Targeting **MPS1** Enhances Radiosensitization of Human Glioblastoma by Modulating DNA Repair Proteins

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**Abstract**

To ensure faithful chromosome segregation, cells use the spindle assembly checkpoint (SAC), which can be activated in aneuploid cancer cells. Targeting the components of SAC machinery required for the growth of aneuploid cells may offer a cancer cell-specific therapeutic approach. In this study, the effects of inhibiting Monopolar spindle 1, **MPS1** (**TTK**), an essential SAC kinase, on the radiosensitization of glioblastoma (GBM) cells were analyzed. Clonogenic survival was used to determine the effects of the **MPS1** inhibitor NMS-P715 on radiosensitivity in multiple model systems, including GBM cell lines, a normal astrocyte, and a normal fibroblast cell line. DNA double-strand breaks (DSB) were evaluated using γH2AX foci, and cell death was measured by mitotic catastrophe evaluation. Transcriptome analysis was performed via unbiased microarray expression profiling. Tumor xenografts grown from GBM cells were used in tumor growth delay studies. Inhibition of **MPS1** activity resulted in reduced GBM cell proliferation. Furthermore, NMS-P715 enhanced the radiosensitivity of GBM cells by decreased repair of DSBs and induction of postradiation mitotic catastrophe. NMS-P715 in combination with fractionated doses of radiation significantly enhanced the tumor growth delay. Molecular profiling of **MPS1**-silenced GBM cells showed an altered expression of transcripts associated with DNA damage, repair, and replication, including the DNA-dependent protein kinase (**PRKDC/DNAPK**). Next, inhibition of **MPS1** blocked two important DNA repair pathways. In conclusion, these results not only highlight a role for **MPS1** kinase in DNA repair and as a prognostic marker but also indicate it as a viable option in glioblastoma therapy.

**Implications:** Inhibition of **MPS1** kinase in combination with radiation represents a promising new approach for glioblastoma and for other cancer therapies. *Mol Cancer Res; 13(5); 852–62.* ©2015 AACR.

**Introduction**

Aneuploidy is a hallmark of cancer cells in which chromosomes are inappropriately partitioned between daughter cells due to aberrant mitosis. Faithful segregation of chromosomes during each cell division is normally ensured by the mitotic spindle assembly checkpoint (SAC; refs. 1, 2). It is a highly robust response and alterations in this pathway are frequently observed in a variety of human cancers (3). It has long been thought that mutation of the genes that control chromosome segregation during mitosis may explain the high rate of chromosomal instability and aneuploidy, which is characteristic of most solid tumors, including glioblastomas (GBM; refs. 4, 5). Monopolar spindle 1 (**MPS1**), an essential SAC kinase, was originally identified as a protein kinase that is overexpressed in human tumor cells and a kinase associated with cell proliferation in mouse embryonic carcinoma cells (3, 4, 6, 7). **MPS1** functions in several aspects of cell-cycle control, including mitotic SAC activation, proper mitotic progression, centrosome duplication, chromosome alignment, error correction of kinetochore-microtubule attachment, and recruitment of SAC components to kinetochores (8–10). Like many cell-cycle regulators, **MPS1** transcription is deregulated in a variety of human tumors and elevated **MPS1** mRNA levels are found in several human cancers, including thyroid papillary carcinoma, breast cancer, gastric cancer, bronchogenic carcinoma, and lung cancers (6, 11, 12). Furthermore, high levels of **MPS1** correlate with a more aggressive histologic grade in breast cancers (13). Several lines of evidence implicate **MPS1** in the genotoxic stress response, such as stress caused by DNA damage. Upon exposure to X-ray or UV irradiation, **MPS1** causes robust mitotic arrest by direct interaction with **CHEK2** and any disruption of the positive feedback loop between these two genes attenuates the DNA damage checkpoint (14, 15).

Approximately 50% of all cancer patients and almost all patients with GBM receive radiotherapy either alone or in combination with other treatment modalities (16, 17). Any improvement in the efficacy of radiotherapy will therefore benefit a large number of patients. Furthermore, one of the biologic factors that...
affects radiotherapy outcome is intrinsic radiation damage repair capacity of tumor cells. Modulating the response to ionizing radiation through the inhibition of DNA repair has been a longstanding focus in translational radiotherapy research and represent an attractive target for new therapeutic modalities (17). In this study, we show that, inhibition of MPS1 abrogates DNA repair following RT allowing an accumulation of DNA damage and, as a consequence, cells eventually undergo mitotic catastrophe. Combination of MPS1 inhibition with irradiation increased the radiosensitivity of GBM cells.

