driving gliomagenesis (9). Consistently, different mouse models analyzing the role of p53 mutations in GB development, either alone or in combination with other molecular alterations, have confirmed a central role of p53 defects in the early stages of gliomagenesis, although additional alterations, such as phosphatase and tensin homolog (PTEN) inactivation, are required to drive p53-mediated GB development (10-13).

Of note, p53 can negatively regulate the expression of O(6)-methylguanine-DNA methyltransferase (MGMT) (14,15), a DNA repair enzyme, whose high level of activity underlies GB resistance to TMZ (16). This suggests that the re-activation of p53 may be an effective strategy with which to overcome both the growth advantage and the tumor resistance to TMZ treatment conferred by p53 inactivation in GB. In addition to p53 inactivation, *TP53* gain-of-function (GOF) mutations are associated with a poor prognosis and response to TMZ in GB (17,18). Thus, restoring wild-type functions in p53 mutants could represent a promising therapeutic approach against GB.

Owing to its crucial role in apoptosis and cell growth control, and considering that its pathway is altered in the majority of human cancers (19), p53 represents one of the most appealing targets for cancer therapy and therefore, to date, several strategies targeting p53 and its pathway have been developed (20-22). Approaches aimed at restoring the oncosuppressive function of p53, through either wild-type *TP53* gene transfer systems (12,23,24) or small molecule/peptide-based methods (25-39), have proven promising for GB treatment, owing not only to their pro-apoptotic and anti-proliferative effects, but also to their ability to sensitize GB cells to TMZ (23,24,28,30,33,37).

The majority of the above-mentioned p53 re-activation strategies are designed to prevent MDM2 from targeting p53 for proteasomal degradation. For instance, the well-known p53 re-activating molecule, nutlin-3, functions by binding MDM2, and thus inducing the release and accumulation of p53. In GB cells, nutlin-3 has been observed to principally cause growth arrest, rather than apoptosis, and to be effective exclusively in p53 wild-type cells (27).

Another molecule, RITA (re-activation of p53 and induction of tumor cell apoptosis), was initially identified as a compound preventing the p53-MDM2 interaction and inducing p53-dependent apoptosis in various tumor cell lines (40). Unlike nutlin-3, RITA was found to bind the p53 N terminus (40) and was suggested to induce a conformational change of p53, thus preventing its binding to MDM2 (41). However, some subsequent studies questioned the inhibitory effects of RITA on the p53-MDM2 interaction (42,43) and it has emerged that the molecular mechanisms underlying the cellular effects of RITA are much more complex than just p53 stabilization. In particular, RITA can function not only through p53-dependent mechanisms, but also through other yet to be fully elucidated p53-independent mechanisms. Indeed, accumulating data have indicated that RITA is also effective on p53 null cells or on cells experimentally depleted of p53 (44-49). Thus, despite the progress in understanding the molecular mechanisms through which RITA functions, mainly involving reactive oxygen species (ROS) induction (48,50,51), c-Jun N-terminal kinase (JNK) signaling activation (48,51,52), DNA damage response (DDR) induction (49,51,53-57) and the suppression of anti-apoptotic and pro-survival factors (48,51,58), the role of p53 in these RITA-triggered events remains controversial. Indeed, RITA has been suggested to induce these processes both dependently (50-52,54-56,58) and independently of p53 (48,49,57).

Although the exact mode of action of RITA warrants further investigation, this small molecule has exhibited several appealing properties, including: i) Its pro-apoptotic rather than growth-arresting effects, which can be crucial for efficiently eliminating cancer cells, while avoiding possible interference with the effects of chemotherapeutic drugs in the clinical setting (59,60); ii) its preferential cytotoxicity to malignant cells (40,48,51,57,58,60-62), which supports the possible use of RITA for a safe anti-cancer treatment; iii) its potential ability to restore wild-type functions in p53 mutants, possibly by stabilizing a wild-type-like conformation (60,63-67), which renders RITA an attractive anti-cancer drug for use against tumors characterized by a high frequency of p53 mutations, such as GB.

Recently, RITA has been shown to reduce the viability of a p53 wild-type GB cell line and to sensitize it to TMZ treatment (68), thus suggesting that RITA may represent, indeed, a novel and feasible approach against GB. In the present study, we evaluated the effects of RITA on different GB cell lines, expressing either wild-type or mutant p53.

Materials and methods

Cell lines and culture conditions. The human GB cell line, PRT-HU2 (69), was kindly provided by Professor Sergio Comincini (University of Pavia, Pavia, Italy), whereas the GB cell line of unknown origin, U-87MG, and the GB cells T98G [Cat. no. ATCC HTB-14 and ATCC CRL-1690, respectively; American Type Culture Collection (ATCC), Manassas, VA, USA] were a kind gift from Professor Annamaria Cimini (University of L'Aquila, L'Aquila, Italy). Normal human astrocytes (NHAs) were purchased from Tebu-Bio (Magenta, Italy; Cat. no. 882-05) and 293FT cells, used to generate lentiviral particles, were purchased from ATCC (Cat. no. ATCC PTA-5077). The GB and 293FT cells were grown in DMEM containing 10% fetal bovine serum, 0.5% penicillin-streptomycin and 1% glutamine at 37˚C in a humidified atmosphere containing 5% CO_2. All cell culture reagents were purchased from Sigma-Aldrich (Milan, Italy).

The NHAs were maintained in astrocyte growth medium (Cat. no. 821-500; Sigma-Aldrich,) in Petri dishes coated with 15 μg/ml poly-L-lysine (Cat. no. P4707; Sigma-Aldrich). All cells were maintained at low passage numbers and periodically tested for the presence of mycoplasma with the PlasmoTest™ Mycoplasma Detection kit (Cat. no. rep-pt1; Invivogen, San Diego, CA, USA).

