Selective Inhibition of Parallel DNA Damage Response Pathways Optimizes Radiosensitization of Glioblastoma Stem-like Cells

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Abstract

Glioblastoma is the most common primary brain tumor in adults and is essentially incurable. Despite aggressive treatment regimens centered on radiotherapy, tumor recurrence is inevitable and is thought to be driven by glioblastoma stem-like cells (GSC) that are highly radioresistant. DNA damage response pathways are key determinants of radiosensitivity but the extent to which these overlapping and parallel signaling components contribute to GSC radioresistance is unclear. Using a panel of primary patient-derived glioblastoma cell lines, we confirmed by clonogenic survival assays that GSCs were significantly more radioresistant than paired tumor bulk populations. DNA damage response targets ATM, ATR, CHK1, and PARP1 were upregulated in GSCs, and CHK1 was preferentially activated following irradiation. Consequently, GSCs exhibit rapid G2-M cell-cycle checkpoint activation and enhanced DNA repair. Inhibition of CHK1 or ATR successfully abrogated G2-M checkpoint function, leading to increased mitotic catastrophe and a modest increase in radiation sensitivity. Inhibition of ATM had dual effects on cell-cycle checkpoint regulation and DNA repair that were associated with greater radiosensitizing effects on GSCs than inhibition of CHK1, ATR, or PARP alone. Combined inhibition of PARP and ATR resulted in a profound radiosensitization of GSCs, which was of greater magnitude than in bulk populations and also exceeded the effect of ATM inhibition. These data demonstrate that multiple, parallel DNA damage signaling pathways contribute to GSC radioresistance and that combined inhibition of cell-cycle checkpoint and DNA repair targets provides the most effective means to overcome radioresistance of GSC. Cancer Res; 75(20); 4416-28. ©2015 AACR.

Introduction

Glioblastoma is the most common primary brain tumor in adults. Despite optimal treatment consisting of surgical resection followed by radiotherapy with concomitant and adjuvant temozolomide chemotherapy, median survival remains dismal at 12 to 15 months (1). Treatment responses are inevitably followed by relapse, typically within the maximally irradiated volume (2, 3). In glioblastoma, tumorigenic cells display complex clonal dynamics in which genetically distinct subclones have variable serial repopulating activity in vivo (4, 5). This readout is likely to represent activity of self-renewing glioblastoma "stem-like" cells (GSC) whose self-renewal ability varies on the basis of frequency and/or quantitative features and underpins the evolution of resistant disease (6). Consistent with this, GSCs that express stem cell markers such as CD133, SSEA-1 (CD15), nestin, SOX2, and OLIG2 (7–10) are more resistant to radiotherapy and conventional chemotherapy than more differentiated "tumor bulk" cells (10–14). There is an urgent need to develop targeted treatment strategies that will overcome the innate resistance of GSCs, improve local tumor control, and extend patient survival.

Radiotherapy is a vital therapeutic modality for glioblastoma that causes single- and double-stranded DNA breaks that evoke a multifaceted DNA damage response (DDR). At the apex of the DDR lie the serine/threonine protein kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), which maintain genomic integrity by activating cell-cycle checkpoints and DNA repair pathways (15). ATM is mainly activated by DNA double-strand breaks (DSB), whereas ATR responds to single-stranded regions of DNA generated at stalled replication forks and during processing of DSBs by nucleases (16–19). The MRN (MRE11–RAD50–NBS1) complex has key roles in sensing and processing DSBs as well as activating ATM and ATR (20). ATR activates cell-cycle checkpoint kinase proteins, including CHK1, whereas ATM functions primarily through activation of CHK2. These downstream checkpoint kinases activate G1 and G2–M cell-cycle checkpoints through phosphorylation of phosphatases CDC25A and CDC25C and kinases CDK1 and WEE1 that regulate cell-cycle progression (21). In addition, ATM promotes repair of a subset of DSBs. PARP facilitates repair of radiation-induced single-strand breaks (SSB), and the radiosensitizing effects of PARP inhibitors are well-characterized (22).
There is growing evidence that DDR signaling is upregulated in glioblastoma and integral to GSC radiosensitivity. Analysis of glioblastoma clinical samples has revealed high levels of p-ATM, p-CHK1, p-CHK2, and PARP1 compared with normal brain tissue (23, 24). Furthermore, basal levels of p-CHK1, p-CHK2, and RAD17 have been shown to be higher in CD133+ GSCs than in nontumorigenic CD133- populations, a finding that was associated with radiosensitivity of the GSC population (10). However, subsequent studies have either failed to show differences in DNA repair capacity based on CD133 status or revealed increased radiosensitivity of CD133+ GSCs compared with established glioblastoma cell lines (25, 26). Such discrepancies may reflect methodologic differences or comparisons between nonisogenic cell lines. Indeed, we have recently demonstrated that radiosensitivity of GSC populations is associated with enhanced ATM-dependent DSB repair proficiency (27).

Currently, there is intense research into the development of small-molecule inhibitors of DDR proteins (DDRi), one aim of which is to increase the therapeutic index of standard treatments (28). This is driven by several factors: (i) DNA is the major target for many anticancer therapies, (ii) constitutive activation of DDR is frequently observed in cancers (29), and (iii) activation of DDR is associated with resistance to cytotoxic therapies. Several preclinical studies have successfully used DDRi to increase chemo- and radiosensitivity in glioblastoma models, but these results were not substantiated in the treatment-resistant GSC population (10). However, this may be remedied by targeting DDR components in GSC models. Indeed, we have recently demonstrated that radioresistance of GSC populations is associated with enhanced ATM-dependent DSB repair proficiency (27).