Materials and Methods

Cell lines and drug treatment

The LN18 (ATCC) and the U251 (National Cancer Institute Frederick Tumor Repository) human GBM cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS), and maintained at 37°C, 5% CO₂, 10% fetal bovine serum (FBS), and maintained at 37°C, 5% CO₂. MRC9 (human fetal lung fibroblasts) were obtained from the ATCC (CCL-212) and maintained in minimum essential medium supplemented with 10% FBS, glutamine, sodium pyruvate, and nonessential amino acids. Human brain astrocytes were purchased from ScienCell (#1800) and grown in Astrocyte Medium with the recommended supplements as per the manufacturer's instructions and used between passages 3 to 6. NMS-P715 was obtained from Nerviano Medical Sciences (18). Drug was reconstituted in dimethyl sulfoxide (DMSO) and stored at -20°C. Cells were plated 24 hours prior to drug treatment and treated with NMS-P715 at the concentrations indicated in each experiment.

siRNA-based analysis, gene expression profiling, and Ingenuity Pathway Analysis

For siRNA transfections, 2-pmol siMPS1 (siMPS1_2_5’-TTGACTATTGATATCTTCGAA-3’, Si00050701; siMPS1_7_5’-TCCGACTTATGATATGAAA-3’, Si02223234; siMPS1_9_5’-CAGAAATAAGGTTACCGGAATT-3’, Si03062745; Qiagen Inc.) were complexed with RNAi Max lipid transfection reagent (Invitrogen) in DMEM media for 15 minutes at ambient temperature. Two-thousand cells suspended in DMEM supplemented with 20% FBS were then added. Plates were maintained at ambient temperature for 15 minutes before being placed at 37°C/5% CO₂. Cell viability was assessed 5 days after siRNA transfection through quantification of ATP (CellTiter-Glo luminescent Reagent; Promega). Untransfected cells and wells transfected with negative [All star siNegative (siNeg); Qiagen] and positive (All star siCelldeath; Qiagen) control siRNAs were used as controls. RNA and protein for quantitative PCR (qPCR) and Western blot analysis were harvested 48 hours after siRNA transfection. To generate a gene expression profile of MPS1-silenced cells, total RNA was extracted (after 6, 24, and 48 hours after transfection) from siMPS1 and siNegative transfected cells and gene expression was assayed using Affymetrix arrays. Analysis was carried out as previously described (19). Data analysis was carried out with the use of R packages from the Bioconductor project (www.bioconductor.org). The datasets were normalized using MAS5 algorithm. Probesets from each array were mapped to official gene symbols. When multiple probesets were mapped to the same gene, the expressions were processed using customized script to rule out combining values with potential splice variants. In short, a Pearson correlation was computed between all probesets for a given gene and the two profiles with highest correlation were averaged to represent the gene. Z-score transformation was used to adjust the systematic bias of datasets. An additional filter with SD < 0.3 was implemented to remove invariant genes. Between group siMPS1 (at 6, 24, and 48 hours), differences were compared using a one-way analysis of variance (ANOVA) with post-hoc Scheffe analysis. Genes changed between siMPS1 and siNeg conditions (P ≤ 0.05 and fold change ≥±1.33) at each time point were further analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems).

Real-time qPCR

qPCR was carried out using Quantitect Reverse Transcription and SYBR Green PCR kits according to the manufacturer’s specifications (Sigma-Aldrich, USA). 100-200ng RNA was used for each reverse transcription reaction. Prevalidated primer sets for MPS1, DNAPK/PKRCDC and TOP2A were used (Sigma). Reactions were run in an Applied Biosystems 7500 Real Time PCR thermal cycler, and the 2⁻ΔΔCt method was used to calculate relative expression.

Western blot analysis

Cell pellets were lysed on ice in RIPA buffer (Pierce, Rockford, IL) supplemented with Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail (Sigma, St. Louis, MO). Nuclear cell extracts used in DNA repair assays were prepared using the NE-PER Nuclear Extraction kit (Thermo Scientific, Maine) and did not contain Phosphatase Inhibitor Cocktail. Protein concentrations were determined by Bradford assay (Bio-Rad). Protein (50 μg) was diluted 1:5 in 5X protein loading buffer (Fermentas, Glen Burnie, MD), boiled at 80°C for 5 minutes, electrophoresed on 4–20% Tris-Glycine gel, and transferred using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% nonfat milk powder (Bio-Rad), incubated with primary antibody overnight at 4°C, incubated with HRP-coupled secondary antibody 1 hour at room temperature, developed with Visualizer Western Blot Detection Kit (Millipore, Billerica, MA) and visualized on a LAS-4000 imager (Fujiﬁlm, Edison, NJ). Membranes were stripped with Re-Blot Plus Mild (Millipore, Billerica, MA) and reblocked and probed for additional proteins of interest. The following antibodies were used at 1:1,000 dilutions: human anti-MPS1 (05-683; Millipore); mouse anti-actin (MAB 1501R; Millipore), human anti-rabbit DNAPK (04-1024; Millipore), pDNAPK S2056 (ab18192; Abcam) and human anti-rabbit TOP2A (4733; Cell Signaling Technology). Secondary antibodies, goat anti-rabbit-HRP and goat anti-mouse-HRP (Santa Cruz Biotechnology) were used at 1:10,000 dilution.

In vitro non-homologous end joining activity and homologous recombination assays

Standard cell free in vitro non-homologous end joining assay (NHEJ) was performed as described previously with some minor modifications (20). Linearized plasmid substrate with cohesive ends was incubated with 1 μg of nuclear protein extracts, 1mmol/L ATP and 1mmol/L DTT in the end joining reaction buffer at 37°C for 1 hour. The reaction mixture was deproteinized with proteinase K at 65°C for 30 minutes, electrophoresed on a 0.7% agarose gel and NHEJ products were detected by staining with SybrGreen (Invitrogen). A detailed protocol is given in the Supplemental Methods. A PCR-based Homologous
Recombination assay was performed using Homologous Recombination Assay Kit according to the manufacturer’s instructions (Norgen Biotek Corp.). A detailed protocol is given in the Supplementary Methods.