TP53 sequencing. Total RNA was extracted using TRIzol reagent (Cat. no. 15596026; Thermo Fisher Scientific, Waltham, MA, USA) and 1 μg of RNA was then retrotranscribed using SuperScript reverse transcriptase III (Cat. no. 18080085; Thermo Fisher Scientific). Full-length *TP53* was amplified by PCR using the *Pfu* DNA polymerase (Cat. no. 600380; Agilent Technologies, Santa Clara, CA, USA) and the following primers: 5'-CGTCCAGGGAGCAGGTAG-3' (forward) and 5'-CAAGCAAGGGTTCAAAGAC-3' (reverse). The reaction mixture was denatured at 94˚C for 2 min and subjected to 40 amplification cycles consisting of 20 sec at 94˚C, 20 sec at 61˚C, 40 sec at 72˚C each, followed by a 3-min extension at 72˚C. PCR products were purified using the QIAquick gel extraction kit (Cat. no. 28704; Qiagen S.r.l., Hilden, Germany). Sequencing reactions were performed by PRIMM S.r.l., and analyzed using Sequencher software 4.10.1 (Gene Codes Corp., Ann Arbor, MI, USA). The human *TP53* wild-type sequence used as a reference was NM_000546.4 (National Center for Biotechnology Information, Bethesda, MD, USA).

Cell treatment with RITA and nutlin-3, MTS and clonogenic assay. RITA (Cat. no. CAY-10006426-5; Vincibiochem S.r.l, Florence, Italy) and nutlin-3 (Cat. no. 675576; Sigma-Aldrich) were dissolved in DMSO as a stock and then diluted in culture medium. The cells were seeded in 96-well plates 24 h prior to treatment with increasing concentrations (0.01-10 μM) of RITA or increasing concentrations (0.62-20 μM) of nutlin-3. As a control, cells were treated with the maximum amount of DMSO used to deliver the compounds. At 72 h after treatment, cell viability was evaluated by MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Cat. no. G3582; Promega, Milan, Italy), following the manufacturer's instructions. The half maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism Software, version 5.01 for Windows. DMSO exhibited no toxic effect on any of the cell lines (data not shown).

For clonogenic assays, $1.5x10^2$ cells were seeded in each well of 6-well plates and, 24 h after seeding, they were treated with RITA for 24 h at its IC_{50} values or DMSO, as a control. The medium containing RITA was then replaced, according to a previously published protocol (63), to avoid excessive cell death in the very sparse cell cultures. After 2 weeks, colonies were fixed with methanol and stained at room temperature for 30 min with crystal violet (Cat. no. HT90132; Sigma-Aldrich).

Cytoflourimetric analyses of cell cycle profile and apoptosis. GB cells were plated in 100-mm diameter Petri dishes and, at 24 h after seeding, were treated with RITA at its IC_{50} values or DMSO. At 48/72 h after treatment, the cells were collected, washed with PBS and then fixed in 70% ice-cold ethanol. The cells were then incubated at 37˚C for 1 h with 50 μg/ml propidium iodide (PI; Cat. no. P4170; Sigma-Aldrich) and 20 μg/ml RNase (Cat. no. 9001-99-4; Sigma-Aldrich) and then analyzed with a BD FACSCalibur flow cytometer (BD Biosciences, Milan, Italy).

Apoptosis was evaluated through FACS analysis following cell staining with Annexin V-FITC and PI (Annexin V-FITC kit; Cat. no. 130-092-052; Miltenyi Biotec Inc., Bologna, Italy) according to the manufacturer's instructions. Pifithrin-α (PFTα; Cat. no. 6320882-82-2; Sigma-Aldrich) was dissolved in DMSO and diluted to 25 μM in culture medium.

Silencing of p53 in T98G, U-87MG and PRT-HU2 cells. To silence *TP53*, $2x10^6$ 293FT cells were transfected with 2.25 μg of PAX2 packaging plasmid (Cat. no. 12260; Addgene, Cambridge, MA, USA), 0.75 μg of PMD2G envelope plasmid (Cat. no. 12259; Addgene) and 3 μg of pLKO.1 hairpin vector, utilizing 30 μl of Attractene (Cat. no. 301005; Qiagen S.r.l). The following pLKO.1 vectors were used: Scrambled short hairpin RNA (shRNA; pLKO.1 shSCR, gift from Professor S. Stewart, Washington University School of Medicine, St. Louis, MO, USA; Cat. no. 17920; Addgene) and p53 shRNA (shp53 pLKO.1 puro, gift from Professor Bob Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, USA; Cat. no. 19119; Addgene). Beginning from 24 h following transfection, supernatants were collected at 24-h intervals for 3 days, filtered and used for the transduction of the T98G, U-87MG and PRT-HU2 cell lines in the presence of 1 μg/ml polybrene (Cat. no. 107689; Sigma-Aldrich). At 3 days post-infection, the cells were selected with 2.5 μg/ml puromycin (Cat. no. P7255; Sigma-Aldrich).

Protein extraction and western blot analysis. The cells were lysed on ice for 30 min in a buffer consisting of 1 mM EDTA, 150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 7.5, and 10 mg/ml each of aprotinin, leupeptin and pepstatin. Proteins were quantified by the Bradford assay (Cat. no. 5000201; Bio-Rad, Segrate, Italy). Equal amounts of proteins (50 μg) per sample were electrophoresed onto 12.5% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (Cat. no. 1620115; Bio-Rad), which were then blocked in 5% non-fat dry milk and incubated at 4˚C overnight with monoclonal antibodies against p53 (Cat. no. sc-126), survivin (Cat. no. sc-17779), MGMT (Cat. no. sc-56432) and GAPDH (Cat. no. sc-32233) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a polyclonal antibody against phospho-histone H2AX (γ-H2AX) (Cat. no. ab11174; Abcam, Branford, CT, USA). The antibodies were diluted according to manufacturers' recommendations. Following incubation at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, Cat. no. sc-2005, and goat anti-rabbit IgG, Cat. no. sc-2004, both from Santa Cruz Biotechnology), signals were detected through ECL (Cat. no. RPN2232; Amersham Biosciences, Little Chalfont, UK).