The observation that GSCs exhibit upregulated DDR signaling provides both opportunities and challenges. While targeting individual DDR components can increase GSC radiosensitivity, the relative impact on radiosensitivity of these and other components of the multiple DDR pathways remains unexplored. We addressed this by using paired, primary, patient-derived glioblastoma cell lines cultured to enrich for or deplete the GSC population (30–34). More recently, we have shown the ATM inhibitor KU55933 to be a potent radiosensitizer of GSC (27), and inhibition of PARP1 has also been shown to overcome radiosensitivity of GSCs (35). These findings corroborate and extend the landmark study by Bao and colleagues in which inhibition of CHK1 and CHK2 was shown to enhance the radiosensitivity of GSCs (10).

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**Materials and Methods**

**Derivation and maintenance of primary patient-derived glioblastoma cell lines**

Primary patient-derived glioblastoma cell lines E2, G7, R10, R15, and R24 were derived in Cambridge, UK, from freshly resected tumor specimens and maintained according to published protocols described previously (22, 36) and in Supplementary Materials and Methods. Patient consent was sought before surgery, and tissue collection was approved by the local regional Ethics Committee (LREC ref 04/Q0108/60) and compliant with the UK Human Tissue Act 2004 (HTA Licence ref 12315). Cell lines were validated in 2014 by expression of glioblastoma and GSC markers and by their capacity to generate orthotopic tumors in CD1 nude mice that recapitulate the key histopathologic features of glioblastoma.

**Generation of orthotopic tumors and immunohistochemistry**

Tumors were generated by injecting $10^6$ cells in CD1 nude mice as described previously (37) and in Supplementary Materials and Methods.

**Drug treatment and radiation**

ATM inhibitor KU-55933 (Tocris Bioscience), ATR inhibitor VE821, PARP inhibitor olaparib, CHK1 inhibitors SCH900776 and CHIR-124 (Selleckchem), hydroxyurea and aphidicolin (Sigma) were all dissolved in DMSO. For drug–radiation combination studies, cells were exposed to fresh media containing the inhibitor or relative DMSO control for 1 hour prior to irradiation (IR) in tissue culture vessels using an XStrahl RS225 cabinet at room temperature with 195 kV/15 mA X rays producing a dose rate of 1.6 Gray per minute. For UV studies, media containing the drug were removed after 1 hour, and cells were irradiated using a UV Stratalinker (Stratagene).

**Clonogenic and neurosphere assays**

Paired GSC and bulk population from E2 and G7 cell lines formed countable colonies and were seeded at a density of 250 cells per well in Matrigel-coated 6-well dishes for 24 hours. Wells were treated with the inhibitors or relative DMSO control for 1 hour followed by mock or 5 Gy IR. Cells were then incubated for a further 24 hours followed by replacement with fresh media. E2 and G7 colonies were fixed after 2 or 3 weeks, respectively, in methanol followed by staining with crystal violet. Colonies consisting of minimum 50 cells were counted manually and using an automated colony counter (GelCount, Oxford Optronix). Clonogenic survival data were fitted using a linear quadratic model, and $S_{ER}$ values were calculated from the fitted curve. ANOVA test was used to analyze differences between clonogenic survival curves.

Neurosphere assay was performed as described previously (27) and in Supplementary materials and Methods.

**Immunofluorescence**

Paired bulk and/or GSCs were plated on coverslips coated with Matrigel and treated with radiation alone or in combination with the inhibitors for 24 hours. Cells were fixed and incubated with γ-H2AX or p-His H3 S10 antibodies overnight at 4°C followed by incubation with secondary conjugated antibodies. γ-H2AX foci data are presented as box and whisker plots from 3 independent experiments unless otherwise stated and analyzed using Mann–Whitney U test as they were not normally distributed.

**Flow cytometry and cell proliferation**

For analysis of mitotic population, cells were treated and fixed with 70% ethanol and incubated with p-His H3 S10 antibody followed by γ-H2AX antibody and analyzed using flow cytometry.
Combined Annexin V and propidium iodide (PI) analysis was carried out according to manufacturer's protocol (BD Biosciences). Analysis of GSC markers was carried out using conjugated CD133 and CD15 (Miltenyi Biotech). FACS was carried out using FACSARia Fusion following labeling with CD133/CD15 phycoerythrin (PE)-conjugated antibodies. Live cells were gated and sorted populations were plated into 6-well plates in identical stem cell culture media for 3 to 7 days before harvesting. Cell-cycle distribution was determined following 40-minute incubation with 10 μmol/L BrdUrd (BD Biosciences). Data were analyzed using FlowJo software (Tristar). Analysis of caspase-3/7 activity was carried out using Caspase-Glo 3/7 kit (Promega).

Cell proliferation was measured in 96-well plates as described in Supplementary Materials and Methods.

Western blotting

For immunoblotting, whole-cell lysates were prepared and processed in SDS buffer, blotted onto membranes, and probed with primary antibodies (Supplementary Materials and Methods) overnight followed by appropriate secondary antibodies for 1 to 3 hours. Bound antibodies were visualized using chemiluminescence and bands quantified using ImageJ.