Clonogenic assay
Cells were seeded into 6-well tissue culture plates and allowed to attach for 6 hours. NMS-P715 or DMSO control was added to the culture media for 2 hours. For combination treatment, NMS-P715 or DMSO control was added to the culture media for 2 hours followed by irradiation with wash after irradiation. Twelve days after seeding, colonies were stained with crystal violet. The number of colonies containing at least 50 cells was determined and the surviving fractions were calculated. For combination treatment, survival curves were generated after normalizing for the cytotoxicity generated by NMS-P715 alone.

Immunofluorescence staining for γH2AX/RAD51 foci
Immunofluorescence staining and counting of γH2AX nuclear foci were performed as previously described (21). The following antibody was used at 1:1,000 dilutions: anti-γH2AX mouse (Millipore; 05-636). Slides were mounted and images captured using Olympus FSX100 fluorescent microscope. For each treatment condition, foci were determined in at least 50 cells. The ImageJ (NIH) software was used to analyze the mean number of foci with combined area of γH2AX foci per nucleus.

Mitotic catastrophe
Immunofluorescence staining and counting of mitotic catastrophe foci were performed as previously described (21). Cells were grown in 4-well chamber slides, fixed with methanol for 15 minutes at −20°C, washed three times with PBS, blocked with 1% BSA three times for 10 minutes, and stained overnight at 4°C with mouse anti-α-tubulin antibody (Sigma) at 1:1,000 dilution. Cells were washed three times with 1% BSA, and were stained with goat anti-mouse Alexa Fluor 594 (Invitrogen) at 1:200 dilution for 2 hours at room temperature. Cells were washed three times with 1% BSA and slides were mounted in Vectashield mounting medium with DAPI (Vector Labs). Images were viewed and captured on a fluorescent microscope (Leica, Leica Microsystems). The presence of giant cells with multilobulated nuclei and aberrant mitoses was the criterion for defining cells undergoing mitotic catastrophe. For each treatment condition, 150 cells were scored; the average of three separate counts of the same cells is reported.

In vivo tumor growth and irradiation
All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals. Four to eight 6-week-old, female, athymic NCr nu/nu, nude mice (NCI Animal Production Program, Frederick, MD) were used for all in vivo studies. Animals were caged in groups of 5 or fewer and fed animal chow and water ad libitum. A single cell suspension (5 × 10^6) of U251 cells was implanted on the lateral aspect of the rear leg. When tumors reached approximately 140 mm^3 ([L × W^2]×π/2), animals were euthanized. Tumors were measured two times per week; animals were euthanized when tumors reached ≥1,600 mm^3. Tumor volumes were interpolated from fit spline curves for all groups the day control tumors had undergone exactly three doublings. Group mean ± standard error is reported.

For drug kinetic studies, tumor-bearing animals were given various doses (either 1 dose, 3 doses, or 5 doses) of NMS-P715 (100 mg/kg) 24 hours apart by oral gavage and tumors were harvested 6 hours after the last treatment.

Bioinformatics analysis of publicly available datasets
MPS1 expression levels were correlated to patient survival by curating publicly available datasets. A detailed analysis with statistical methods is given in the Supplementary Methods.

Statistical analysis
Data presented are the mean ± SD from three independent experiments unless indicated otherwise. All statistical tests were two-sided. For comparisons between groups, a Student t test was used. All analyses were completed using GraphPad Prism software (GraphPad Prism Inc.).

Results
MPS1 inhibition affects cell proliferation in GBM cells
Previously, we conducted a siRNA-based screen focused on the human kinome in GBM cells and identified the genes required for viability of GBM cells, including the mitosis-associated kinase PLK1 (23). Another mitosis-associated kinase gene this siRNA screen identified as potentially required for GBM cell survival was MPS1. Confirmation of this observation is shown in Supplementary Fig. S1. We used three nonoverlapping siMPS1 siRNAs. A substantial decrease in MPS1 mRNA (Supplementary Fig. S1A) and protein (Supplementary Fig. S1B) mediated by siRNAs corresponding to MPS1 (siMPS1) was observed with all the three siRNAs. In all the subsequent siMPS1 experiments, we used siMPS1_2 siRNA. The decreased expression of MPS1 transcript and subsequent reduction in protein levels was associated with a significant reduction in the viability of the GBM cell line U251 (Fig. 1A).

Pharmacologic MPS1 inhibition affects cell proliferation by induction of mitotic catastrophe in GBM cells
MPS1 encodes the mitotic kinase, MPS1. Recently, an ATP-competitive inhibitor of MPS1, NMS-P715 has been developed (18). To confirm and extend our siRNA-based findings, we exposed two GBM tumor cell lines, LN18 and U251, a normal fibroblast line (MRC9) and a normal astrocyte line, to increasing concentrations of NMS-P715. GBM cells LN18 and U251 treated from Monday through Friday, followed by 4-Gy local irradiation 2 hours after the fifth dose. The treatment was repeated one more time with a 2-day gap in between. Irradiation treatment was performed with mice placed in well-ventilated plexiglass jigs with shielding for the entire torso of the mouse along with critical normal structures of the head (e.g., ears, eyes, and neck) using a XRad 320 Xirradiator (Precision X-Ray, Inc.) at 320-kV X-ray and a dose rate of 289.8 cGy/min. Sham-irradiated mice served as the control group. To obtain tumor growth curves, perpendicular diameter measurements of each tumor were made twice a week with digital calipers, and volumes were calculated using formula, ([L × W^2]×π/2). Tumors were measured two times per week; animals were euthanized when tumors reached ≥1,600 mm^3. Tumor volumes were interpolated from fit spline curves for all groups the day control tumors had undergone exactly three doublings. Group mean ± standard error is reported.
with NMS-P715 showed a statistically significant inhibition of cell proliferation with a concentration-dependent decrease in cell viability of GBM cells over time and a minimal effect on the viability of normal cells (MRC9 and normal astrocytes; Fig. 1B).