Drug combination studies. For drug combination studies, we first determined the 72-h IC_{50} values of TMZ (Cat. no. 85622-93-1; Santa Cruz Biotechnology) through MTS assay in the GB cells, as described above for RITA. Subsequently, based on the RITA and TMZ IC_{50} values, we challenged the GB cells for 72 h with the two drugs, both alone and in combination

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Cell lines	p53 status	IC_{50} (μM)	
		RITA	Nutlin-3
T98G	Mutant (c.711G>A; p.M237I)*	0.06	ND
U-87MG	Wild-type*	5.81	ND
PRT-HU2	Wild-type	1.45	ND

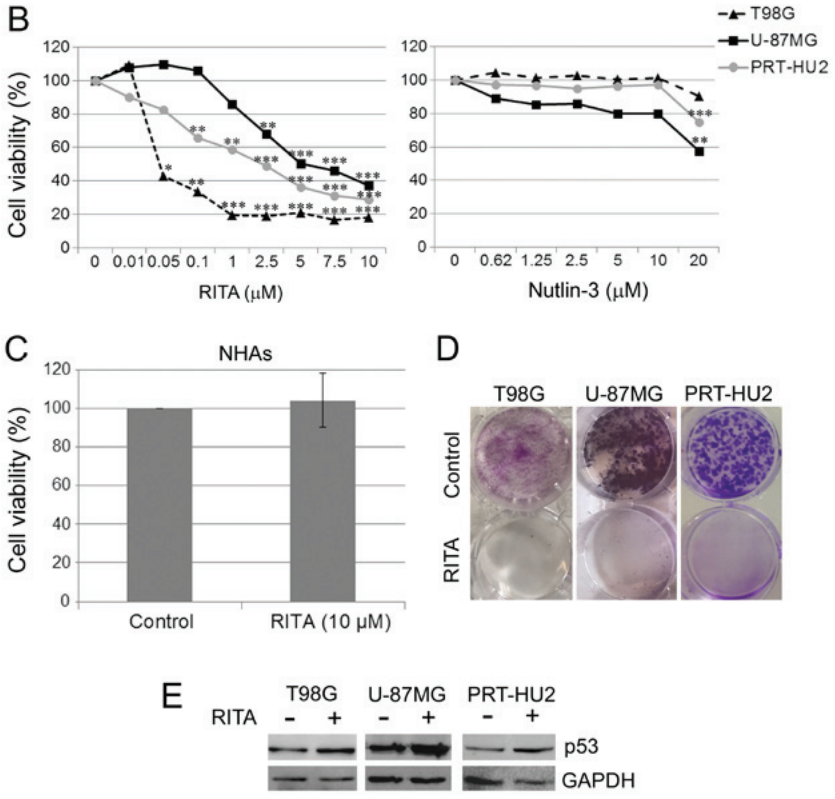

Figure 1. Effect of RITA and nutlin-3 on glioblastoma (GB) cell viability. (A) Table reporting the p53 mutational status in T98G, U-87MG and PRT-HU2 cell lines, and RITA IC_{50} values, as determined through MTS assay in these cell lines at 72 h after treatment. Nutlin-3 IC_{50} values were not determinable (ND) at the range of concentrations used. The asterisks indicate *TP53* mutational status, which was previously reported (Sanger Institute, Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/) (70). (B) Dose-response curves obtained through MTS assay in T98G, U-87MG and PRT-HU2 cell lines at 72 h after treatment with either RITA or nutlin-3 at the indicated concentrations. Results are reported as the means of at least 2 independent experiments, each conducted in triplicate, and expressed as percentages of cell viability calculated with respect to the control cells treated with DMSO alone. The absorbance values of the treated and control samples were subjected to one-way ANOVA with Dunnett's post hoc test. Statistically significant differences between the treated and control cells are indicated as follows: $^{*}P<0.05$, significant; $^{**}P<0.01$, very significant; and $^{***}P<0.001$, extremely significant. (C) Histogram showing that 72 h of treatment with 10 μM RITA had no toxic effect on normal human astrocytes (NHAs), as determined by MTS assay. Results are reported as the mean of 4 independent experiments and expressed as percentage of cell viability calculated with respect to control cells treated with DMSO alone. (D) Representative dishes, out of 2 independent clonogenic assays, showing the long-term effect of RITA treatment on T98G, U-87MG, and PRT-HU2 cell lines. Control cells were treated with DMSO alone. (E) Western blot analysis of p53 in T98G, U-87MG and PRT-HU2 cell lines treated for 24 h with RITA or DMSO, as a control. An anti-GAPDH antibody was used for a loading control. A representative experiment, out of at least 2 independent ones, is shown.

at various concentrations in a constant ratio (2-fold serial dilutions above and below the RITA and TMZ IC_{50} values), and assessed cell viability through MTS assay. Synergism, additivity or antagonism were determined calculating the combination index (CI) according to the Chou-Talalay equation, using CalcuSyn software 1.1.1 (BioSoft, Cambridge, UK). CI <1 indicates synergism, CI = 1 additive effect, and CI >1 antagonism. The r value represents the linear correlation coefficient of the median-effect plot, which indicates the conformity of the data to the mass-action law.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Software, version 5.01 for Windows. Statistically significant differences were evaluated by one-way repeated measures ANOVA with Dunnett's post hoc test, to compare all data vs. the controls. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Anti-proliferative effects of RITA on GB cell lines. We examined the effects of RITA on 3 GB cell lines (T98G, U-87MG and PRT-HU2), expressing either wild-type or mutant p53 (Fig. 1A). In particular, the *TP53* mutational status was evaluated in previous studies in both T98G cells, in which *TP53* was found to be mutated (Sanger Institute, Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/) (70) and in U-87MG