Statistical analyses

All experiments were repeated three times unless otherwise stated and data points reported as mean ± SEM. Statistical analysis and graphs were produced using Minitab 16 and GraphPad Prism 6. Unpaired t test or one-sample t test were used to generate P values.

Results

GSC populations are radioresistant

We previously characterized two primary glioblastoma cell lines (E2 and G7; refs. 27, 37). Using the same techniques, additional primary glioblastoma cell lines (R10, R15, and R24) were expanded by culturing under selective media to enrich for or deplete the GSC population. In immunodeficient mice, GSC-enriched populations of E2, G7 (27, 37), R10, and R15 cells generated orthotopic tumors that recapitulated the human disease whereas GSC-depleted tumor bulk populations did not (Supplementary Fig. S1). While the neural stem cell markers nestin, SOX2, CD133, Olig2, and CD15 were heterogeneously expressed across GSC cultures, expression of these markers was consistently increased in GSCs compared with bulk populations (Fig. 1A and B). Clonogenic survival assays in E2 and G7 cells confirmed GSC cultures to be more radioresistant than paired bulk populations. Dose modifying factors calculated at 37% survival (DMF0.37) showed that E2 and G7 GSCs were more radioresistant than corresponding bulk populations by factors of 1.36 (P = 0.001) and 1.44 (P < 0.001), respectively (Fig. 1C–E). Radioresistance of GSC populations was confirmed by comparing surviving fraction at 4 Gy (SF4.0, Supplementary Table S1). These findings are consistent with the observation that GSCs were refractory to cell death after high radiation doses (15 and 30 Gy) as measured by Annexin V and PI staining (Fig. 1F and Supplementary Fig. S2) and caspase-3/7 activity assays (Fig. 1G).

GSCs express high levels of DDR targets under basal conditions and activate CHK1 rapidly following IR

DDR targets have previously been shown to be upregulated and associated with GSC radioresistance (10, 27, 35). We explored this further in our GSC and bulk culture models and observed an overall pattern of higher levels of total and phosphorylated CHK1 (S345) and ATR (S428) in GSCs compared with bulk populations (Fig. 2A). Importantly, this was not an artifact of culture conditions, as CD133- or CD15-positive sorted cells exhibited significantly higher levels of phosphorylated CHK1 than negative sorted populations of E2, R24, and G7 cell lines cultured in the same conditions (Fig. 2B). Higher levels of total CHK1 were also observed in R24 and G7 cells expressing the relevant stem cell marker.

We next explored whether differences in cell-cycle distribution could account for the disparate CHK1 levels observed in GSC and bulk populations, as CHK1 is typically expressed in S and G2 phases of proliferating cells. Analysis of BrdUrd incorporation indeed revealed significant differences in cell-cycle profiles (Fig. 2C and Supplementary Fig. S3) with GSCs exhibiting a marked increase in the percentage of cells in S and G2 phases of the cell cycle. Interestingly, the differences in cell-cycle distribution had no significant impact on cell doubling times, which were similar in GSC and bulk populations in both E2 and G7 cell lines (Supplementary Fig. S3). While these results might explain the observed increase in CHK1 levels in GSC, they also indicate that enhanced radioresistance of GSCs is not a consequence of reduced proliferation.

Further interrogation of DDR markers revealed higher levels of PARP1 and phosphorylated ATM (p-ATM, S1981) in most GSC populations (Fig. 2A). These findings were verified in vivo in our E2 and G7 GSC orthotopic xenograft models. Analysis of tumor sections revealed heterogeneous staining for p-ATM (S1981) and PARP1, highlighting differential protein expression between different cell populations (Fig. 2D). PARP1 staining in G7 tumors was abundant and homogeneous, consistent with previous reports proposing PARP1 as a glioblastoma marker (24). These findings were reproduced in a clinical glioblastoma specimen, which exhibited marked p-ATM and PARP1 expression in the majority of tumor cells (Fig. 2D). These data illustrate collective upregulation and/or activation of DDR targets in GSC populations under basal conditions in vitro and in a subpopulation of glioblastoma cells in vivo.

We next sought to investigate how differences in basal CHK1 levels between GSC and bulk populations affect the DDR. Irradiation of E2 GSCs evoked more pronounced activation of CHK1 than in corresponding bulk populations as demonstrated by a significantly greater increase in the phospho-CHK1 S345:total CHK1 ratio within 15 minutes that was maintained for at least 3 hours (Fig. 2E and F). Reduced CHK1 activation in E2 bulk cells occurred despite similar levels of DNA damage induction (γ-H2AX). While the relative extent of radiation-induced CHK1 activation was similar in bulk and GSC populations of G7 cells (Supplementary Fig. S4), absolute levels of CHK1 and p-CHK1 were significantly higher in G7 GSCs than in paired bulk cells both under basal conditions and following IR. CHK2 activation (T68 phosphorylation) was similar in E2 GSC and bulk populations, although differences were observed between G7 bulk and GSC populations (Fig. 2E and Supplementary Fig. S4). We explored the possibility that enhanced activation of CHK1 in GSC could be caused by “priming” of the MRN DNA damage sensor complex under basal conditions. No differences in total or phosphorylated MRE11 and NBS1 between GSC and bulk populations were observed under basal conditions or following IR, but modest upregulation of RAD50 was observed in E2 and G7 GSCs.
Radiosensitization of Glioblastoma Stem-like Cells