As a functional assay for MPS1 kinase inhibition, we determined whether NMS-P715–treated GBM cells and normal astrocytes exhibited mitotic catastrophe. Cells with nuclear fragmentation, defined as the presence of two or more distinct nuclear lobes within a single cell, were classified as being in mitotic catastrophe. As shown in Fig. 2A, inhibition of MPS1 resulted in a significant increase in the percentage of cells undergoing mitotic catastrophe at 24 hours in both LN18 and U251 GBM cells as compared with untreated cells (P = 0.00006 and 0.003, respectively), indicating one of the mechanisms of cell kill is induction of mitotic catastrophe. Consistent with this, normal astrocytes treated with NMS-P715 did not undergo mitotic catastrophe.

MPS1 inhibition enhances the radiosensitivity of GBM cells

Next, we measured the effect of the MPS1 inhibitor NMS-P715 on the enhancement of radiosensitivity by clonogenic survival analysis in three different cell lines (Fig. 2B). Cells were plated at specific clonogenic density, allowed to attach (6 hours), and 1 μmol/L NMS-P715 inhibitor (Fig. 2B) was added 2 hours before irradiation. After irradiation, fresh drug-free medium was added, and colonies were stained 12 days later. The survival efficiencies were 35% and 32% in LN18 and U251 cells, respectively. NMS-P715 treatment resulted in an increase in the radiosensitivity of each of the two GBM cell lines tested. The dose enhancement factors (DEF) at a surviving fraction of 0.1 for LN18 and U251 were 1.40 and 1.32, respectively (Fig. 2B). The same experiment was performed using the normal lung fibroblast line, MRC9 (Fig. 2B). NMS-P715 alone or in combination with irradiation had no effect on MRC9 survival and radiosensitization. These results suggest that MPS1 is required for cell survival following irradiation of GBM cells but is not required for the survival of normal cells.

Effect of MPS1 inhibition on repair of irradiation induced DNA double-strand breaks

To assess the effects of NMS-P715 treatment on DNA damage and repair, we analyzed irradiation induced double-strand breaks (DSB) by γH2AX foci formation (Fig. 3A and B). At 0.5 and 6 hours, cells treated with radiation or the combination of NMS-P715 and radiation had similar significant elevations in γH2AX levels compared with control or drug-treated cells. At 24 hours, γH2AX level had returned to near baseline in the cells treated with radiation alone; however, γH2AX levels were significantly higher in the cells treated with combination NMS-P715 and radiation. On the contrary, normal astrocytes treated with NMS-P715 showed similar number of γH2AX foci at 24 hours as untreated astrocytes. Moreover, irradiated LN18 and U251 cells treated with 1 μmol/L NMS-P715 showed...
Pharmacologic MPS1 inhibition induced mitotic catastrophe and decreased clonogenic survival in GBM cells. A, to assess mitotic catastrophe represented as the number of cells with multinucleated giant nuclei and cells with abnormal mitoses, LN18 and U251 cells and normal astrocytes stained with antitubulin antibody and nuclei were visualized with DAPI staining. Values represent the mean SD for three independent experiments; P < 0.05, according to the Student t test. B, MPS1 inhibition enhances the radiosensitivity of GBM cells. Cells were exposed to 1 μmol/L NMS-P715 or DMSO control and clonogenic survival was assessed. Colony-forming efficiency was determined in GBM tumor cells LN18, U251, and normal fibroblast cell line (MRC9). Ten to 14 days later, survival curves were generated after normalizing for the cytotoxicity induced by NMS-P715 alone, and surviving fraction (SF) and DEF were calculated. Values represent the mean SD for three independent experiments; P < 0.05 according to the Student t test.

Statistically more γH2AX foci (P = 0.005), indicating persistence of radiation-induced DNA-damaged lesions and enhancement of radiation sensitivity in GBM cells but not in normal cells (Fig. 3A). We also observed some induction of γH2AX foci by NMS-P715 treatment alone. We attribute the DNA damage caused by NMS-P715 to the segregation defects caused by MPS1 inhibition. Representative images of U251 GBM cells and normal astrocytes at 24-hour time point depicting γH2AX foci cells stained with anti-γH2AX antibody followed by Alexa Fluor 488-labeled secondary antibody (Invitrogen; green) and nuclei with DAPI (blue) are shown (Fig. 3B). Given the role of MPS1 as a mitotic kinase, next we looked at mitotic catastrophe as a mechanism of cell death. In addition, LN18 and U251 cells when treated with NMS-P715 and 4-Gy irradiation, showed significant increases in postirradiation mitotic catastrophe at 48 hours compared with NMS-P715-treated cells (P = 0.0004 and 0.002, respectively; Fig. 3C and D). As shown in the representative photomicrograph (Fig. 3D), U251 cells undergoing mitotic catastrophe are visualized by staining with anti-tubulin antibody (red) and nuclei with DAPI (blue). These results indicate that inhibition of MPS1 can increase radiosensitivity of GBM cells by enhancing the number of cells undergoing mitotic catastrophe. The increase in radiosensitivity following MPS1 inhibition could involve the abrogation of DSB repair and increase in the number of cells undergoing mitotic catastrophe, contributing to decrease in clonogenic survival.

