Preclinical Efficacy of the MDM2 Inhibitor RG7112 in MDM2-Amplified and TP53 Wild-type Glioblastomas

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Abstract

Purpose: p53 pathway alterations are key molecular events in glioblastoma (GBM). MDM2 inhibitors increase expression and stability of p53 and are presumed to be most efficacious in patients with TP53 wild-type and MDM2-amplified cancers. However, this biomarker hypothesis has not been tested in patients or patient-derived models for GBM.

Experimental Design: We performed a preclinical evaluation of RG7112 MDM2 inhibitor, across a panel of 36 patient-derived GBM cell lines (PDCL), each genetically characterized according to their p53 pathway status. We then performed a pharmacokinetic (PK) profiling of RG7112 distribution in mice and evaluated the therapeutic activity of RG7112 in orthotopic and subcutaneous GBM models.

Results: MDM2-amplified PDCLs were 44 times more sensitive than TP53-mutated lines that showed complete resistance at therapeutically attainable concentrations (avg. IC50 of 0.52 μmol/L vs. 21.9 μmol/L). MDM4-amplified PDCLs were highly sensitive but showed intermediate response (avg. IC50 of 1.2 μmol/L), whereas response was heterogeneous in TP53 wild-type PDCLs with normal MDM2/4 levels (avg. IC50 of 7.7 μmol/L). In MDM2-amplified lines, RG7112 restored p53 activity inducing robust p21 expression and apoptosis. PK profiling of RG7112-treated PDCL intracranial xenografts demonstrated that the compound significantly crosses the blood–brain and the blood–tumor barriers. Most importantly, treatment of MDM2-amplified/TP53 wild-type PDCL-derived model (subcutaneous and orthotopic) reduced tumor growth, was cytotoxic, and significantly increased survival.

Conclusion: These data strongly support development of MDM2 inhibitors for clinical testing in MDM2-amplified GBM patients. Moreover, significant efficacy in a subset of non–MDM2-amplified models suggests that additional markers of response to MDM2 inhibitors must be identified. Clin Cancer Res; 1–12. © 2015 AACR.

Introduction

Primary glioblastoma (GBM) is the most frequent and aggressive form of brain tumors in adults. Approximately 3 to 4 new cases per 100,000 people are diagnosed every year (1). Despite intensive treatment, including surgery, radiotherapy and cytotoxic chemotherapy, the prognosis of GBM patients remains poor, with a median survival of 12 to 24 months (2). One promising strategy to improve therapy is to use small pharmacologic molecules to target key molecular events driving gliomagenesis. In this context, a major effort has been conducted by several teams worldwide, including The Cancer Genome Atlas (TCGA), to identify pivotal molecular alterations in GBM with basic and clinical perspectives (3, 4).
The TCGA has demonstrated that the vast majority of GBM exhibits, through various genetic mechanisms, disruption of three major signaling pathways (4): (i) the tyrosine kinase receptor pathway, (ii) the retinoblastoma pathway, and (iii) the p53 pathway. Deregulation of the core p53 pathway occurs in 43% of cases due to: (i) p53 gene mutation or homozygous deletion in 27.9% of cases, (ii) focal MDM2 amplification in 8% to 9% of cases, and (iii) focal MDM4 amplification in 7% to 9% of cases. However, it is hypothesized that restoration of p53 function may have significant therapeutic benefit in those patients without direct mutations in the P53 gene.

p53 is a transcription factor that responds to several forms of cellular stress (5). The quantity of functional p53, together with a variety of factors that affect other signaling pathways, promotes events such as cell-cycle arrest, DNA repair, cellular senescence, or apoptosis to prevent tumor initiation or progression (5). p53 activates a large number of targets, including the cell-cycle regulator p21WAF1 (p21; refs. 6, 7). One of the main modulators of functional p53 level is MDM2 proto-oncogene, E3 ubiquitin protein ligase (a.k.a. MDM2), which binds to p53 and induces its proteasome-mediated degradation (8). Expression of the MDM2 gene is p53 dependent; thus, p53 controls the level of its own regulator MDM2 through a feedback loop (8). MDM4 (also known as MDMX) is another regulator of p53 structurally related to MDM2, but acts differently on p53 by regulating its transcriptional activity rather than its protein level (9).