Figure 1.
GSCs are radioresistant with defective DDR. A, enrichment of stem cell markers SOX2, nestin, CD133, and Olig2 under GSC culture conditions compared with paired bulk populations of primary, patient-derived glioblastoma (GBM) cell lines. B, flow cytometric plots and summary showing relative expression of CD133 and CD105 in E2 and G7 GSC and bulk populations. C and D, clonogenic survival assays showing E2 and G7 GSCs are more radioresistant than paired bulk populations. E, calculation of DMF at 37% survival comparing E2 and G7 GSC versus bulk populations and P values associated with comparisons of DMF. F and G, plots summarizing combined Annexin V and PI staining (F) or fold induction in caspase-3/7 activity at 48 hours in E2 and G7 GSC and bulk populations following treatment with 15 or 30 Gy ionizing radiation (G). Error bars show mean ± SEM from n = 3 independent experiments. ∗, P < 0.05; NS, nonsignificant.

following IR (Fig. 2E, Supplementary Fig. S4). The possibility that CHK1 activation was delayed (rather than reduced) in bulk cells was excluded by demonstrating that S296 phosphorylation of CHK1 was attenuated in E2 and absent in G7 bulk cells but maintained in both GSC cultures at 6, 12, and 24 hours after IR (Fig. 2G). While some variation in the kinetics of CHK1 activation was observed between different experiments, attributable to the use of different batches of frozen primary patient-derived cells, both phosphorylated and total CHK1 levels were consistently higher in GSCs than in bulk cells and were reproducibly augmented at all time points following radiation. Finally, enhanced CHK1 activation in GSC was not only a radiation-

specific phenomenon: E2 and G7 GSC also exhibited markedly enhanced activation of CHK1 (S345 and S296 phosphorylation) in response to UV, hydroxyurea, or aphidicolin treatments despite levels of γ-H2AX induction that were comparable to bulk populations (Fig. 2H, Supplementary Fig. S4).

Rapid induction of CHK1 in GSCs is associated with enhanced G2–M cell-cycle checkpoint activation

We next investigated the impact of enhanced CHK1 activation on cell-cycle checkpoint responses of GSCs. Both E2 and G7 cell lines were refractory to radiation-induced G1–S checkpoint activation, consistent with frequent deregulation of p53 signaling...
Figure 2.
GSCs have upregulated DDR proteins under basal condition and active CHK1 rapidly following IR. A, analysis of multiple DDR proteins under basal conditions in GSC or bulk populations in a panel of primary, patient-derived glioblastoma (GBM) cell lines. Loading control as shown in Fig. 1A. B, analysis of total and phosphorylated CHK1 in CD133 or CD15 sorted cell populations. Flow cytometric plots show post-sort analysis of G7 cells sorted by CD15 expression. C, cell-cycle distribution and profiles of E2 GSC and bulk populations. D, immunohistochemical analysis of orthotopic tumor sections generated from E2 and G7 orthotopic xenografts and a GBM patient specimen showing PARP1 and p-ATM staining. E, Western blots showing rapid activation of DDR markers at early time points in E2 GSC compared with bulk populations following 5-Gy IR. F, fold induction in CHK1 activation relative to untreated cells following quantification of p-CHK1(S345):CHK1 ratios from immunoblots after radiation. Error bars show mean ± SEM from three independent experiments. G, twenty-four hour time course highlighting CHK1 and phosphorylated CHK1 levels in E2 and G7 paired GSC and bulk populations following 5-Gy IR. H, response of E2 bulk and GSC populations to various activators of CHK1: IR, 5 Gy, 1 hour; UV, 10 J/m², 1 hour; hydroxyurea (HU), 10 mmol/L, 3 hour; aphidicolin, 1 mmol/L, 3 hours (h, longer exposure).
pathways in glioblastoma (Supplementary Fig. S5; ref. 38). However, analysis of the G2–M checkpoint by flow cytometric quantification of mitotic cells identified by S10 phosphorylation on histone H3 (p-His H3) revealed that GSC activated G2 arrest significantly more efficiently than paired bulk populations, requiring less time to reduce the mitotic cell population by 50% following IR treatment (Fig. 3A and Supplementary Fig. S6). Consistent with this, E2 CD133+ sorted cells activated G2 arrest more rapidly and completely than CD133−/CD0 cells cultured in the same conditions (Supplementary Fig. S7). Intriguingly, E2 bulk cells failed to fully activate the G2–M checkpoint at any time point (Fig. 3C). Dose–response studies revealed that depletion of mitotic cell fraction by 75% occurred after significantly lower radiation doses in E2 and G7 GSC compared with bulk populations (Fig. 3B and Supplementary Fig. S6) and that E2 and G7 bulk cells were released from the G2–M checkpoint significantly quicker than the paired GSC population as measured by the time required for mitotic cell population to return to baseline levels. Plots, mean ± SEM; n ≥ 3 independent experiments.