Molecular profiling reveals changes in genes associated with DNA replication, recombination, and repair

To characterize the molecular mechanism underlying the enhanced radiosensitivity exhibited by GBM cells after MPS1 inhibition, we used gene expression profiling of MPS1-silenced U251 cells. Microarray analysis was used to compare the mRNA expression profile of siMPS1-silenced U251 cells compared with siRNA control (siNeg) transfected cells at 6, 24, and 48 hours after transfection (Gene Expression Omnibus accession number GSE57091). Two-way hierarchical clustering of mRNA expression profile of siMPS1-silenced U251 cells compared with siNeg-transfected or untransfected cells (Control) at 6, 24, and 48 hours after transfection is shown (Supplementary Fig. S2A). Values were derived using a cutoff P ≤ 0.05 and fold change of ≥1.33. We observed 237, 391, and 891 genes as differentially expressed in siMPS1-silenced U251 cells compared with siNeg-transfected or untransfected cells (Control) at 6, 24, and 48 hours after transfection, respectively. Computational analysis of these differentially expressed genes identified enrichment for genes associated with the neurological disease, nervous system development, DNA replication, recombination, and repair pathway (DRRRp; Supplementary Fig. S2B and S2C). DRRRp was one of the top identified pathways (IPA score, 29), as well as the top molecular and cellular function with 20 molecules affected by down-regulation of MPS1 (Supplementary Fig. S2B and S2C). Genes identified as deregulated following MPS1 loss-of-function that are associated with DNA damage and repair were DNAPK [also
known as protein kinase DNA-activated (PRKDC), and topoisomerase II alpha (TOPO2A).

MPS1 modulates DNA repair molecules in vitro and in vivo

We confirmed that silencing of MPS1 induces a reduction in DNAPK and TOPO2A mRNA (Supplementary Fig. S2D) and protein levels (Fig. 4A) in U251 GBM cells and that NMS-P715 also induced a dose-dependent decrease in DNAPK and TOPO2A protein in treated U251 cells (Fig. 4A). Both siRNA-mediated silencing of MPS1 and small-molecule inhibition of MPS1 activity reduced total and phosphorylated DNAPK levels, as well as TOPO2A protein levels (Fig. 4A). MPS1 inhibition also downregulated irradiation-induced phosphorylated and total DNAPK levels in U251 cells (Supplementary Fig. S3A). To determine whether inhibition of MPS1 can modulate DNA repair molecules in vivo, U251 cells grown as xenografts were treated with NMS-P715 (100 mg/kg) by oral gavage. Twenty-four hours later, tumors were collected and subjected to immunoblot analysis. As shown in Fig. 4B and C), NMS-P715–treated tumors showed statistically significant reduction in DNAPK (P = 0.002) and TOPO2A (P = 0.0003) levels, compared with vehicle-treated tumors (Fig. 4B and C).

MPS1 regulates NHEJ and HR DNA repair pathways

Given the altered expression in key members of the DNA damage response—DNAPK and TOPO2A following MPS1 loss of function—we next investigated whether MPS1 affects DNA repair efficacy, by examining two major DNA DSB repair pathways: NHEJ and homologous recombination (HR; Fig. 4D and E and Supplementary Figs. S3–S5). To assay NHEJ DNA repair pathway, EcoRI endonuclease digested and gel-purified plasmid substrate was incubated with nuclear extracts derived from either siNeg- or siMPS1–treated U251 cells (Fig. 4D, i) or NMS-P715–treated U251 cells (Fig. 4D, ii). Nuclear extracts with functional NHEJ activity can end-join linearized plasmid to form dimers and multimers visualized by agarose gel electrophoresis. In the absence of nuclear extract, a single band of the 3.8-kb plasmid substrate was visualized (Fig. 4D, lane 1). Nuclear extracts from siNeg–transfected U251 cells with intact NHEJ activity showed the presence of ligated dimers and multimers (Fig. 4D, i and ii, lane 1). Nuclear extracts from siMPS1–transfected U251 cells inhibited formation of ligated products, indicating impaired NHEJ DNA repair activity (Fig. 4D, i, lane 3). Similarly, nuclear extracts from cells with no NMS-P715 treatment showed higher-

Figure 3.
Effect of MPS1 inhibition on repair of radiation-induced DNA DSBs. A and B, immunocytochemical staining to detect γH2AX foci was performed. LN18, U251 tumor cells, and normal astrocytes were plated in 4-well chamber slides, allowed to attach (overnight) and then treated with NMS-P715 for 2 hours prior to irradiation (4 Gy). A, foci were counted at 0.5, 6, and 24 hours after radiation in at least 150 cells per treatment per experiment. Data presented are the mean ± SD from at least three independent experiments. B, representative images of U251 GBM cells and normal astrocytes showing γH2AX foci, cells stained with anti-γH2AX antibody followed by Alexa Fluor 488–labeled secondary antibody (green) and nuclei were visualized with DAPI (blue) staining. C and D, LN18 and U251 cells growing in chamber slides were exposed to NMS-P715 for 2 hours, irradiated (4 Gy), and 24 and 48 hours after irradiation processed for immunocytochemical analysis of mitotic catastrophe. C, nuclear fragmentation (defined as the presence of two or more distinct lobes within a single cell) was evaluated in at least 150 cells per treatment per experiment. *, P < 0.05, comparing cells in the combination group compared with either drug or radiation alone groups at the same time point. D, representative images of U251 GBM cells showing mitotic catastrophe, cells stained with antitubulin antibody (red) and nuclei were visualized with DAPI (blue) staining.