Several strategies to restore p53 tumor-suppressive function, including MDM2 small-molecule inhibitors, have been explored (10). RG7112 (MDM2 Kd of 10.7 nm) has improved potency and pharmacologic properties compared with Nutlin-3. It is the first MDM2 inhibitor to enter clinical trials, where its tolerability was confirmed in liposarcoma patients and was also recently reported to have clinical efficacy in leukemia (11, 12). Tolerability was also assessed in a phase I trial in patients with advanced solid tumors and hematological neoplasms (NCT00559533), and results are pending. On the basis of their mechanisms of action, RG7112 and other agents in this class might be effective in patients with MDM2 amplification and wild-type TP53 tumor. However, studies to test this hypothesis have not been broadly performed in large numbers of genetically characterized patient-derived cell lines (PDCL) or in the clinical trials performed to date. The study presented here aims to test the therapeutic activity of RG7112, a member of MDM2/Nutlin (cis-imidazoline) inhibitors (13), in GBM PDCLs and animal models of GBM carrying various alterations of the core p53 pathway (MDM2 amplification, MDM4 amplification, TP53 mutation).

Materials and Methods

GBM cell lines and human GBM samples
U251, U87, and LN229 GBM cell lines were purchased from the ATCC and were cultured in DMEM supplemented with 10% FBS (Gibco; Life Technologies). The SJA-1 osteosarcoma cell line (formerly OsA-CL) was purchased from the ATCC and included in the present study as an MDM2-amplified–positive control (14, 15). Cells were cultured in DMEM/F12 + GlutaMax (Gibco; Life Technologies) supplemented with 10% FBS and were used within 6 months of culture after purchase of the lines. 3731, 4339, 7015, and 6215 PDCLs were established by the GlioTex team (Glioblastoma and Experimental Therapeutics) in the Institut du Cerveau et de la Moelle épinière (ICM) laboratory and maintained in neurosphere growth conditions using DMEM/F12 (Gibco; Life Technologies) culture medium supplemented with 1% penicillin–streptomycin, B27 (Gibco), EGF (20 ng/mL), and FGF (20 ng/mL; Preprotech). All PDCLs with a name starting with BT were established from tumors resected at Brigham and Women’s Hospital and Boston Children’s Hospital (Boston, MA) and were maintained in neurosphere growth conditions using the NeuroCult NSA Proliferation Kit (Stemcell Technologies) supplemented with 0.0002% heparin (Stemcell Technologies), EGF (20 ng/mL), and FGF (10 ng/mL; Miltenyi). The identity of all cell lines established at the ICM or the Brigham and Women’s Hospital and Boston Children’s Hospital were confirmed by short tandem repeat assay and validated within 6 months of their use for the studies presented here. Cell line gene statuses are listed in Supplementary Materials and Methods §4. ** indicates cell lines from the cohort #1 only, whereas the *** indicates cell lines included in both cohort #1 and #2. * indicates cell lines that were included for the in vivo studies only. All other cell lines were part of the cohort #2.

Human GBM tissue samples were selected from OncoNeuroThèque (Institut du Cerveau et de la Moelle épinière, Paris, France) tumor tissue bank. Tumor and annotations was collected with informed consent and with the relevant ethical board approval in accordance with the tenets of the Declaration of Helsinki. All cell lines were derived from patients with consent or waiver of consent by the appropriate Institutional Review Board.

Proliferation assay
For drug sensitivity assays of the cohort #1 cell lines, 96-well plates were coated with 10 µg/mL laminin (cat no. L2020, Sigma-Aldrich) at 37°C for 1 hour. Three thousand cells per well were then plated. RG7112 (gift from Roche TCRC) was resuspended as a 10 mmol/L stock solution in DMSO and was added 24 hours after plating. Seventy-two hours after drug addition, WST-1 reagent (Roche) was added according to the manufacturer's instructions. WST-1 salt is cleaved to a soluble formazan dye by a NAD(P)H-dependent reaction in viable cells. Plates were incubated for 3 hours and read by spectrophotometry at 450 nm wavelength. For cohort #2 cell lines, cells were plated in 384-well format and a pin transfer robot (Epson) was used to transfer the compound solution (MedChemExpress) into each well, with
three replicates per condition. Cell viability was measured after 72 hours of continuous drug exposure by CellTiter-Glo luminescence assay (Promega). IC₅₀, IC₉₀, and IC₁₀₀ (concentrations that induce a 75%, 99%, and 100% decrease in cell viability, respectively) were determined by least squares curve fitting using GraphPad Prism 6.

In vivo efficacy orthotopic and heterotopic studies
All protocols involving work with live animals were reviewed and approved by the Ministère de l’Enseignement Supérieur et de la Recherche (Paris, France) under the protocol #0113.02 or in accordance with Dana-Farber Cancer Institute animal facility regulations and policies under the protocol #09-016. For the orthotopic model, before inoculation, GBM cells were transduced with the luciferase gene (Gentaur). GBM cells were implanted (1.4 x 10⁵ cells/2 µL) into the brain of Athymic Nude mice (7-week-old females, 10 animals/group). A stereotaxic injection frame (David Kopf Instruments) was used to inject cells into the right caudate nucleus-putamen (ML +0.15 mm; AP +1.0 cm; DV –0.25 mm). Animals were imaged weekly using the IVIS Spectrum (Perkin-Elmer) 10 minutes after injection of 2 mg luciferin (Promega). Animals were randomly assigned to treatment or vehicle arm when a signal of 1.106 photon/second was measured (Promega). Animals were randomly assigned to treatment or vehicle arm when a signal of 1.106 photon/second was measured (Promega). Animals were randomly assigned to treatment or vehicle arm when a signal of 1.106 photon/second was measured (Promega). Animals were randomly assigned to treatment or vehicle arm when a signal of 1.106 photon/second was measured (Promega). Animals were randomly assigned to treatment or vehicle arm when a signal of 1.106 photon/second was measured (Promega).

