

Enhanced radiosensitization of human glioma cells by combining inhibition of poly(ADP-ribose) polymerase with inhibition of heat shock protein 90

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Abstract

Glioblastoma multiforme (GBM) are the most common primary brain tumor and are resistant to standard therapies. The nondividing nature of normal brain provides an opportunity to enhance the therapeutic ratio by combining radiation with inhibitors of replication-specific DNA repair pathways. Based on our previous findings that inhibition of poly(ADP-ribose) polymerase (PARP) increases radiosensitivity of human glioma cells in a replication-dependent manner and generates excess DNA breaks that are repaired by homologous recombination (HR), we hypothesized that inhibition of HR would amplify the replication-specific radiosensitizing effects of PARP inhibition. Specific inhibitors of HR are not available, but the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) has been reported to inhibit HR function. The radiosensitizing effects of 17-AAG and the PARP inhibitor olaparib were assessed, and the underlying mechanisms explored. 17-AAG down-regulated Rad51 and BRCA2 protein levels, abrogated induction of Rad51 foci by radiation, and inhibited HR measured by the I-Sce1 assay. Individually, 17-AAG and olaparib had modest, replication-dependent radiosensitizing effects on T98G glioma cells. Additive radiosensitization was observed with combination treatment, mirrored by increases in γ H2AX foci in G₂-phase cells. Unlike olaparib, 17-AAG did not increase radiation sensitivity of Chinese hamster

ovary cells, indicating tumor specificity. However, 17-AAG also enhanced radiosensitivity in HR-deficient cells, indicating that its effects were only partially mediated by HR inhibition. Additional mechanisms are likely to include destabilization of oncoproteins that are up-regulated in GBM. 17-AAG is therefore a tumor-specific, replication-dependent radiosensitizer that enhances the effects of PARP inhibition. This combination has therapeutic potential in the management of GBM. [Mol Cancer Ther 2009;8(8):2243–54]

Introduction

Treatment options for glioblastoma multiforme (GBM) are limited by the innate resistance of these tumors to standard therapy (i.e., radical radiotherapy with concomitant and adjuvant temozolomide chemotherapy; ref. 1). At least half of GBM express the DNA repair protein O⁶-methylguanine-DNA-methyltransferase; this phenotype is associated with resistance to chemoradiation and with poor prognosis (2). Escalating radiation dose does not improve outcome (3) and exacerbates the risk of late, irreversible neurotoxicity (4). Novel treatment approaches are urgently required to increase radiosensitivity in a tumor-specific manner.

We have previously shown that chemical inhibition of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) increases the radiation sensitivity of a panel of human glioma cell lines, an effect that is dependent on DNA replication (5). Because GBM are rapidly proliferating tumors within a nonreplicating normal tissue environment—the brain—this finding suggests that PARP inhibitors may enhance the therapeutic ratio achieved by radiotherapy. The radiosensitizing effect of PARP inhibition is relatively modest, however, and thus we have investigated approaches to increase the effect while maintaining tumor specificity.

Synthetic lethality involves targeting parallel DNA repair pathways so that lesions generated by inhibition of one pathway are rendered lethal by obstructing another. This paradigm is illustrated by the marked sensitivity of BRCA1- or BRCA2-deficient breast or ovarian cancers to chemical inhibition of PARP. This was observed initially in the laboratory (6, 7) and has yielded promising results in patients (8). The underlying mechanism involves accumulation of endogenously arising DNA damage that would normally be repaired. In replicating cells, such single-strand DNA breaks (SSB) can generate collapsed replication forks that are repaired exclusively by the homologous recombination (HR) repair pathway (9). Because BRCA1 and BRCA2 are key components of HR, tumor cells deficient in either protein are unable to repair the collapsed forks and the resulting double-stranded DNA breaks (DSB) are cytotoxic.