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order ligated products, whereas nuclear extracts from cells treated with NMS-P715 inhibited formation of higher-order ligated products over time (Fig. 4D, ii, lanes 3–5 vs. lane 2). However, under similar NMS-P715 treatment conditions, U251 cells showed minimal mitotic catastrophe (Supplementary Fig. S3C).

Next, we analyzed NHEJ activity in the total extracts prepared from U251 xenografts treated with NMS-P715 (Fig. 4D, iii). In the absence of tumor extract, a single band of the 3.8-kb plasmid is detected (Fig. 4D, iii, lane 1). However, tumor extracts from vehicle-treated U251 tumors showed the presence of ligated dimers (Fig. 4D, iii, lanes 2–4), whereas tumor extracts from NMS-P715–treated tumors inhibited formation of ligated dimers, indicating impaired NHEJ DNA repair activity (Fig. 4D, iii, lanes 5–9). These observations indicate that intact or active MPS1 is necessary for a functional NHEJ pathway in vitro and in vivo in GBM. To assay HR pathway, we used a rapid and sensitive PCR-based method. The quality control data for the HR assay is shown in the Supplementary Fig. S3B. U251 cells were first transfected with siMPS1/siNegative, followed by a second cotransfection with the two HR plasmids (dl-1 and dl-2) 24 hours after the first transfection. Plasmid DNA was extracted 24 hours after the second transfection and HR activity was quantified using qPCR according to the manufacturer's instructions. The supplied negative and positive primer pairs amplified the expected size of amplicon of similar intensity (Supplementary Fig. S3B). As shown in Fig. 4E, plasmid DNA extracted from U251 cells treated with different treatments generated a plasmid recombination amplicon of 420 base pairs (bps) as a result of HR activity. The intensity of amplicon is directly correlated to the efficiency of HR activity in the template DNA. The intensity of PCR product generated from siMPS1- transfected cells was lower compared with siNeg-transfected cells (Fig. 4E, lane 4 vs. lane 2). A similar decrease was observed in NMS-P715–treated cells compared with DMSO-treated cells (Fig. 4E, lane 4 vs. lane 1), indicating MPS1 inhibition–mediated decrease in HR activity.

We also examined HR and NHEJ assay using DR-GFP plasmid as given in the Supplementary Methods (Supplementary Figs. S4 and S5). The HR recombination assay relies on the two inactivated tandem repeated (DR)-GFP transfected cells to express GFP, if HR activity is functional. The results in Supplementary Fig. S4A–S4D clearly show significant (P < 0.05) decrease in percentage GFP+ cells with MPS1 inhibition both by using siMPS1 and NMS-P715, compared with untreated...
cells. A RAD51 inhibitor B02 was used as a positive control. A decrease in GFP\(^*\) cells indicates inhibition of HR activity. This decrease in HR activity was further confirmed using HR-specific primers to amplify the recombined region. As shown, we observed a significant (\(P < 0.05\)) decrease in amplification of recombinant PCR product in cells with MPS1 inhibition (Supplementary Fig. S4E). A RAD51 inhibitor B02 was used as a positive control. The PCR amplification results are quantified (Supplementary Fig. S4F).

Using this well-established HR repair system, we also verified NHEJ activity in these cells by a PCR-based method as described in Supplementary Materials and Methods. As shown in the figure, the enzyme-resistant 650-bp PCR fragment represents functional NHEJ activity (Supplementary Fig. S5). MPS1 inhibition by both siMPS1 and NMS-P715 treatment showed lower intensity of enzyme-resistant 650-bp product compared with control-transfected cells. A DNAPK inhibitor, Nu7441, was used as a positive control for NHEJ repair activity showing similar decrease in enzyme-resistant 650-bp PCR product. The differences were statistically significant (\(P < 0.05\); Supplementary Fig. S5A). The PCR amplification results are quantified (Supplementary Fig. S5B).

These results indicate MPS1 inhibition can alter HR as well as NHEJ-driven DNA repair activity in GBM cells. This further confirms our observations that inhibition of MPS1 leads to the persistence of DNA lesions normally repaired by HR/NHEJ repair pathways.

**MPS1 abrogation inhibits GBM tumor growth in vivo**

We next determined whether the enhancement of tumor cell radiosensitivity could also extend to an in vivo model using U251 GBM xenografts (Fig. 5). To ensure the right dosing schedule, mice bearing U251 GBM xenografts were treated with multiple doses (1–5) of NMS-P715 at 100 mg/kg concentration and tumors were examined for pDNAPK and total DNAPK levels by an immunoblot analysis (Fig. 5A). We observed decrease phosphorylation of DNAPK with all the three tested doses of NMS-P715 (1 dose, 3 doses, and 5 doses); however, administration of five doses of NMS-P715 was significantly better in targeting pDNAPK. We also observed downregulation of total DNAPK in treated tumors.