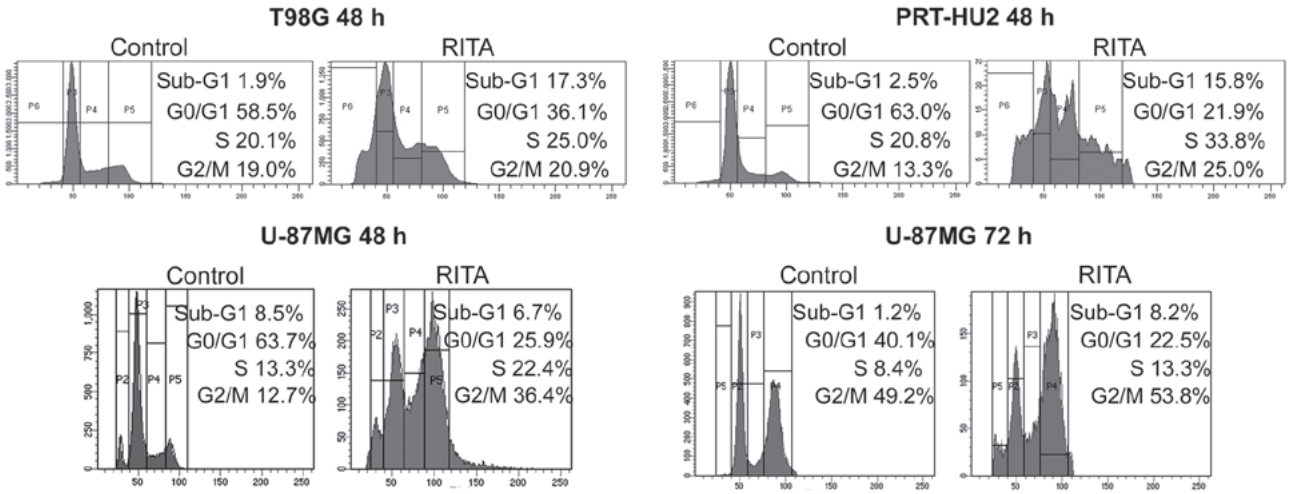

Figure 2. Effects of RITA on cell cycle progression. Representative cell cycle profiles, out of 2 independent FACS analyses of RITA-treated glioblastoma (GB) cells. For the T98G and PRT-HU2 cells, data obtained at 48 h after treatment are shown, whereas for the U-87MG cells, the reported data were obtained at both the 48- and 72-h time points, since a sub-G1 peak was still not evident at 48 h after treatment. DMSO alone was added to untreated control cells.

cells, in which it was found to be wild-type (70). Since the data on *TP53* mutational status were, conversely, not available for the PRT-HU2 cells, we performed *TP53* cDNA sequencing in these cells, which were found to express wild-type *TP53* (data not shown).

We treated the 3 GB cell lines with RITA at concentrations ranging from 0.01 to 10 μM. After 72 h, we evaluated cell viability by MTS assay and observed that RITA exerted significant cytotoxic effects on all GB cell lines (Fig. 1B). Therefore, as expected, RITA was effective not only on p53 wild-type cells, but also on p53 mutant cells. We also compared the anti-proliferative effects of RITA with those of the more well-studied p53 re-activating molecule, nutlin-3, and observed that nutlin-3 was much less effective than RITA in exerting anti-proliferative effects on the GB cell lines, only affecting the viability of the p53 wild-type cell lines (U-87MG and PRT-HU2) at the highest concentration used (Fig. 1B). These data are consistent with those of a previous study reporting that nutlin-3 was ineffective on the T98G cells, whereas it affected U-87MG cell viability (27). We calculated the RITA IC_{50} values for the 3 GB cell lines at 72 h after treatment, whereas the nutlin-3 IC_{50} values were not determinable under our experimental conditions (Fig. 1A).

To rule out the possible cytotoxic effects of RITA on non-neoplastic brain cells, we treated NHAs with RITA at a concentration of 10 μM, which corresponds to the maximum amount of RITA used in the MTS assays on the GB cells. After 72 h, we evaluated cell viability by MTS assay and observed no toxic effect on the NHAs (Fig. 1C).

To evaluate whether RITA was able to exert a long-term inhibitory effect on GB cell growth, we performed clonogenic assays in the T98G, U-87MG and PRT-HU2 cells and observed that treatment with RITA markedly inhibited colony formation in all the cell lines (Fig. 1D).

We also verified the effects of RITA on p53 protein levels through western blot analysis of total protein extracts from GB cells treated with RITA for 24 h at its IC_{50} values. We observed that treatment with RITA increased the p53 levels in both wild-type and mutant GB cells (Fig. 1E).

RITA affects cell cycle progression and induces p53-dependent apoptosis of GB cell lines. To assess the effects of RITA on GB cell cycle progression, we analyzed by FACS the cell cycle profiles of the T98G, U-87MG and PRT-HU2 cell lines treated with RITA at the IC_{50} values reported in Fig. 1A. We observed an increase in the sub-G1 peak, which could be indicative of apoptosis, at 48 h following treatment with RITA in the T98G and PRT-HU2 cells, and at 72 h following treatment in the less RITA-responsive U-87MG cells (Fig. 2). Moreover, we observed cell accumulation in the S and/or G2/M phases in all cell lines, consistent with the findings of previous studies showing that RITA can stall replication fork elongation (55,56) and induce G2 arrest (56).