For the heterotopic (subcutaneous) model, 2 x 10⁵ cells were resuspended in Hank’s Buffered Salt Solution (LifeTechnologies), mixed with an equal volume of Matrigel (BD Biosciences), and injected into both flanks of 8-week-old NU/NIU mice (Charles River Laboratories). Animals were randomly assigned to treatment or vehicle arm when tumors measured a volume of 200 mm³.

For both orthotopic and heterotopic models, animals were treated by gavage with 100 mg/kg of RG7112 formulation (100 mg/mL RG7112, 2% hydroxypropylcellulose, 0.1% Tween 80, 0.09% methylparaben and 0.01% propylparaben in water) or vehicle once per day, 5 days per week for 3 weeks. For the evaluation of GBM blood–brain barrier (BBB) integrity only, 1.2 mg of Hoechst 33342 (Sigma) diluted in PBS was injected i.v. before termination. Mice were terminated by asphyxiation when they showed signs of tumor-associated illness or before reaching maximum subcutaneous tumor burden.

Pharmacokinetics studies
GBM cells were inoculated in the brain of Athymic Nude mice (Harlan) as described below and animals were assigned to different pharmacokinetics (PK) time points when bioluminescence signal reached 1.10⁸ photon/second. This threshold was selected to ensure that tumor volumes were as significant as possible without causing symptoms of pain or illness. The dose treatment solution of RG7112 (100 mg/mL RG7112) was prepared in a vehicle composed of 2% hydroxypropylcellulose, 0.1% Tween 80, 0.09% methylparaben, and 0.01% propylparaben in water. Mice were sacrificed at 0, 1, 2, 4, 8, 24, and 48 hours post-gavage (3 mice/time point). Blood was collected via cardiac puncture in polyethylene tubes using a heparinized syringe. Samples were immediately centrifuged at 5,000 rpm for 15 minutes and plasma was removed and stored at –80°C until analysis. Whole brains were collected, rinsed with 0.9% sodium chloride. The right and left brain hemispheres were harvested separately and labeled as tumor hemisphere and counter hemisphere, respectively, and were frozen at –80°C. RG7112 levels in mice plasma, and brains were measured using validated liquid chromatography coupled with mass tandem spectrometry methods (TQS Quantum Ultra; Thermo Fisher Scientific).

Live cardiac puncture exsanguination was shown to remove most of the blood and intravascular drug from functional vessels, suggesting that perfusion before brain harvesting has limited impact on PK measurements (16). In addition, others (17) have suggested that no correction of apparent drug tissue concentrations for blood contamination was necessary for brain tissue, unless the brain-to-plasma ratio is below 0.1. The PK parameters Tₘₐₓ (the time to reach peak concentration) and Cₘₐₓ (the peak concentration) were, thus, calculated accordingly by non-compartmental analysis of mean concentration time data with WinNonlin (version 5.2; Pharsight). It should be noted that this was a destructive sampling study design, hence, variability associated with the PK parameters other than AUC₀₋ₜ could not be determined, and thus no statistical test was performed on these parameters.

AUC₀₋ₜ for brain and plasma samples were calculated by using the trapezoidal rule. In this serial sampling design, only one measurement was available per animal, so the method developed by Bailer for destructive sampling was used to compare the AUCs ratios of brain/plasma concentrations between tumor and contralateral brain hemispheres (18).

The SEM concentrations p_i at time t_i, denoted sem_i was estimated as the empirical SD divided by n_i. Thus, the SE of the AUC was estimated according to the equation:

\[ \text{SE}^2 [\text{AUC}_i] = \left(0.5 \times (t_i - t_0) \times \text{sem}_i\right)^2 + \sum_{i=2}^{n} \left(0.5 \times (t_i - t_{i-2})\right)^2 + 
\left(0.5 \times (t_n - t_{n-1}) \times \text{sem}_n\right)^2. \]

The test for equality of the mean AUCs between animal groups A and B was performed using the standard Wald statistic:

\[ z_{obs} = \frac{[\text{AUC}_A - \text{AUC}_B]}{\sqrt{\text{SE}^2 (\text{AUC}_A) + \text{SE}^2 (\text{AUC}_B)}}. \]