Next, to check the efficacy of MPS1 target in vivo, mice bearing U251 GBM tumors (~140 mm\(^3\)) were randomized into four groups: vehicle-treated controls, NMS-P715 100 mg/kg (18), fractionated irradiation (RT), and NMS-P715 plus irradiation (NMS-P715+RT). On the basis of our dosing schedule data (Fig. 5A) in the drug cohort, NMS-P715 was delivered (oral gavage) for five doses twice with a 2-day gap after the first five doses (total 10 doses). Local irradiation of 4 Gy was given 2 hours after the fifth and the tenth dose of NMS-P715 to the NMS-P715+RT group and to the RT-only group. The growth rates of U251 tumors in different groups are shown (Fig. 5B). For each group, the time to grow from 140 mm\(^3\) (volume at time of treatment initiation) to \(\geq 1,600\) mm\(^3\) was calculated using the tumor volumes from the individual mice in each group (mean ± SEM). The tumor growth rates were similar for
U251 tumors treated with either vehicle or NMS-P715, and by day 20, all the mice in control, and NMS-P715 groups had reached the tumor volume of 1,600 mm\(^3\) and were euthanized. The other two groups, RT and combination (NMS-P715 + RT) group, were followed until day 33, when at least one mouse in each group reached the tumor volume of 1,600 mm\(^3\). The absolute growth delays were calculated when mice reached \(1600 \text{ mm}^3\) tumor volume. The vehicle-treated control group took 12.4 days, the drug-treated group took 13.4 days, the RT group took 26.4 days, and the NMS-P715 + RT group took 32.4 days. At this tumor volume, the growth delay for the NMS-P715 alone and radiation alone groups were 1 day and 14 days, respectively. The growth delay in mice treated with the combination of NMS-P715 and radiation was 20 days, which is greater than the sum of the growth delays caused by NMS-P715 alone and radiation alone. Thus, NMS-P715 delivered alone was not sufficient to have an effect on U251 tumor growth; however, in combination with irradiation, the MPS1 inhibitor enhanced radiation-induced tumor growth delay.

**MPS1 expression is associated with decreased survival in GBM and other cancer histologies**

We evaluated the prognostic effect of MPS1 expression on overall patient survival using a Cox proportional hazard model and found lower MPS1 expression to be a significant marker of better prognosis in GBM (197 patients), breast (1,115 patients), and lung (1,405 patients) cancers (Fig. 6 and Supplementary Fig. S6). Patients with neural GBM classification showed the widest range of MPS1 expression (Fig. 6B). High MPS1 expression was associated with a hazard ratio of 2.80 and 1.56 in neural and proneural GBM subtypes, respectively. On the contrary, low MPS1 expression is associated with better survival in both GBM subtypes. However, only the neural subtype reached the statistical significance \((P = 0.03; \text{Table 1})\). Differences in median survival were also observed when patients were stratified using only MPS1 expression status, with prolonged overall survival of 4.1 months in patients with neural GBM (476 days in low expressers versus 354 days in high expressers; Fig. 6B). However, exclusion of age and MGMT promoter methylation status, two important prognostic factors in GBM, resulted in statistically insignificant \(P\) values. Next, we compared whether the same trend is followed in other cancer types. Univariate survival analysis was performed using data derived from individuals with breast or lung cancer. The Kaplan–Meier curve analysis showed that patients with MPS1 expression above the median had significantly poorer survival compared with those with MPS1 expression below the median, both in breast cancer \((P = 2E^{-04})\) and lung cancer \((P = 1.5E^{-08}; \text{Supplementary Fig. S6})\). The survival analysis clearly shows that individuals with tumors expressing low levels of MPS1 had a significant survival advantage when compared with individuals with tumors expressing high levels of MPS1. This confirms MPS1 expression as a potential prognostic marker and as a putative molecular target for cancer therapy.

**Discussion**

This study demonstrates that inhibition of MPS1 enhances radiosensitivity of human GBM cells both in vitro and in vivo. The

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<th>Table 1. Multivariate survival analysis for various GBM subtypes</th>
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<td>Log-rank (P) value</td>
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<td>Exp. HR ((P) value)</td>
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Abbreviations: C, classical; M, mesenchymal; P, proneural; N, neural.
potential for MPS1 inhibition as an effective strategy for cancer treatment has been suggested previously (11, 13). However, this is the first study to successfully demonstrate that targeting MPS1 causes radiation sensitization in GBM cells and sheds light on the molecular mechanism of how inhibition of MPS1 activity modulates the growth of cancer cells. The MPS1 kinase has multiple roles in mitosis; these include spindle pole assembly, mitotic exit, cytokinesis, and response to genotoxic stress. This implies that MPS1 is near the top of the checkpoint-signaling pathway (8). Upregulation of MPS1 has been correlated with increase in aneuploidy, higher histologic grade, and lower survival in human cancers, including GBM (13, 25, 26).