To verify the ability of RITA to induce apoptosis of GB cells, we analyzed cell staining with Annexin V-FITC and PI by FACS analysis at 72 h following treatment with RITA at its IC_{50} values. These analyses revealed that RITA induced a massive apoptosis of all GB cell lines (Fig. 3A). Moreover, to assess whether p53 has a role in the observed RITA-induced apoptosis of GB cells, we treated these cells with PFTα, an agent reported to prevent p53 transcription-dependent apoptosis (71). PFTα markedly decreased the RITA-induced apoptosis of all GB cell lines (Fig. 3A), thus suggesting that p53 transcriptional activity could be involved in the apoptotic program triggered by RITA. To confirm the role of p53 in RITA-induced apoptosis, we used this compound (at its 72-h IC_{50} values) to treat the T98G, U-87MG and PRT-HU2 cells in which p53 expression was stably silenced through transduction with lentiviral vectors expressing *TP53*-specific shRNAs. We performed Annexin V assays at shorter treatment times (24/48 h) as the transduced cells underwent apoptosis earlier than the non-transduced parental cells upon RITA treatment and also in order to analyze early apoptotic events, which, compared with later apoptotic events, are better distinguishable from possible necrotic processes. We observed that in all the p53-silenced cells, the percentage of apoptosis upon RITA treatment was markedly decreased compared with that observed in the control p53-expressing cells, stably transduced with non-targeting shRNAs (Fig. 3B). In addition, the p53 levels in the GB cells expressing either