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Ionizing radiation exerts its cytotoxic effects by inducing a spectrum of lesions in DNA. Whereas DSB are more lethal, SSB are about 25 times more numerous (10). Our previous work has shown that PARP inhibition increases the cytotoxicity of radiation by amplifying levels of unrepaired DSB in a replication-dependent manner. The proportion of DSB repaired by HR is increased in this setting (5). These observations suggest that, in the presence of a PARP inhibitor, radiation-induced SSB generate an increased burden of collapsed replication forks. We hypothesized that simultaneous inhibition of HR repair would increase the cytotoxicity of these lesions and enhance the radiosensitizing effect of PARP inhibitors on replicating cells.

Currently, no specific inhibitor of HR repair is available. However, the heat shock protein 90 (Hsp90) inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) exhibits indirect inhibitory effects on HR function that have been attributed to destabilization of factors including BRCA2 and Rad51 (11). Hsp90 is a chaperone protein that stabilizes a number of proteins, including the products of oncogenes such as *src* and *erbB2*, which are involved in pro-survival and antiapoptotic pathways, metastasis, and angiogenesis (reviewed in ref. 12). HR deficiency is also associated with radiosensitization; as with PARP inhibition, this occurs specifically in replicating cells (13, 14). Inhibitors of Hsp90 such as 17-AAG are active against a range of preclinical tumor models, including GBM, and are now undergoing clinical testing with promising results (15). Hence, 17-AAG is a potentially useful agent in the treatment of GBM by a variety of mechanisms: direct anti-oncogenic activity, radiosensitization via inhibition of HR, and potentiation of the radiosensitizing effects of PARP inhibition.

A potential drawback is that simultaneous inhibition of PARP and HR might prove toxic to replicating normal tissues. However, there is considerable evidence to indicate that Hsp90 inhibitors possess intrinsic tumor specificity. Hsp90 is up-regulated in tumor tissue, causing selective accumulation of inhibitors including 17-AAG. Furthermore, in tumor cells, Hsp90 exists in "super-chaperone" complexes that are up to 100-fold more sensitive to inhibitors than are Hsp90 in isolation (16). Finally, the cytotoxic effects of PARP inhibitors on HR-deficient cells are only manifest when cells are subjected to continuous exposure during multiple rounds of cell division (6, 7). This is consistent with the proposed mechanism whereby low levels of endogenously arising DNA damage eventually accumulate to lethal levels. In the context of radiation-induced damage, we have shown that exposure to PARP inhibition for only 2 hours is sufficient for full radiosensitization (5). Thus, it may be possible to limit the duration of either PARP inhibition or HR inhibition and, by appropriate scheduling with radiation therapy, achieve a beneficial therapeutic ratio.

In this study, we tested the capacity of 17-AAG to inhibit HR repair in human glioma cells. We have focused on the T98G cell line, which expresses high levels of *O*⁶-methylguanine-DNA-methyltransferase and is representative of GBM that are resistant to current treatments. We show that a nontoxic

dose of 17-AAG enhances the radiosensitizing effects of the PARP inhibitor olaparib (AZD2281, previously KU-0059436; AstraZeneca) in a replication-dependent manner. To understand the mechanisms and potential therapeutic implications, we also investigated the relative contributions of HR-dependent and HR-independent actions of 17-AAG on cellular responses to radiation and olaparib.

Materials and Methods

Cell Culture

Cells were maintained as monolayers at 37°C in 5% CO₂. Human glioblastoma cell lines T98G and U87-MG and HeLa cells were obtained from the European Collection of Animal Cell Cultures. The human fibroblast cell line NF-DRGFP was a gift from Prof. Simon Powell (Washington University School of Medicine, St. Louis, MO), and the Chinese hamster ovary (CHO) cell lines *irs1SF* (*XRCC3*^{-/-}) and *CXR3* (*irs1SF* cells complemented with human *XRCC3* cDNA; ref. 17) were provided by Dr. L.H. Thompson (Livermore National Laboratory, Livermore, CA). Cells were cultured in EMEM supplemented with 10% FCS (T98G, U87-MG) or in DMEM supplemented with 12% FCS (HeLa) or 10% FCS (NF-DRGFP, CHO cell lines).