Under the null hypothesis that the mean AUCs are equal, this statistic follows a normal distribution. The null hypothesis was rejected if \( |z| > 1.96. \)

A separate distribution study on subcutaneous tumor was also conducted. GBM cells were implanted s.c. in Athymic Nude mice (Harlan; and ICR SCID, Taconic). The RG7112 treatment solution (100 mg/mL) was prepared as described above and sacrificed was performed 48 hours after gavage. Blood was collected via cardiac puncture in polyethylene tubes using a heparinized syringe. Samples were immediately centrifuged at 5,000 rpm for 15 minutes and plasma was removed and stored at –80°C until analysis. The subcutaneous tumor was collected, rinsed with 0.9% sodium chloride and was frozen at –80°C. RG7112 levels in mouse plasma and tumor were measured using validated liquid chromatography coupled with mass tandem spectrometry methods (TQS Quantum Ultra; Thermo Fisher Scientific) and compared with plasma and brain hemispheres of GBM orthotopic-grafted mice sacrificed 48 hours after drug administration.
Magnetic resonance imaging in mice

The MR images were acquired with a 11.7-T system (Bruker Biospec 117/16 USR horizontal bore, 750 mT/m gradients, Paravision 5.1) and a CryoProbe (Bruker Biospin). Anatomical T1-weighted images were acquired 3 weeks after bioluminescence signal reached 1.10³ photon/second, before and after i.v. gadolinium injection with a 2D rapid acquisition relaxation enhanced (RARE) sequence; TR = 500 ms; TE = 10 ms; Matrix (Mtx) = 128 × 128; field-of-view (FOV) = 12.8 × 12.8 mm (resolution = 100 × 100 μm²); slice thickness = 400 μm; number of excitations (Nex) = 1; RARE factor = 1; scan time (Tacq) = 1 min. Anatomical T2-weighted images were acquired before initiation of treatment (when bioluminescence signal reached 1.10³ photon/second) and at the end of the 3 weeks treatment period with a 3D gradient-echo sequence with TR = 16 ms; TE = 4.7 ms; Mtx = 192 × 192 × 192; FOV = 19.2 × 19.2 × 19.2 mm (resolution 100 μm isotropic voxel); Nex = 3; Tacq = 20 min. Tumor volumes were estimated using the formula V = 5/3π(a × b × c) where a, b, and c are the three radii (19).

Statistical analysis

All statistical data were collected using GraphPad Prism software. For protein and RNA level comparisons, P values were calculated by comparing data measured from three independent experiments from each individual cell line with data from all the other cell lines. Parametric analysis was done using SEM, mean experiments from each individual cell line with data from all the calculated by comparing data measured from three independent posttests for multiple comparisons, and the Student's test assessed in 11 cell lines. The two MD2-amplified cell lines (i.e., 3731 and BT484) overexpressed MDM2 mRNA and protein. Levels of MDM4 protein were statistically greater in the wild-type cell line and differences between curves were statistically different from the other wild-type lines. Although a trend for increased MDM4 protein expression was observed in BT112 (P = 0.16), the levels were not statistically different from the non-amplified lines. TP53 homozygous mutant 4339 showed a significantly higher p53 protein expression, but the slight increase observed in the TP53-mutant U251 line was not found to be statistically different from the other wild-type lines. Accumulation of p53 protein was not detected in TP53-mutant BT216.

Results

MDM2 amplification does not affect prognosis in newly diagnosed GBM patients and is maintained at tumor recurrence

Datasets from the 2013 GBM TCGA database (4) (publically available on cBio portal; MSKCC) were extracted and analyzed. TP53, MDM2, and MDM4 were found to be genetically altered (i.e., gene copy number or sequence abnormalities) in 22%, 9%, and 9%, respectively, of GBM from TCGA 2013 database. Graphs can be seen in Supplementary Materials and Methods S1. MDM2/MDM4 alteration with concurrent TP53 mutation was found to be a rare event detected in 1.37% of cases. Moreover, MDM2 and MDM4 amplifications were found to be mutually exclusive. Finally, as expected, MDM2 mRNA was found to be overexpressed in MDM2-amplified tumor (Supplementary Materials and Methods S1).

From the OncoNeuroTheque database, 696 newly diagnosed GBM with genomic profiling and clinical annotations were identified. Forty-three tumors of 696 (6.2%) were MDM2 amplified. No statistical significance was detected between both groups for median OS (15.5 months in non-amplified vs. 15.5 months in MDM2-amplified), age at diagnosis (57.4 vs. 58.9 years in MDM2 amplified), and sex ratio (1.43 vs. 1.39 in MDM2-amplified). Survival curve is provided in Supplementary Materials and Methods S2.