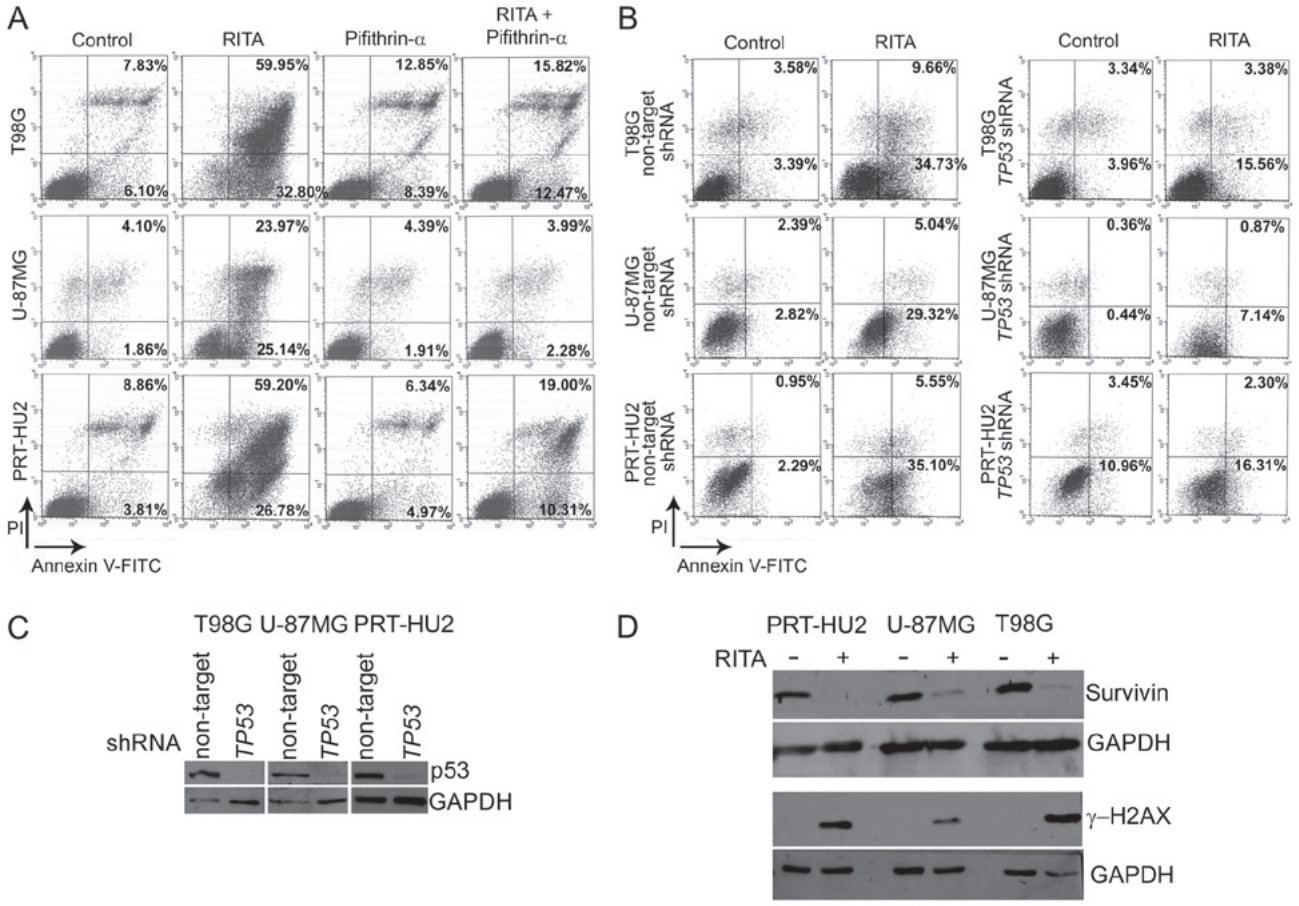

Figure 3. Induction of apoptosis of the RITA-treated glioblastoma (GB) cell lines. (A) FACS analysis to investigate apoptosis by cell staining with Annexin V-FITC and propidium iodide (PI) in the T98G, U-87MG and PRT-HU2 cells at 72 h after treatment with RITA and/or pifithrin-α or DMSO, as a control. The graphs show the percentages of early apoptosis (Annexin V-positive and PI-negative) and late apoptosis/necrosis (Annexin V-positive and PI-positive). A representative experiment, out of two independent ones, is shown. (B) Representative Annexin-V assays (out of 2 independent experiments) in T98G, U-87MG and PRT-HU2 cells expressing either non-targeting or *TP53*-specific shRNAs and treated for 48 h (T98G) and 24 h (U-87MG and PRT-HU2) with RITA or DMSO, as a control. The graphs show the percentages of early apoptosis and late apoptosis/necrosis. (C) Western blot analysis of p53 in T98G, U-87MG and PRT-HU2 cells expressing either non-targeting or *TP53*-specific shRNAs. An anti-GAPDH antibody was used for a loading control. A representative experiment, out of 2 independent ones, is shown. (D) Western blot analysis of survivin and γ-H2AX expression in PRT-HU2, U-87MG and T98G cell lines after 24 h of RITA treatment. Control cells were treated with DMSO alone. An anti-GAPDH antibody was used for a loading control. A representative experiment, out of 2 independent ones, is shown.

non-targeting or *TP53*-specific shRNAs were assessed by western blot analysis in parallel experiments, which revealed that p53 was efficiently knocked down by the shRNAs (Fig. 3C).

Both wild-type p53 (72,73) and RITA/nutlin-3-activated p53 (27,58) have previously been shown to inhibit the expression of the anti-apoptotic protein, survivin, which has been implicated in GB cell growth (74), prognosis (75,76) and resistance to treatment (77-79), and also seems a promising target for immunotherapeutic approaches against gliomas (80). Thus, in this study, we examined survivin expression in the GB cell lines treated with RITA at its 72-h IC_{50} values. We observed, through western blot analysis, that the survivin levels were sharply decreased after 24 h of treatment with RITA in all GB cell lines (Fig. 3D), thus suggesting that the decrease in survivin expression may be involved in RITA-triggered apoptosis.

Accumulating data have also suggested that RITA activity largely depends on DDR induction (49,51,53-57). Therefore, in this study, we examined the effect of RITA on γ-H2AX, a marker of DNA damage, and found, indeed, that RITA increased the γ-H2AX levels in all GB cell lines (Fig. 3D). This observation is consistent with the S-phase cell accumulation described above, which can indeed depend on RITA ability to activate an S-phase DNA damage checkpoint, as previously described (55,56).

RITA synergizes with TMZ in GB cell lines. We then examined whether RITA synergizes with TMZ, a chemotherapeutic agent currently used in GB therapy. We first determined the 72-h IC_{50} values of TMZ on the T98G, U-87MG, PRT-HU2 cell lines by MTS assay (Fig. 4A). In line with what has been previously reported (81), we observed that the T98G cells were resistant to TMZ, as revealed by its high IC_{50} value (987.4 μM). Subsequently, based on these IC_{50} values and those previously calculated for RITA (Fig. 1A), we challenged the 3 GB cell lines for 72 h with the two drugs, both alone and in combination at various concentrations in a constant ratio (Fig. 4B). MTS data analysis through the Chou-Talalay method (82) revealed CI values <1 for all cell lines (Fig. 4C), which indicate synergism between RITA and TMZ, although

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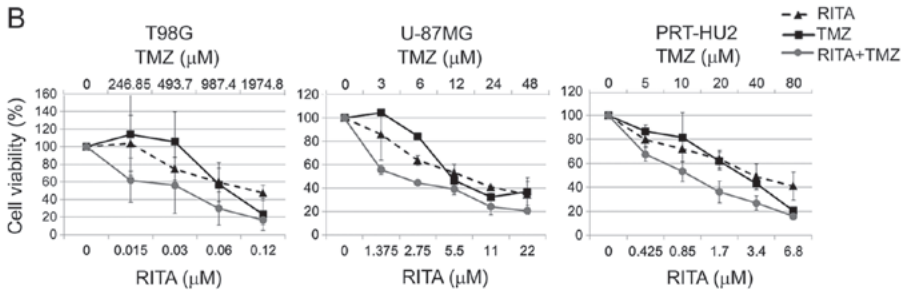
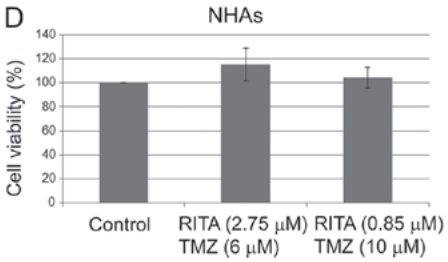
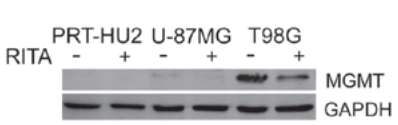
Cell lines	TMZ IC_{50} (μM)
T98G	987.40
U-87MG	12.06
PRT-HU2	19.71



C

Cell lines	RITA+TMZ	
	CI_{50}	r
T98G	0.57±0.22	0.97±0.0193
U-87MG	0.46±0.15	0.98±0.0001
PRT-HU2	0.79±0.14	0.99±0.0035