Radiation and Drug Treatments

Adherent cells were irradiated in medium with 250-kVp X-rays (dose rate, 0.5 Gy/min). The PARP inhibitor olaparib (gift of Kudos Pharmaceuticals/AstraZeneca) was dissolved in DMSO/PBS (50:50) and administered in cytotoxicity assays at 1 nmol/L to 10 μmol/L, otherwise at 1 μmol/L, a noncytotoxic dose that was previously shown to abolish PARP activity in the cell lines used. The Hsp90 inhibitor 17-AAG (InvivoGen) was dissolved in DMSO and administered in cytotoxicity assays at 1 nmol/L to 10 μmol/L, otherwise at 100 or 500 nmol/L. The phosphoinositide 3-kinase inhibitor caffeine was administered at a final concentration of 2 mmol/L. The DNA polymerase-α inhibitor aphidicolin was dissolved in DMSO/PBS (50:50) and administered at 2 μmol/L. For all drug treatments, the final concentration of DMSO did not exceed 0.2%.

Flow Cytometry

For cell cycle experiments, cells were treated with 100 nmol/L 17-AAG ± 1 μmol/L olaparib 1 h before irradiation and until harvesting, then fixed in ice-cold 70% ethanol and stained for DNA content with propidium iodide (0.45 μg/mL), RNase (0.45 mg/mL), and 0.045% Tween before analysis on a FACSCanto flow cytometer (Becton Dickinson). Cell cycle profiles were obtained using FACSDiva software (Becton Dickinson). For *I-Sce1* assays, unfixed cells were analyzed directly for green fluorescent protein (GFP) expression.

Transient Gene Targeted Knockdown of Rad51 Using Short Interfering RNA Oligonucleotides

On-TARGETplus SMARTpool duplex short interfering RNA (siRNA), consisting of four siRNA sequences targeting *Rad51* mRNA (target sequences: 5'-UAUCAUCGCCCAUGCAUCAUU-3', 5'-CUAAUCAGGUGGUAGCUCAUU-3', 5'-GCAGUGAUGUCCUGGAUAAUU-3', and 5'-CCAACGAUGUGAAGAAUUUU-3'), and control siRNA targeting