Among these patients, five MDM2-amplified GBM patients were re-operated at tumor recurrence once (4 patients) or twice (1 patient). Interestingly, MDM2 amplification and TP53 mutational status were maintained over time for all patients (Supplementary Materials and Methods S3).

PDCLs maintain genetic TP53 status heterogeneity observed in human GBM tumors

Thirty-six GBM cell lines, including 32 PDCLs from two sets (cohort #1 and cohort #2, 4/32 PDCLs were used in both cohorts) and four commercially available cell lines were used in the present study. Data acquired from both cohorts were obtained from two independent laboratories. A summary of PDCL genetic status can be seen in Supplementary Materials and Methods S4. MDM2 and MDM4 high-level amplifications were detected in 2 of 32 (BT484 and 3731) and 2 of 32 (BT112 and BT216) GBM PDCLs, respectively, roughly equivalent with the incidence rate reported in patient samples in the TCGA dataset. MDM2 and MDM4 gain was seen in 3 of 32 and 2 of 32 PDCLs cell lines, respectively. Fourteen of the total cohorts of 36 GBM lines carry TP53 point mutations altering p53 functions. Most mutations were amino acid substitutions leading to a non-functional p53 protein. One cell line (BT320) exhibited TP53 homozygous deletion. Although none of the MDM2-amplified cell lines carried TP53 mutations, MDM4-amplified BT216-harbored TP53 mutation.

MDM2, MDM4, and TP53 expressions at the mRNA level (Supplementary Materials and Methods S5) and at the protein level were also quantified (Supplementary Materials and Methods S6) in 11 cell lines. The two MDM2-amplified cell lines (i.e., 3731 and BT484) overexpressed MDM2 mRNA and protein. Levels of MDM4 protein were statistically greater in the MDM4-amplified line BT216 compared with non-amplified cell lines. Although a trend for increased MDM4 protein expression was observed in BT112 (P = 0.16), the levels were not statistically different from the non-amplified lines. TP53 homozygous mutant 4339 showed a significantly higher p53 protein expression, but the slight increase observed in the TP53-mutant U251 line was not found to be statistically different from the other wild-type lines. Accumulation of p53 protein was not detected in TP53-mutant BT216.

RG7112 exhibits greatest efficacy in MDM2-amplified GBM cell lines

The impact of RG7112 on cell viability was first assessed in 11 GBM cell lines (cohort #1, AI). The IC₅₀ after exposure for 72 hours were shown in Fig. 1B, and were derived from dose-response curves (Fig. 1A). The IC₅₀ of both cell lines carrying MDM2 gene amplification and wild-type TP53 (3731 and BT484) are 37 times lower than that of TP53 mutated and/or MDM2/4 non-amplified cell lines. MDM4-amplified BT112 and MDM2-gained DKMG lines were 7 and 3.5 times more sensitive than TP53-mutant and/or MDM2/4 nonamplified cell lines, respectively. The IC₅₀ of TP53 homozygous mutant cell lines (4339, U251, and BT216) were not different from those of TP53 wild-type/MDM2/4 non-amplified lines (7015, 6215, LN229, and U87).

Validation experiments were conducted in an independent lab (cohort #2, KL). MDM2-amplified cell lines (3731 and
BT484) were found to be highly sensitive to the inhibitor (average IC_{50} of 0.52 \mu mol/L), whereas all cell lines carrying a TP53 genetic alteration showed marked resistance to the inhibitor, with an average IC_{50} value of 21.9 \mu mol/L (42-fold increase). The MDM4-amplified line BT112 together with all lines carrying gains in MDM2 or MDM4 or TP53 showed intermediate sensitivity (average IC_{50} of 1.5 \mu mol/L). Overall, MDM2/4-amplified/gained lines, TP53-mutated/deleted cell lines, or cell lines with none of these alterations (MDM2/4_NL/TP53_Wt lines) were 18- and 2.8-fold, respectively, more sensitive than the TP53-mutated/deleted cell lines (Fig. 1C).

RG7112 restores the p53 signaling pathway in MDM2-amplified cell lines in vitro

Protein levels of p53 and its downstream effector p21 were assessed by immunofluorescence in 3731^{MDM2_Amp/TP53_Wt}, 4339^{MDM2/4_NL/TP53_Mt}, and 7015^{MDM2/4_NL/TP53_Wt} (Fig. 2A and B). A 24 hours exposure to IC_{100} restored p53 and p21 expression in MDM2-amplified 3731. This time point was selected because signs of cell death started to occur only 20 to 25 hours after addition of RG7112, an observation that is consistent with a previously published report (20). In 4339^{MDM2/4_NL/TP53_Mt}, p53 protein level was also increased by the treatment, whereas p21 level was not significantly affected. Finally, in 7015^{MDM2/4_NL/TP53_Wt}, p53 level remained unchanged but p21 protein level was found to be decreased.