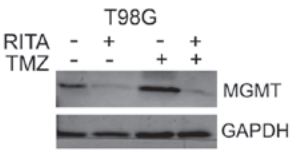


Figure 4. Synergistic effects of RITA-TMZ combination on glioblastoma (GB) cell lines. (A) The table reports the TMZ IC_{50} values on GB cell lines. These values were calculated from cell viability data obtained through MTS after 72 h of treatment with TMZ. (B) Dose-response curves for RITA alone, TMZ alone and RITA-TMZ combinations in T98G, U-87MG and PRT-HU2 cell lines at 72 h after treatment. Results represent the means of 2 independent experiments, each conducted in triplicate, and are expressed as percentages of cell viability calculated with respect to control cells treated with DMSO alone. (C) Table reporting the means ± standard deviations of combination index (CI) and r values of RITA-TMZ combination at 50% of cell killing (CI_{50}) following 72 h of treatment, calculated by the CalcuSyn software for each of the 2 independent experiments. CI values <1 indicate synergism. (D) Histogram showing that 72 h of treatment with RITA-TMZ at the indicated combination doses had no toxic effect on normal human astrocytes (NHAs), as determined through MTS assay. Results are reported as the means of 2 independent experiments and expressed as percentages of cell viability calculated with respect to control cells treated with DMSO alone. (E) Western blot analysis of MGMT expression in PRT-HU2, U-87MG and T98G cell lines at 24 h after RITA treatment. Control cells were treated with DMSO alone. An anti-GAPDH antibody was used for a loading control. A representative experiment, out of 3 independent ones, is shown. (F) Western blot analysis of MGMT expression in T98G cells at 24 h after treatment with RITA and TMZ, both alone and in combination. Control cells were treated with DMSO alone. An anti-GAPDH antibody was used for a loading control. A representative experiment, out of 3 independent ones, is shown.

high concentrations of TMZ were still required to markedly reduce the viability of resistant cells (T98G).

To rule out possible cytotoxic effects of this drug combination on non-neoplastic cells, we treated the NHAs with two different RITA-TMZ combination doses, which corresponded to the two-drug concentrations leading to a ~50% reduction in the viability of the two TMZ-sensitive GB cell lines (U-87MG and PRT-HU2), respectively (as shown by the MTS assays in Fig. 4B). Through MTS assay, we observed no toxic effect of these drug combinations on NHAs 72 h following treatment (Fig. 4D).

Considering the crucial role of MGMT in conferring resistance to TMZ in GB cells (16), we examined, through western blot analysis, the MGMT expression levels in GB cell lines, both untreated and treated for 24 h with RITA at its 72-h IC_{50} values. Consistent with what has previously been reported for T98G and U-87MG cells (83), in this study, we observed that MGMT was expressed only in the TMZ-resistant T98G cells, whereas it was not expressed at detectable levels in the TMZ-sensitive U-87MG and PRT-HU2 cell lines (Fig. 4E). Importantly, RITA treatment decreased MGMT expression in T98G cells. Moreover, we observed that RITA was able to decrease the MGMT levels in the T98G cells also when combined with TMZ (following 24 h of treatment with both drugs used at their IC_{50} values), inhibiting the increase in MGMT expression, which was observed upon treatment with TMZ alone (Fig. 4F). Thus, these data further suggest the potential of RITA to overcome TMZ resistance in GB cells.

Discussion

Despite current intensive treatment regimens, consisting of surgical resection followed by radiotherapy with concomitant and adjuvant TMZ chemotherapy (3,4), GB remains universally fatal, with a median survival ranging between 12 and 15 months from diagnosis (5).

Several advances have been achieved in the understanding of the molecular mechanisms leading to GB development and resistance to therapy, which are crucial for identifying specific therapeutic targets. An appealing target for GB therapy is the tumor suppressor p53, since the inactivation of its pathway plays a key role in both GB development (6,8,12) and resistance to TMZ treatment (14-16). Moreover, *TP53* GOF mutations have been shown to be associated with a poor prognosis and response to TMZ in GB (17,18). Therefore, the re-activation of p53 wild-type functions holds the potential to be an effective therapeutic approach for GB.

In the present study, we tested RITA, a molecule originally identified as a p53 re-activator preventing the p53-MDM2

interaction (40), on 3 GB cell lines, expressing either wild-type or mutant p53. In particular, the T98G cells have previously been reported to carry a homozygously mutated form of *TP53* (Sanger Institute, Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/) (70). Conversely, both the U-87MG and PRT-HU2 cells bear wild-type *TP53*, as previously reported for U-87MG cells (70) and found in the present study for PRT-HU2 cells.

In this study, we observed that RITA increased the p53 levels and reduced the viability and clonogenic potential of all GB cell lines, irrespectively of the *TP53* mutational status. This observation is consistent with the findings of previous studies by both our group and others, showing that RITA was effective not only on p53 wild-type cells, but also on p53 mutant cells (57,60,63-67,84).

To date, different other compounds targeting the MDM2-p53 interaction have been designed, which have proven to be promising also for GB treatment (27-33,35-39). In this study, we compared the anti-proliferative effects of RITA with those of the well-known p53 re-activating molecule, nutlin-3, and observed that nutlin-3 was much less effective on GB cell lines under our experimental conditions, significantly reducing only the viability of the p53 wild-type cell lines (U-87MG and PRT-HU2) at the highest concentration. These observations are consistent with those of a previous study showing that nutlin-3 was ineffective on T98G cells, whereas it affected U-87MG cell viability, although with a seemingly higher efficacy with respect to that observed in the present study, owing to the longer treatment time used in the previous study (27).

Importantly, in this study, we observed that RITA, at the maximum concentration used on GB cells, had no toxic effect on NHAs, consistent with the findings of previous studies showing that RITA was preferentially cytotoxic to malignant cells (40,48,51,57,58,60-62). Conversely, nutlin-3 has previously been shown to reduce the viability of both NHAs (27) and other non-cancer cells (60), suggesting that RITA may be more tumor-selective with respect to nutlin-3.