Radiosensitization effects of CHK1 inhibition are more pronounced in glioblastoma bulk cells than GSCs
Reasoning that radioresistance of GSCs might be driven by enhanced CHK1-mediated activation of the G2–M checkpoint, we hypothesized that inhibition of CHK1 would abrogate the G2–M checkpoint and increase radiosensitivity. To investigate this, we used SCH 900776 (SCH), a well-characterized CHK1-specific inhibitor, which had no significant effect on CHK2 activity as highlighted by

Figure 3.
Rapid activation of G2–M cell-cycle checkpoint in GSCs following IR. Summary of flow cytometry data analyzing mitotic p-His H3 S10 cell population to measure G2–M cell-cycle checkpoint in paired E2 and G7 GSC and bulk populations. A, 3-hour time course following 5-Gy IR treatment showing a rapid activation of G2–M checkpoint in GSCs as measured by the time required to reduce mitotic population by 50%. B, IR dose response showing E2 and G7 GSC populations require significantly lower dose of radiation compared with the bulk cells to activate G2–M checkpoint by 75% relative to unirradiated cells. C, twelve hour IR time course showing E2 and G7 bulk cells exit the G2–M checkpoint significantly quicker than the paired GSC population as measured by the time required for mitotic cell population to return to baseline levels.
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Figure 4. GSCs are radiosensitized by the CHK1 inhibitor SCH900776 (SCH). A, inhibition of CHK1 in E2 GSCs following 1-hour pretreatment with various concentrations of SCH followed by 120 J/cm² UV for 1 hour. B, time course showing abrogation of IR-induced CHK1 activation by pretreatment of E2 GSCs with 3 μmol/L of SCH for 1 hour. C, analysis of multiple biomarkers of CHK1 inhibition at 24 hours in E2 GSCs treated with various concentrations of SCH followed by 1-hour pretreatment with various concentrations of SCH for 1 hour followed by 5-Gy IR. D, plots summarizing flow cytometry data showing inhibition of IR-induced G2–M checkpoint activation in E2 and G7 GSCs treated with 3 μmol/L SCH for 1 hour followed by 5-Gy IR (mean ± SEM from n ≥ 3 independent experiments; **P < 0.01; ***P < 0.001 relative to IR alone). E, clonogenic survival curves showing radiosensitization of E2 and G7 GSC and bulk populations following 3 μmol/L of SCH treatment. F, summary of clonogenic data showing SER of GSC and bulk populations at 37% survival and SF at 4 Gy in the presence or absence of SCH. *, P < 0.05 based on the 95% confidence intervals between SER of GSCs and bulk population or SF of GSC or bulk populations plus or minus SCH treatment. NS, nonsignificant.

unaltered phosphorylation of T68 (Fig. 4A–C; refs. 39, 40). As expected, SCH treatment alone inhibited CHK1 S296 autophosphorylation in a dose-dependent manner and induced CHK1 S345 phosphorylation through CHK1-dependent inhibition of the PP2A phosphatase feedback loop (Fig. 4A and C; Supplementary Fig. S8; ref. 41). Treatment of E2 GSCs with SCH resulted in enhanced induction of CHK1 S345 phosphorylation by UV or IR along with increased γ-H2AX levels reflecting increased DNA damaging signaling in response to CHK1 inhibition (Fig. 4A–C and Supplementary Fig. S8). Induction of CHK2 T68 phosphorylation was also observed following combined SCH/IR treatment, suggesting compensatory activation of CHK2 following CHK1 inhibition (Fig. 4C; ref. 42). In addition, SCH treatment alone generated a γ-H2AX signal highlighting a basal function of CHK1 in maintaining genomic stability (43). Additional biomarkers of CHK1 inhibition included downregulation of phosphorylated WEE1 (S642) and CDC25C (T48), whereas upregulation of CDC25A was observed following SCH treatment alone and augmented in combination with radiation, consistent with enhanced cell-cycle progression despite the presence of damaged DNA (Fig. 4B and C and Supplementary Fig. S8). Because our earlier results showed that GSCs rapidly activate the G2–M cell-cycle checkpoint in response to IR (Fig. 3), we
Inhibition of CHK1 increases DNA damage, induces mitotic catastrophe, and leads to genomic instability in irradiated GSC. A, representative plots showing the total percentage of \( \gamma \)-H2AX–positive cells in E2 GSC 24 hours after 3 \( \mu \)mol/L SCH treatment for hour followed by 5-Gy IR. B, bar chart summarizing the percentage of total \( \gamma \)-H2AX–positive cells at 24 hours following treatment of E2 GSC with various concentrations of SCH + 5 Gy; error bars show mean ± SEM, \( n \geq 3 \) independent experiments; *, \( P < 0.05 \). C, representative histogram plots from flow cytometry data showing the percentage of mitotic cells with high \( \gamma \)-H2AX at 24 hours following treatment with 3 \( \mu \)mol/L SCH and 5 Gy IR. D, summary of flow cytometry data showing the percentage of mitotic cells with high \( \gamma \)-H2AX at 24 hours following treatment of E2 GSC with various concentrations of SCH; error bars show mean ± SEM, \( n \geq 3 \) independent experiments; **, \( P < 0.01 \). E, representative immunofluorescent images showing p-His H3–positive mitotic cells (green) with no/low \( \gamma \)-H2AX (red, top) or high \( \gamma \)-H2AX (middle), image of cell undergoing mitotic catastrophe as marked by nuclear blebbing and high \( \gamma \)-H2AX (bottom). DAPI nuclear stain is shown in blue. Bar charts summarizing immunofluorescent data from analysis of about 75 mitotic cells. F, representative image and summary of the percentage of E2 GSCs with micronuclei (arrows) 24 hours following treatment with 3 \( \mu \)mol/L SCH + 5 Gy; error bars show mean ± SEM from scoring ~500 nuclei; *, \( P < 0.05 \); **, \( P < 0.01 \).