Protein levels of p53 and p21 as well as MDM2 were also assessed by Western Blot analysis in 3731^{MDM2_Amp/TP53_Wt} following a 24 hours exposure to 0.5 \mu mol/L, 1.5 \mu mol/L and 4 \mu mol/L RG7112 which were calculated to be the IC_{75}, IC_{99}, and IC_{100}, respectively, when cells are exposed over 72 hours (Fig. 2C). p21, p53, and MDM2 protein levels were significantly increased by RG7112.
RG7112 crosses the BBB and the blood–tumor barrier

A key question related to the imidazole class of MDM2 inhibitors has been to what extent their relatively larger size might limit their penetration across the BBB. First, to assess the permeability of the BBB in GBM tumor generated by 3731 MDMS2 _Amp/TP53_Wt PDCL, this line was engrafted in the putamen of mice. T1-weighted MRI (Fig. 3A and B) showed marked gadolinium contrast enhancement 130 days after inoculation, consistent with a partial disruption of the BBB in this model. Further assessment of BBB disruption was also performed with Hoechst 33342 injected i.v. in animals before sacrifice. Whereas Hoechst staining was not detected in normal brain tissue, staining was found in tumor tissue, mainly in the center of tumors and in the vicinity of tumor blood vessels (Fig. 3C and D). Taken together, these data suggest a partial disruption of the BBB in this MDM2-amplified GBM model compared with the normal brain.

PK profiling of RG7112 in the plasma, tumor cells grafted hemisphere and contralateral hemisphere was conducted in 3731 MDMS2 _Amp/TP53_Wt GBM-bearing mice (treatment started on an average of 80 days after inoculation; Fig. 3E). T max were 2 to 8 hours, 2 and 8 hours in the plasma, grafted, and contralateral hemispheres, respectively. C max were 17,178 ng/mL, 3328 ng/g (equivalent to 4.8 μmol/L) and 2025 ng/g (equivalent to 2.9 μmol/L) in plasma, grafted and contralateral hemispheres, respectively. All of these C max were well above the mean IC 50 value calculated from _in vitro_ studies for the 3731 MDMS2 _Amp/TP53_Wt cell line (0.5 μmol/L). Finally, brain tissue-to-plasma AUCs ratio was significantly greater (1.9-fold) in tumor-grafted hemispheres compared with contralateral non-tumor hemispheres (0.198 vs. 0.106 ng·h/g/ng·h/mL in grafted and contralateral hemispheres, respectively, z = −4.07; Figure 3, panel F). Interestingly, RG7112 distribution was 22-fold higher in subcutaneous tumor than in tumor-grafted hemispheres with a subcutaneous tumor-to-
plasma ratio reaching 6.08 48 hours after drug administration (Fig. 3G). This observation suggests that the BBB is only partially disrupted in the grafted hemisphere.

RG7112 reduces tumor growth rate and increases survival in heterotopic and orthotopic animal models bearing MDM2-amplified GBM

The in vivo efficacy of RG7112 was first assessed in subcutaneous GBM (3731MDM2_Amp/TP53_Wt, BT139MDM2/4_NL/TP53_Wt, BT182MDM2/4_NL/TP53_Wt), and 4339MDM2/4_NL/TP53_Mt) and osteosarcoma (SISA5MDM2_Amp/TP53_Wt) models (Fig. 4). Interestingly, similarly to what was observed with the SISA5MDM2_Amp/TP53_Wt model, a complete tumor growth suppression, and even a 80% regression, was achieved in the 3731MDM2_Amp/TP53_Wt model during the 21-day treatment period (P = 0.003), but tumors rapidly re-emerged within days of withdrawing treatment. A slight but not statistically different reduction in growth rate was also observed in the TP53 wild-type models (BT139 and BT182); however, the treatment did not induce tumor regression. Finally, RG7112 appeared to be inactive in a TP53-mutant model (4339). Taken together, these observations correlate with the data observed in vitro.

The efficacy of RG7112 (100 mg/kg) was then assessed in the MDM2-amplified 3731 orthotopic GBM in vivo model. RG7112 decreased tumor progression rate as shown by bioluminescence imaging curves (Fig. 5A–C). This decrease of tumor growth was validated on T2-weighted MRI anatomical images (Fig. 5D). The tumor volume decreased from 4.91 mm³ before RG7112 treatment to 4.28 mm³ at the end of treatment (13% decrease). In contrast, tumor volume increased from 5.97 to 84.84 mm³ in vehicle-treated tumors, representing a 14.2-fold volume increase in these animals. Finally, RG7112 prolonged survival of tumor-bearing mice treated with RG7112 versus vehicle-treated mice (P = 0.0003; Fig. 5B).