We then investigated whether the cytotoxic effects of RITA on GB cells were due to cell cycle arrest or to cell death and, in line with what has previously been reported (55,56), we found that RITA induced cell accumulation in the S and G2/M phases, but not in the G1 phase. Moreover, RITA induced massive apoptosis of all GB cell lines. This is noteworthy, considering the intrinsic resistance to apoptosis of GB cells, which is a key mechanism whereby these cells evade death induced by anticancer treatments (85).

Although RITA was initially identified as a compound preventing the p53-MDM2 interaction and inducing a p53-dependent apoptosis (40), subsequent studies failed to demonstrate RITA inhibitory effect on the p53-MDM2 interaction (42,43) and indicated that RITA can act also through p53-independent mechanisms (44-49,57,84). Thus, in this study, to investigate the role of p53 in RITA-induced apoptosis in GB cells, we treated these cells with RITA in combination with the p53 inhibitor, PFTα, and observed that this inhibitor markedly decreased the RITA-induced apoptosis of all GB cells. Although this observation suggests that p53 can contribute to the apoptotic program triggered by RITA, PFTα has also been shown to be able to inhibit p53-independent apoptotic processes induced by DNA damage (86). Therefore, the ability of PFTα to inhibit apoptosis induced by RITA may also be due to a PFTα action against p53-independent RITA pro-apoptotic effects, as previously observed (48). In this study, to confirm the role of p53 in RITA-induced apoptosis, we used this compound to treat GB cells in which p53 was stably silenced and observed a markedly decreased apoptosis of all p53-silenced cell lines. Thus, RITA-induced apoptosis was at least in part dependent on p53 in both p53 wild-type and mutant GB cells.

To further dissect the molecular mechanisms underlying the pro-apoptotic effects of RITA on GB cells, we analyzed the expression of the anti-apoptotic protein, survivin, in RITA-treated GB cell lines and observed a sharp decrease in its levels in all cell lines. This observation is consistent with previous data showing the ability of both wild-type p53 (72,73) and RITA/nutlin-3-activated p53 (27,58) to inhibit the expression of survivin. In particular, a similar decrease in survivin levels was previously observed in nutlin-3-treated GB cells, although the decrease in survivin expression was only found in p53 wild-type cells (27). The ability of RITA to decrease the survivin levels is noteworthy, considering the involvement of this protein in GB cell growth (74), prognosis (75,76) and resistance to treatment (77-79).

In line with the S-phase cell accumulation observed in this study, it has previously been reported that RITA is able to activate an S-phase DNA damage checkpoint, stalling replication fork elongation (55,56). DDR induction has been suggested to be the primary RITA mechanism of action, leading to an indirect p53 stabilization or operating also independently of p53 (49,57). Therefore, in this study, we analyzed the effect of RITA on the DNA damage marker γ-H2AX and found, indeed, that RITA increased its levels in all GB cell lines. Thus, although further studies are required to elucidate the mechanisms of action of RITA in GB cells, the effects of this molecule could, at least in part, depend on the induction of DDR also in these cells.

Given that resistance to TMZ is a major cause of GB treatment failure (16), we then assessed whether RITA could increase GB cell sensitivity to TMZ. We observed that RITA synergized with TMZ in TMZ-sensitive cells (U-87MG and PRT-HU2) and enhanced the sensitivity of TMZ-resistant cells (T98G) to this agent, although high TMZ concentrations were still required to markedly reduce the viability of resistant cells. Moreover, we also observed that the two-drug combination had no toxic effects on NHAs, at least at the same concentrations causing approximately a 50% reduction in the viability of the two TMZ-sensitive GB cell lines.

Considering the crucial role of MGMT in the TMZ resistance of GB (16), we analyzed the expression of this DNA repair enzyme in GB cell lines and observed that it was expressed only in T98G cells, consistent with what has been previously reported (83). Notably, RITA was able to reduce MGMT expression in T98G cells, not only when used as a single agent, but also when combined with TMZ. Thus, RITA proved to be able to overcome the increase in MGMT expression observed upon TMZ treatment, which was previously described as a response to the TMZ-induced DNA damage (87). These data further support the possible use of RITA to sensitize GB cells to TMZ treatment. Of note, it was previously suggested that GOF activities of mutant p53 could contribute to TMZ resistance

in T98G cells (70). Indeed, the knockdown of mutant p53 in these cells sensitized them to TMZ and reduced their MGMT expression (70). Since we observed that RITA was similarly able to sensitize T98G cells to TMZ and reduce MGMT expression, despite the RITA-induced increase in mutant p53 levels in these cells, we can speculate that RITA stabilized a wild-type-like conformation of mutant p53 and restored its wild-type functions in T98G cells. Analogously, another study demonstrated that in pancreatic adenocarcinoma cells, in which a gemcitabine (GEM)-induced adverse stabilization of mutant p53 was shown to produce chemoresistance, either the knockdown of mutant p53 or treatment with p53 re-activating molecules, including RITA, sensitized these cancer cells to GEM (66). Such data suggest that the used compounds stabilized a wild-type-like conformation of mutant p53, which enabled cells to respond to GEM treatment (66). However, the mechanisms through which RITA could help overcome resistance to chemotherapy warrant further investigation.

In conclusion, in this study, we observed that RITA induced massive p53-dependent apoptosis of both p53 wild-type and mutant GB cell lines and synergized with TMZ, without affecting non-neoplastic brain cells. Therefore, although additional studies are required to fully clarify the mechanisms of action of RITA, our data suggest a potential application of this approach in GB therapy.

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Availability of data and materials

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

Authors' contributions

IMF performed most of the experiments, contributing to the experimental design and data analysis; PI defined the experimental design, analyzed the data and wrote the manuscript; FP conceived and supervised the study; AG supervised the whole work and critically contributed to the study development; CAI, DC, DDM, DB and FC each performed some of the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors report no conflict of interest.

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