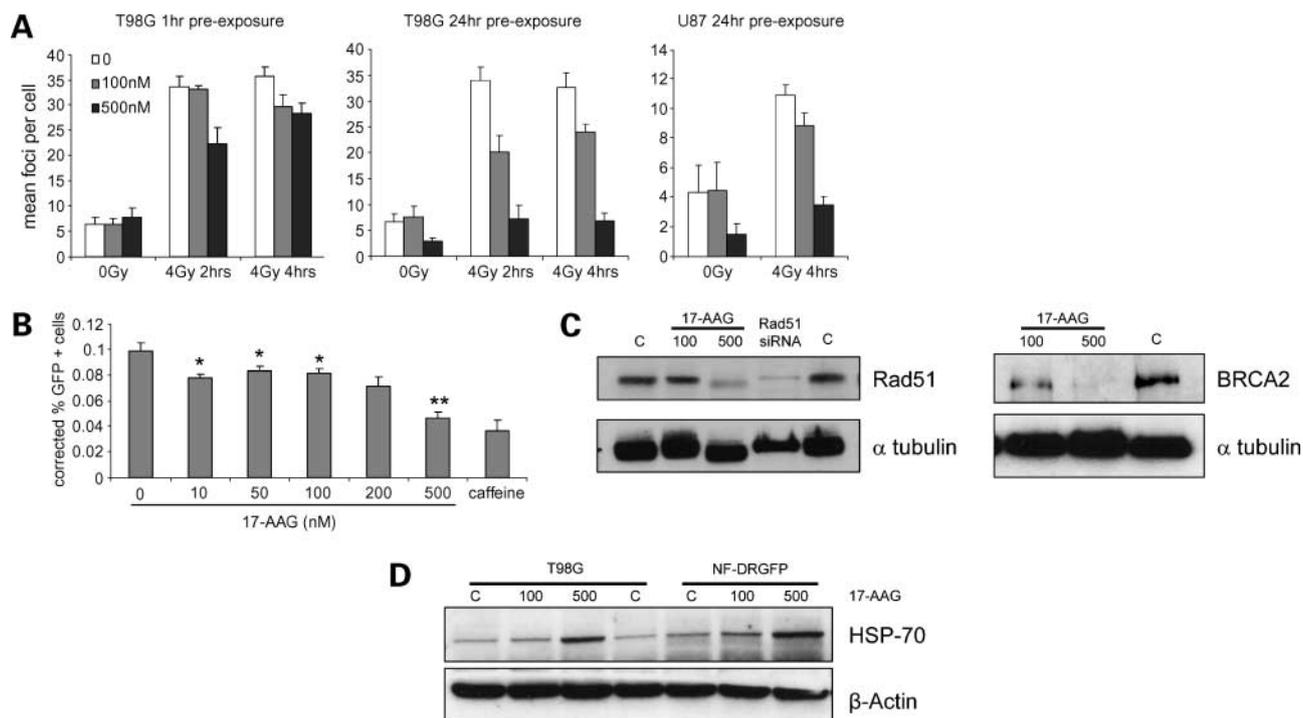


Figure 1. **A**, exponentially growing T98G and U87-MG cells were exposed to 17-AAG for 1 or 24 h before 4-Gy X-irradiation, fixed, and immunostained with anti-Rad51 monoclonal and anti-CENP-F polyclonal antibodies. *Columns*, mean number of Rad51 foci per CENP-F positive nucleus from at least four experiments; *bars*, SE. **B**, effect of 17-AAG delivered 1 h before transfection on HR repair of I-Sce1-induced DSB in NF-DRGFP cells, shown as percentage of GFP-positive NF-DRGFP cells corrected for transfection efficiency. Caffeine (2 mmol/L) treatment 3 h after transfection was done as indicated. Means of at least three independent experiments, except for 200 nmol/L 17-AAG (two repeats from one experiment). *, $P < 0.05$; **, $P < 0.01$, 17-AAG versus no drug. **C**, immunoblot analysis of Rad51 and BRCA2 in T98G cells that were untreated (C), pretreated for 24 h with 17-AAG, or transfected by electroporation with siRNA targeting Rad51. **D**, immunoblot analysis of Hsp70 in T98G and NF-DRGFP cells either untreated or pretreated for 24 h with 100 or 500 nmol/L 17-AAG.

luciferase (target sequence: 5'-CUUACGCUGAGUA-CUUCGAdTdT-3') were commercially synthesized (Dharmacon). T98G cells were transfected with siRNA (40 or 80 pmol per reaction) using electroporation and Nucleofector kit V according to the manufacturer's instructions (Amaxa Biosystems). The GFP-expressing pMAX vector (Amaxa Biosystems) was transfected in parallel to control for transfection efficiency. Rad51 protein levels were analyzed by immunoblot to establish optimum knockdown conditions. For clonogenic survival assays, cells were plated 24 h after transfection.