In this study, we show that MPS1 mediates regulation of key DNA repair and damage genes, including PRKDC (DNA-PK) and TOP2A. TOP2A is highly conserved among eukaryotes and is absolutely required for chromosomal segregation and cell viability (27). Decreased expression of TOP2A in GBM cells following MPS1 inhibition could be attributed to segregation errors, leading to DNA damage and decreased DNA repair efficacy. Inhibition of MPS1 downregulated both phosphorylated and total levels of DNA-PK in vitro and in vivo. DNA-PK is essential for the repair of DNA DSBs and has been implicated in NHEJ and HR repair pathways. Thus, DNA-PK is necessary for cellular resistance to ionizing radiation (28). DNA-PK plays an instrumental role in many cell processes, including cell-cycle control, regulation of mitosis, microtubule dynamics, and proper chromosomal segregation. Majority of these cell functions are also regulated by MPS1 (29), it was therefore not surprising to see MPS1-mediated DNA-PK regulation in our study.

The direct consequence of MPS1-mediated DNA-PK downregulation was inhibition of NHEJ and HR DNA repair pathways. Eukaryotic cells rely on two highly regulated DSB repair pathways: the NHEJ and HR. Although both NHEJ and HR contribute to DSB rejoining, their involvement varies during the different cell-cycle phases as NHEJ is active throughout the cell cycle while HR is active during the S and G2 phases when sister chromatids are available (30). The DNA-PK catalytic subunit and Ku heterodimer form the biologically critical DNA-PK complex that plays a crucial role in the repair of ionizing radiation-induced DNA DSBs through NHEJ pathway (31). DNA-PK phosphorylation/autophosphorylation facilitates NHEJ (32). Phosphorylation of DNA-PKcs is complex and occurs at probably more than 40 sites with distinct phosphorylation events resulting in distinct functional consequences (33, 34). The observed NHEJ deficits are likely the consequence of decreased DNA-PK levels. Although the role of DNA-PK in NHEJ is clear, its role in regulation of HR has not been elucidated. Nonetheless, a recent study showed that NHEJ and HR pathways cooperate in G2-phase cells in order to repair DSB and the impairment of NHEJ by DNA-PK inhibitor affects RAD51 recruitment by HR to DSB sites (30). Therefore, our observation of decrease HR activity could be a direct consequence of decreased expression of DNA-PK by MPS1 inhibition, as these cells get arrested in G2-phase of cell cycle upon MPS1 abrogation (18).

The reduced DNA repair efficiency in MPS1-inhibited GBM cells was associated with increased retention of γH2AX foci and induction of mitotic catastrophe. This effect was enhanced in irradiated cells. Inhibition of MSP1 decreased clonogenic survival ability of GBM cells in conjunction with irradiation. These effects were specific to GBM tumor cells, as normal cells treated with NMS-P715 did not show inhibition of either cell proliferation or decrease in DNA repair and survival potential. Similarly, Slee and colleagues (26) showed that pancreatic ductal adenocarcinoma cells are more sensitive to NMS-P715 inhibition than the adipo-derived normal mesenchymal stem cells. Consequently, the differences in sensitivity between tumor and normal cells could provide a favorable therapeutic window in the treatment of GBM.

To further evaluate the clinical potential of NMS-P715 delivered in combination with radiotherapy, we evaluated this treatment strategy in a preclinical model system. U251 tumor xenografts treated with NMS-P715 showed significant increase in radiation-induced tumor growth delay. This coincided with the downregulation of DNA-PK in tumor xenografts. This is important as DNA-PK is required to prevent mitotic catastrophe in response to DNA damage and is associated with radioresistance in glioma (28, 35).

Our model for sensitivity to MPS1 inhibition depends on NHEJ and HR deficiency in MPS1-depleted cells. Therefore, this approach may be more widely applicable in the treatment of variety multitude of cancers. In order to test this, we looked at MPS1 expression and patient survival in GBM patient samples from The Cancer Genome Atlas (TCGA) database stratified according to their GBM molecular subtypes (classical, mesenchymal, proneural, and neural; ref. 36) and two other tumor histologies, breast cancer and lung cancer. Using a Cox proportional hazards model, we observed that lower MPS1 expression is a significant marker of better prognosis in GBMs of neural classification, and breast and lung cancers. Similar findings have been reported recently where it was shown that MPS1 inhibition enhances chemosensitivity of GBM to antimitotic drugs (25). Together, these findings suggest that MPS1 is a promising molecular target in the treatment of GBM. In addition, MPS1 has prognostic value not only in a subtype of GBM patients but also in patients with other cancer histologies. Many of the conventional approaches to the treatment of advanced cancers are genotoxic and therefore trigger a DNA-damage response leading to cell-cycle checkpoint response (37). Intact checkpoint responses can reduce sensitivity to these agents (38, 39). The reduced repairability of DNA damage using specific repair pathways in MPS1-deficient tumor cells could be exploitable in combination with radiotherapy for the treatment of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: U.B. Maachani, N.J. Caplen, K. Camphausen, A. Tandle
Development of methodology: U.B. Maachani, T. Kramp, A. Tandle
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Kramp, R. Hanson, S. Zhao, A. Tandle
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): U.B. Maachani, R. Hanson, O. Celikcu, U. Shankavaram, K. Camphausen, A. Tandle
Writing, review, and/or revision of the manuscript: U.B. Maachani, R. Hanson, R. Colombo, N.J. Caplen, K. Camphausen, A. Tandle
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Targeting \textit{MPS1} Enhances Radiosensitization of Human Glioblastoma by Modulating DNA Repair Proteins

Uday Bhanu Maachani, Tamalee Kramp, Ryan Hanson, et al.


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