Figure 5.

Inhibition of CHK1 increases DNA damage, induces mitotic catastrophe, and leads to genomic instability in irradiated GCS. A, representative flow cytometric plots showing the total percentage of \( \gamma \)-H2AX–positive cells in E2 GSC 24 hours after 3 \( \mu \)mol/L SCH treatment for hour followed by 5-Gy IR. B, bar chart summarizing the percentage of total \( \gamma \)-H2AX–positive cells at 24 hours following treatment of E2 GSC with various concentrations of SCH + 5 Gy; error bars show mean ± SEM, \( n \geq 3 \) independent experiments; *, \( P < 0.05 \). C, representative histogram plots from flow cytometry data showing the percentage of mitotic cells with high \( \gamma \)-H2AX at 24 hours following treatment with 3 \( \mu \)mol/L SCH and 5 Gy IR. D, summary of flow cytometry data showing the percentage of mitotic cells with high \( \gamma \)-H2AX at 24 hours following treatment of E2 GSC with various concentrations of SCH; error bars show mean ± SEM, \( n \geq 3 \) independent experiments; **, \( P < 0.01 \). E, representative immunofluorescent images showing p-His H3–positive mitotic cells (green) with no/low \( \gamma \)-H2AX (red, top) or high \( \gamma \)-H2AX (middle), image of cell undergoing mitotic catastrophe as marked by nuclear blebbing and high \( \gamma \)-H2AX (bottom). DAPI nuclear stain is shown in blue. Bar charts summarizing immunofluorescent data from analysis of about 75 mitotic cells. F, representative image and summary of the percentage of E2 GSCs with micronuclei (arrows) 24 hours following treatment with 3 \( \mu \)mol/L SCH + 5 Gy; error bars show mean ± SEM from scoring ~500 nuclei; *, \( P < 0.05 \); **, \( P < 0.01 \).

hypothesized that CHK1 inhibition would abolish this effect and thus overcome the radioreistant GSC phenotype. Indeed, SCH treatment abrogated radiation-induced G2 arrest in E2 and significantly attenuated it in G7 GSCs, whereas SCH treatment in the absence of radiation was sufficient to drive E2 and G7 GSCs into mitosis (Fig. 4D). Clonogenic survival assays confirmed significant radiosensitization of E2 and G7 GSC by CHK1 inhibition as illustrated by sensitization enhancement ratios at 37% survival (SERR) of 1.9 and significant reductions in surviving fractions at 4 Gy (SF4 0.7) in both cell lines (Fig. 4E and F). Despite the limited ability of bulk populations to phosphorylate CHK1 and induce G2–M checkpoint activation in response to radiation, a similar or greater magnitude of radiosensitization (SERR) of 2.3–2.4 was observed in bulk populations treated with SCH. This unexpected observation was confirmed using an alternative CHK1 inhibitor CHIR-124 (Supplementary Fig. S9; ref. 44). While these data support the assertion that CHK1 inhibition has therapeutic potential in glioblastoma, the observation that overexpression and constitutive activation of CHK1 in GSCs was not associated with increased radiosensitization by CHK1 inhibitors indicates that additional mechanisms could be responsible for limiting the radiosensitization effects of CHK1 inhibition in the GSC population.

Inhibition of CHK1 radiosensitizes GSC through a mechanism involving mitotic catastrophe

Our subsequent studies investigated the mechanisms responsible for (i) GSC radiosensitization by CHK1 inhibition and (ii) enhanced radiosensitization of bulk cells. Initial analysis of DNA damage in E2 GSCs using flow cytometry highlighted a significant increase in the \( \gamma \)-H2AX–positive cell population following...
combined SCH and IR treatment compared with individual treatments alone (Fig. 5A and B). SCH treatment alone induced a γ-H2AX response, confirming our earlier observations (Fig. 4A–C). Because this signal was observed predominantly in S-phase cells, we inferred that CHK1 inhibition exacerbates replication stress in GSCs. Because SCH treatment also inhibited the G2–M checkpoint (Fig. 4D), we investigated whether treated GSCs would enter mitosis with damaged DNA. Indeed, following SCH treatment, we observed a distinct population of mitotic cells with high γ-H2AX signal that was significantly increased after combined treatment with SCH and IR (Fig. 5C and D). Immunofluorescent analysis confirmed these results, demonstrating a distinct mitotic cell population with intense, pan-nuclear γ-H2AX staining (middle row, Fig. 5E). This is likely to represent cells in the initial stages of mitotic catastrophe, which was clearly visible in a separate population of mitotic cells characterized by loss of membrane integrity and fragmented morphology (bottom row). Both populations were augmented in GSCs following combined treatment with SCH and IR relative to either treatment alone. In addition, we evaluated the consequence of mitotic division in cells with damaged DNA and found a significant increase in the percentage of GSCs harboring one or more micronuclei following combined treatment (Fig. 5F). These data demonstrate that CHK1 inhibition sensitizes GSC to IR by preventing G2–M checkpoint activation, allowing mitotic entry of cells bearing damaged DNA, resulting in mitotic catastrophe and genomic instability.