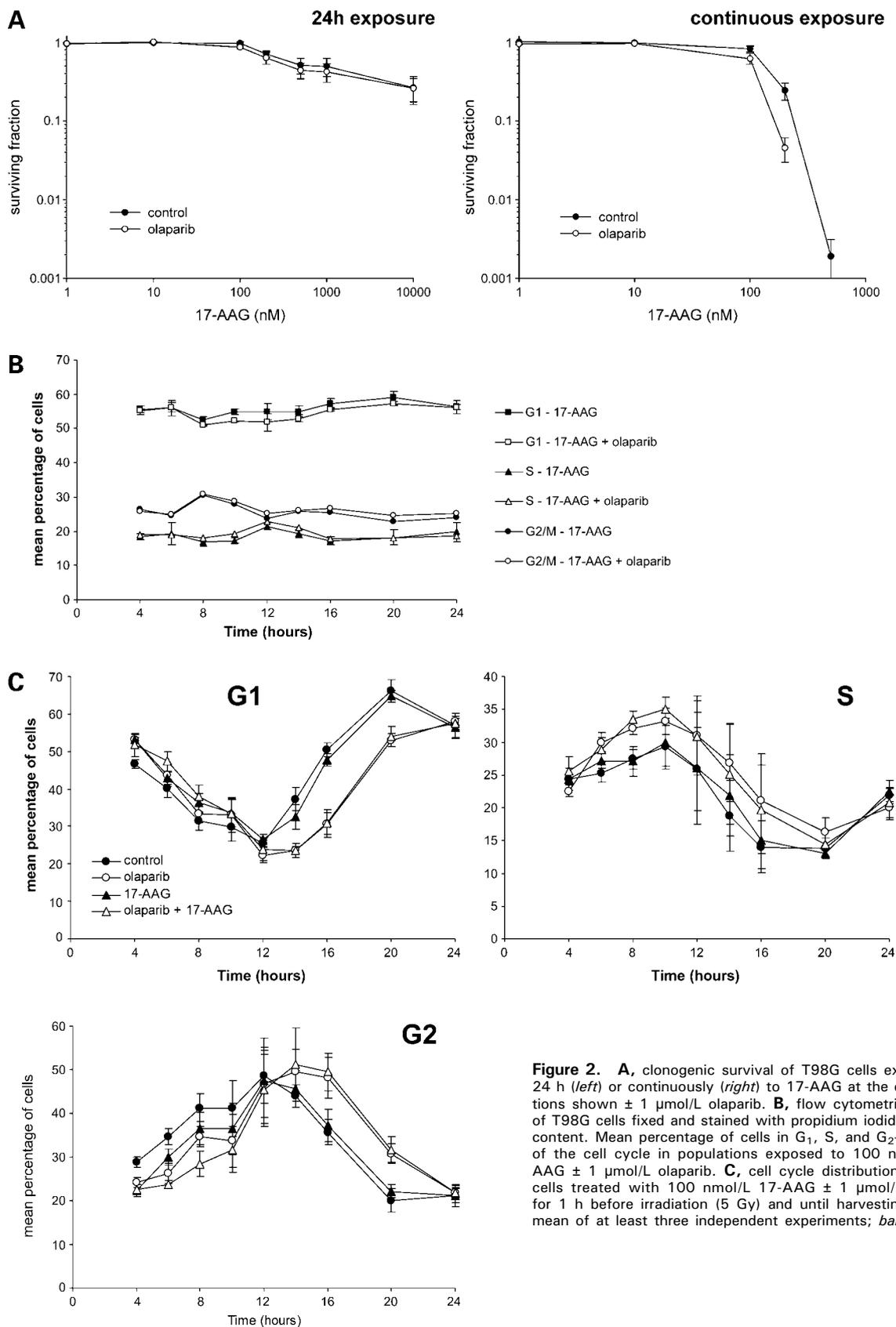
I-Sce1 Assay of HR Repair Activity

NF-DRGFP cells, stably transfected with a pDR-GFP plasmid containing a mutated GFP gene with an 18-bp I-Sce1 site and in-frame termination codon, were maintained under puromycin selection for at least 1 wk before use. Transient expression of I-Sce1 in NF-DRGFP cells creates a DSB at the relevant site in the integrated GFP gene. HR repair of this break restores gene expression (18). Plasmids containing expression sequences for I-Sce1 (pCMV3xnlsl-I-Sce1) and controls (pCMV) were gifts from Prof. Simon Powell; pEGFP plasmids were a gift from Prof. Penny Jeggo (University of Sussex, Brighton, UK). Exponential-phase NF-DRGFP cells were exposed to 17-AAG for 1 h in antibiotic-free medium. Cells were then transfected with pI-Sce1,