Pharmacodynamic studies

A pharmacodynamic study was also conducted in subcutaneous GBM models, where tumor-bearing animals were treated for 48 hours with RG7112 and subsequently analyzed for effect on pathway dynamics (Fig. 6). The expression of Ki67, a proliferation
marker, was reduced by the treatment in all models. Cleaved caspase-3 (CC3), an apoptosis marker, was increased by the treatment in all models, indicating a strong cytotoxic effect (Fig. 6A–D, F). Finally, protein expression of MDM2, p53, and p21 was also increased by the treatment in all models compared with untreated (vehicle) animals as shown by IHC (Fig. 6A–D, F) and Western blot analysis (Fig. 6E).

p53 and p21 protein expression was also assessed in the orthotopic model at the end of the 21-day treatment period (Fig. 6G). However, none of these markers were statistically different in treated animals compared with untreated ones.

Orthotopic tumors at recurrence after RG7112 treatment were characterized as well. Treated and untreated animals were sacrificed when they showed signs of illness, which occurred at 19 to 31 days and 3 to 19 days after the end of the treatment period, respectively. Figure 6H shows that p53 protein level only (and not p21) was increased in recurrent tumors of treated animals compared with untreated tumors. The \( TP53 \) gene was sequenced by Sanger sequencing in these tumors, but no mutation was detected. MDM2 amplification level was quantified by TaqMan assay and the amplification level was unchanged between treated and untreated animals.

Discussion

Translational research is now largely influenced by the approach of molecular personalized therapy. Indeed, molecular targeting has dramatically changed the prognosis of multiple aggressive cancers (e.g., gastrointestinal stromal tumor or metastatic melanoma) exhibiting a specific actionable molecular alteration (21, 22).

In this study, we have evaluated the activity of one of the most promising MDM2 inhibitor, RG7112, in a panel of GBM PDCL carrying different molecular alterations of the p53 core pathway that are frequently found in human GBM. The experiments were conducted on a large set of GBM PDCL in two independent laboratories to validate the findings.

The GBM PDCL used in the present study carried alterations of the p53 core pathway in proportions that were similar to what is found in human GBM. Indeed, \( TP53 \) mutation/deletion, MDM2/4 amplifications or gains, which are generally mutually exclusive, were observed in 41.7%, 13.9%, and 13.9% of our cell lines, respectively. MDM2/4 amplifications were associated with increased protein expression. \( TP53 \) mutation was associated with accumulation of p53 protein in two of the three \( TP53 \)-mutated cell lines assessed. The absence of detected p53 protein in BT216 can be explained by the fact that deletion mutations resulting in truncated protein do not lead to detectable protein accumulation (23, 24).

In vitro studies revealed that PDCL\( ^{MDM2/Amp} \) were approximately 40 times more sensitive to RG7112 than the other lines. A 24-hours exposure to RG7112 restored p53 and p21 protein expressions in PDCL\( ^{MDM2/Amp} \). In addition, MDM2 expression was also increased, probably due to an increased transcription of the gene induced by p53 (8). It can, thus, be presumed that cell death occurred through a p53-driven mechanism in these cells. Although PDCL\( ^{TP53/\text{Wt}} \) showed intermediate and variable sensitivity, PDCL\( ^{TP53/\text{Mt}} \) or PDCL\( ^{TP53/\text{Deleted}} \) were resistant to RG7112.
The IC50s reported in this study are consistent with previous reports showing that MDM2 amplified cancer cell lines (e.g., liposarcoma and osteosarcoma lines) are more sensitive to RG7112 or Nutlin-3 (20, 25–27). Interestingly, PDCL MDM4 Amp/TP53 Wt and PDCL MDM4 Gain/TP53 Wt also showed considerable sensitivity to the compound, which was unexpected, as Nutlin compounds have a 100-fold greater affinity for MDM2 over MDM4 (13). MDM4 amplification was even shown to reduce sensitivity of cells to Nutlin-3 (28–30).

However, as suggested (13), p53 induced upregulation of MDM2 might promote MDM4 degradation in some cell lines, which, in turn, would activate apoptosis cascade. These observations will need to be confirmed using additional cell lines harboring these alterations.

Overall, our in vitro data suggested that RG7112 is active in GBM cells. We thus proceeded with in vivo PK and efficacy studies. We showed that the compound absorption from oral administration was fairly rapid, as the plasma Cmax was achieved within 2 hours. Plasma AUC0–48 h (420,253 ng·h/mL) following a single dose of 100 mg/kg RG7112 was consistent with a previous study that reported an AUC of 250,000 ng·h/mL preclinically in mice with a dose of 50 mg/kg (31). PK study conducted in contralateral hemispheres of tumor-bearing mice showed that RG7112 crosses the normal BBB with a brain-to-plasma AUC ratio of 0.106, which is consistent with preliminary PK data obtained in healthy mice in our laboratory (data not shown).