Limited radiosensitization of GSCs by CHK1 inhibition is explained by enhanced DNA repair

We next sought to identify the mechanism responsible for limiting the radiosensitizing effects of CHK1 inhibition in GSCs by comparing effects of IR plus SCH with those observed in paired bulk cell populations in which SCH had shown more pronounced radiosensitizing effects (Fig. 4E and F). Analysis of G7 cells revealed a significant increase in both total and mitosis-specific γ-H2AX–positive cells in the bulk population compared with

Figure 6.

GSCs show enhanced DNA repair capacity following IR and/or CHK1 inhibition. Summary of flow cytometry data showing the percentage of γ-H2AX–positive cells (A), mitotic cells with high γ-H2AX (B), and mitotic catastrophe (arrows; C) in paired G7 bulk and GSC populations at 24 hours following treatment with 3 μmol/L SCH and/or 5 Gy IR. Error bars show mean ± SEM, n ≥ 3 independent experiments, **, P < 0.01; *** P < 0.001. D, bar chart summary of the percentage of G7 bulk and GSC with micronuclei 24 hours following the indicated treatment combinations. Error bars show mean ± SEM from scoring ~600 nuclei. ***, P < 0.001. E, representative immunofluorescent images of E2 GSC and bulk populations at 24 hours following 10-Gy IR, γ-H2AX foci (green), CENPF (red), and DAPI (blue). F, plots showing median γ-H2AX foci per nucleus in E2 bulk and GSC CENPF–positive or -negative cells populations from scoring a minimum 65 (CENPF–positive) or 300 (CENPF-negative) nuclei. *, P < 0.05; ***, P < 0.001.
Optimum radiosensitization of GSCs through parallel inhibition of DDR pathways

To identify the optimum targets for GSC radiosensitization, we evaluated small-molecule inhibitors of additional DDR proteins that were upregulated in GSCs (Fig. 2A). DNA repair was targeted using the PARP inhibitor olaparib, cell-cycle checkpoints were inhibited using the ATR inhibitor VE821, and combined targeting of cell-cycle checkpoints and DNA repair was evaluated using the ATM inhibitor KU55933. Results in E2 cells showed that ATR inhibition sensitized GSC and bulk populations equally, although there was a trend toward increased radiosensitization of bulk cells (Fig. 7A and B). \( \text{SER}_{37} \) values for ATR and CHK1 inhibition were comparable. In contrast, inhibition of PARP or ATM revealed a trend toward increased radiosensitization of GSCs. The ATM inhibitor KU55933 proved to be the most potent radiosensitizer of GSC (\( \text{SER}_{37} = 2.60 \), Fig. 7B).

To understand the reasons for the different magnitudes of GSC radiosensitization by the DDR inhibitors, we investigated their effects on cell-cycle checkpoints and DNA repair. Whereas inhibition of either ATR or CHK1 completely ablated radiation-induced G2-M checkpoint activation (Fig. 7C), inhibition of ATM had only a partial effect. In contrast, ATM inhibition significantly impaired repair of radiation-induced DSBs as demonstrated by a significant increase in the number of unresolved \( \gamma-H2AX \) foci 24 hours post-IR (Fig. 7D). A similar effect on DNA repair was observed following PARP inhibition. Considering that ATM inhibition was associated with the greatest radiosensitization of GSCs, we hypothesized that dual inhibition of cell-cycle checkpoint activation and DNA repair might provide optimum radiopotentiation. This was investigated by concomitant inhibition of DNA repair and cell-cycle checkpoint function by combined treatment with inhibitors of PARP and ATR. As predicted, the radiosensitizing effect of this combination in GSCs exceeded that of any of the inhibitors individually (\( \text{SER}_{37} = 3.20 \), Fig. 7E) and was significantly greater than that observed in the paired bulk cells. This is likely to be due to increased unrepaired DSBs, as combined inhibition of ATR and PARP was shown to be associated with a significant increase in \( \gamma-H2AX \) foci in GSCs compared with the bulk population (Supplementary Fig. S11). To support the clinical relevance of this result, we performed neurosphere formation assays using E2 GSCs and confirmed that dual inhibition of ATR and PARP achieved maximum potentiation of the inhibitory effects of radiation on neurosphere formation (Fig. 7F). Taken together, our results show that optimal radiosensitization of GSCs is achieved through parallel inhibition of DDR pathways.

Discussion

Glioblastomas are heterogeneous tumors that are thought to be dependent on a cellular hierarchy that includes a privileged GSC subpopulation resistant to conventional therapy and capable of tumor propagation. Uncertainty over the ideal model(s) in which to study radiation responses of the GSC population may account for the disparities in the existing published studies (10, 25, 26). We generated paired cultures of primary, patient-derived glioblastoma cell lines (10, 35) in which the GSC population was either enriched or depleted (27). While the use of sorted cell populations on the basis of a specific stem cell marker such as CD133 has been questioned because both CD133<sup>+</sup> and CD133<sup>–</sup> cells have been capable of generating tumors in vivo (45), we used this technique to validate observations made in our enriched populations and to exclude the potential confounding effects of different cell culture conditions. The GSC “signature” is likely to be complex, modulated by the cellular microenvironment and governed by multiple parameters, including epigenetic and genetic aberrations, that impact upon the expression of key regulatory proteins. In keeping with this interpretation, a recent publication highlights 4-core neurodevelopment transcription factors that are crucial to GSC maintenance and tumorigenicity (46).

Our study shows that GSCs are more radiosensitive than paired bulk populations and exhibit higher expression of total and activated DDR targets under basal conditions. The reason for this is unclear; we speculate that it may reflect an endogenous response of neural stem cells to DNA damage, which is subsequently maintained as part of mutagenic selection and the malignant phenotype. In addition, genetic differences between GSCs and bulk cells may affect cellular biochemistry, possibly accounting for lower proteasomal activity in GSCs contributing to higher levels of DDR targets (47). More recently, increased levels of reactive oxygen species have been proposed as a mechanism responsible for upregulated PARP1 in GSCs (35).

One surprising observation was the apparent lack of CHK1 activation in bulk cells exposed to IR. This observation and the slower DNA repair kinetics of these cells might explain why they are significantly more radiosensitive than the GSC population. While our results support this, it should be emphasized that the bulk cells remain relatively radiosistent, suggesting that alternative pathways might compensate for the lack of CHK1 activation and attenuated DDR. Indeed, noncanonical DDR pathways, including NOTCH, TGFβ, and receptor tyrosine kinase signaling, have all been associated with glioblastoma radioresistance and...
Figure 7.
Enhanced radiosensitization of GSCs by parallel inhibition of DNA repair and cell-cycle checkpoint pathways. A and B, clonogenic survival curves in E2 GSC and bulk populations following treatment with multiple DDRi in combination with radiation. B, summary of SER at 37% survival in paired populations with 95% confidence intervals. *, P < 0.05 and nonsignificant (NS) between GSC and bulk population. CHK1i data as shown previously in Fig. 4E and F. C, inhibition of radiation-induced G2–M checkpoint activation in E2 GSCs following 5-Gy IR treatment in the presence of various DDRi; significance relative to DMSO treatment (IR alone). *, P < 0.05; **, P < 0.01; ***, P < 0.001. D, plots showing median number of γ-H2AX foci in CENPF-positive cells at 24 hours following treatment with IR in combination with DDRi, minimum of 65 nuclei scored. ***, P < 0.001. E, clonogenic survival curves in E2 cells showing significant radiosensitization of GSC over the bulk population as shown by the SER following combined treatment with ATR + PARP inhibitors in combination with radiation. ATMi data as shown in A and B. F, neurosphere formation assay showing maximum reduction in the number of neurospheres at 4 weeks following ATR + PARPi inhibition in combination with radiation. **, P < 0.01; ***, P < 0.001.
may be activated in these cells (reviewed in ref. 48). Alternatively, bulk cells may rely on DNA damage tolerance (DDT) mechanisms, which use translesion synthesis polymerases to bypass lesions for repair at later time points (49). This provides a mechanism to tolerate DNA damage allowing cells to continue replicating and might explain survival of bulk cells following IR despite the lack of checkpoint activation and early checkpoint release.

Our study shows that multiple DDR targets, including ATR, ATM, CHK1, and PARP1, are upregulated in GSCs compared with the bulk population and that some are also preferentially activated. Although some of these targets have overlapping functions, individually they have distinct roles, which suggest that multiple DDR pathways contribute to GSC radioresistance. We showed that both GSC and bulk populations were radiosensitized by CHK1 or ATR inhibition despite higher expression and activity of these proteins in GSCs. This led us to propose that alternative DDR pathways might mitigate the radiosensitizing effects of CHK1 or ATR inhibition in the GSC populations. In support of this explanation, inhibition of ATM, with its dual functions in DNA repair and cell-cycle checkpoint activation, yielded the most potent single-agent radiosensitization of GSCs. We confirmed this hypothesis by demonstrating that combined inhibition of ATR and PARP generated maximum radiosensitization and that this effect was of significantly greater magnitude in GSCs than in bulk populations. Inhibition of PARP resulted in a significant increase in DNA DSBs, likely generated during S-phase from unrepair DNA SSBs. In the context of ATR inhibition, these cells were unable to activate the G2/M cell-cycle checkpoint and thus entered mitosis carrying high levels of unresolved DSBs. We therefore propose that GSC radioresistance is driven by both enhanced cell-cycle checkpoint activation and DNA repair and that optimal radiosensitization can only be achieved by dual inhibition of both pathways.

Our data strongly support further preclinical evaluation of ATR and PARP inhibitors in combination with IR as a potential treatment for glioblastoma. This is an entirely novel approach that to our knowledge has not been investigated in any cancer models. Clearly, there will be concerns over the potential in vivo toxicity of this combination treatment modality. ATR is essential for tissue homeostasis, highlighted by embryonic lethality of ATR-knockout mice, whereas PARP1 knockout is less deleterious. However, clinical experience with PARP inhibitors has been extremely promising; it is tolerated well as a single agent and will have particular impact on the radioresistant GSC population.

In summary, our study provides the first detailed examination of DDR responses in GSCs and has important implications for the management of glioblastoma. Our results support the notion that GSCs are profoundly radioresistant and identify a novel drug combination strategy targeting both cell-cycle checkpoint and DNA repair functions that has potential to overcome this. Future studies will focus on in vivo characterization of this strategy and will identify optimal radiation–drug combination scheduling to take forward to phase I clinical trials in patients with glioblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.U. Ahmed, R. Carruthers, A.J. Chalmers

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.U. Ahmed, R. Carruthers

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