A lack of data on the degree to which existing MDM2 inhibitors might penetrate the BBB or blood–tumor barrier and have efficacy has limited studies in GBM. After demonstrating that the BBB is partially disrupted in our GBM mouse models using Hoechst dye (32, 33) and brain MRI with gadolinium infusion, we showed that RG7112 crosses pathologic blood–brain and blood–tumor barriers with a brain-to-plasma AUC ratio of 0.198. Interestingly, the Tmax in the tumor hemisphere was achieved within 2 hours compared with 8 hours in the contralateral hemisphere, and the AUC in the grafted hemisphere was almost twice the AUCcontralat, suggesting that RG7112 penetrates more easily the tumor tissue. However, the much higher concentration in the subcutaneous tumor suggests that the BBB is only partially disrupted in the brain tumor. It is important to note that, because of the nature of GBM tumor vasculature, which was shown to comprise between 30% and 50% of poorly perfused vessels (34), we cannot exclude the possibility of a contribution from intravascular drug and blood in our brain tissue AUCs calculations.

Most importantly, we demonstrated that RG7112 is active in our in vivo MDM2-amplified GBM models. The 3-week treatment
period led to a 50% increase in median survival of orthotopic tumor-bearing animals. Whereas a stabilized disease was observed in the orthotopic 3731\textsuperscript{MDM2\_Amp} model, a complete inhibition of tumor growth was observed in the subcutaneous model, suggesting a greater efficacy of RG7112 in the subcutaneous model compared with the orthotopic model. This increased efficacy could be explained by the much greater drug quantity measured in subcutaneous tumors compared with orthotopic tumors. In the clinic, RG7112 treatment of MDM2-amplified liposarcoma patients resulted in a stable disease (14/20 patients) or a partial response (1/20 patients; ref. 11). The data reported here in the orthotopic model are coherent with this observation, as tumor growth was inhibited by the treatment, but seemed to be reinitiated when the treatment was halted. In this same clinical study, increased p53, p21, and MDM2 protein expressions and decreased Ki67 expression were observed in the tumor after the first 6 to 10 days cycle of daily RG7112 administration (11). In our study, increased expression of p53, p21, MDM2 and decreased expression of Ki67 were also measured in subcutaneous treated tumors 48 hours after RG7112 dosing.

In both orthotopic and subcutaneous models, it appears that inhibition of MDM2 prevented tumor growth. However, when the treatment was stopped, tumor growth was reinitiated, suggesting that RG7112 is efficacious but of limited duration. Moreover, the expression of MDM2, p53, p21, Ki67, and CC3 markers was assessed in orthotopic tumors that were treated for 3 weeks and, although trends were observed, no statistically significant changes were observed. This suggests that tumor concentration of RG7112 was not high enough at that point to induce detectable changes in the expression of these markers, or that resistance mechanisms had been activated. Panel 6H proposes a possible mechanism of resistance to MDM2 inhibition. An increase in p53 expression (but not p21 nor MDM2) was observed in tumors at relapse, potentially associated with the emergence of TP53-mutant tumor.

Figure 6.
Quantification of markers of response to RG7112. A to D, quantification of Ki67, CC3, MDM2, p53, and p21 protein level by immunohistochemistry of: A and B, MDM2/4 and TP53 wild-type cell line; C, MDM2 amplification and TP53 wild-type cell line; and D, MDM2-amplified osteosarcoma-positive control, harvested 48 hours after one dose of RG7112. E, Western immunoblot analysis of subcutaneous tumors showing MDM2 75 and 90 kDa forms, p53 and p21 proteins. F, representative images showing marker staining in brown and nuclei in blue of vehicle and RG7112 treated 3731\textsuperscript{MDM2\_Amp/TP53\_Wt} tumor. G, quantification of p53 and p21 protein levels on the day after the 21-day treatment period; or H, at the moment of termination when animals showed signs of tumor-associated illness due to relapse after treatment.
cell subpopulations, as it was reported previously (reviewed in ref. 35). However, TP53 sequencing of these tumors did not reveal any mutations, possibly because these cells were not abundant enough in the tumor tissue. In vitro studies, including a comprehen-
sive assessment of molecular characteristics of cells exposed to
RG7112 for an extended period of time, could help identify
resistance mechanisms and propose combination therapies to
improve RG7112 therapeutic efficacy. For example, temozolo-
mide was previously shown to act synergistically with RG7112
(36). Other MDM2 inhibitors were shown to enhance sensitivity
towards MDM2, whose tumor carries this genomic alteration.

Discussion of Potential Conflicts of Interest

P.Y. Wen reports receiving speakers bureau honoraria from Merck, and is a
consultant/advisory board member for Cavion, Cubist, Foundation Medicine,
Midatech, Novartis, Regeneron, Roche, and Vascular Biogenics. A. Idbaih
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