pEGFP, or pCMV using GeneJuice (Novagen). Caffeine (2 mmol/L) was added 3 h after transfection, where appropriate, as a positive control for HR repair inhibition. Drug concentrations were maintained by 24-hourly medium changes. After 72 h, cells were harvested by trypsinization; the percentage of GFP-expressing cells was measured by flow cytometry; and frequency of recombination events was calculated as the mean percentage of GFP-positive cells transfected with pI-Sce1 divided by the mean percentage of GFP-positive cells transfected with pEGFP.

Clonogenic Cell Survival Assays

Exponential-phase cells were plated 2 h before exposure to 17-AAG and/or olaparib and incubated at 37°C. Confluence-arrested cells had been plated at high density 4 d before trypsinization; $\geq 75\%$ G₁ arrest was confirmed by flow cytometry (data not shown). Drug-free medium was replaced after 24 h or, for long-term cytotoxicity studies, drug-containing medium was replaced every 48 h. Control flasks underwent the same medium changes. After 7 to 9 d, plates were stained with 1% methylene blue, and visible colonies counted manually. For radiation sensitivity assays, cells were allowed to adhere for at least 1 h before irradiation. Cells were exposed to olaparib for 1 h before and 2 h after irradiation (minimum exposure sufficient for radiosensitization in previous studies; ref. 5) or for 1 h before and 23 h after



irradiation. Cells were exposed to 17-AAG for 1 h before and 23 h after irradiation, the minimum exposure required for a radiosensitizing effect (data not shown). Aphidicolin exposures lasted 1 h before and 2 h after irradiation. Survival curves were derived from a minimum of three independent experiments, each done in triplicate. Surviving fraction was calculated using mean unirradiated, drug-free plating efficiency as the denominator, unless otherwise stated. The linear quadratic equation (19) was fitted to data sets to generate survival curves; sensitizer enhancement ratios (SER) for drugs were calculated at 37% survival (20).

Immunofluorescent Detection of γ H2AX and Rad51 Foci in G₁- and G₂-Phase Cells

To detect induction and resolution of γ H2AX and Rad51 foci, exponential-phase HeLa, U87-MG, and T98G cells were plated on coverslips and allowed to adhere for 48 h before drug treatments, as described, and irradiation with 1 Gy (γ H2AX assays) or 4 Gy (Rad51 assays). Cells were fixed 2, 4, or 24 h after irradiation using ice-cold methanol/acetone (50:50) for 5 min (γ H2AX) or 3.5% paraformaldehyde and 0.4% Triton at room temperature for 15 min (Rad51). Unirradiated samples were fixed at the earliest time point for that experiment. Coverslips were blocked with 2% bovine serum albumin and probed with primary antibodies to γ H2AX (1:800; mouse monoclonal clone JBW301, Upstate) or Rad51 (1:1,000; mouse monoclonal 14B4, Genetex) and centromere protein F (CENP-F; 1:1,000; rabbit polyclonal, Abcam), then Alexa-488- and/or Alexa-594-conjugated secondary antibodies (1:1,500; Molecular Probes). Coverslips were mounted in Gel Mount (Sigma) containing 4',6-diamidino-2-phenylindole (1:5,000) and imaged at $\times 100$ magnification on a Zeiss Axioplan 2 fluorescence microscope fitted with a Hamamatsu Orca digital camera and Simple PCI imaging software.

G₂ and G₁ cells were identified by intense or negative nuclear staining for CENP-F, respectively. Nuclei with intermediate CENP-F staining were assumed to be in S phase and excluded. Mean number of foci per cell was determined from at least 30 CENP-F-positive or CENP-F-negative nuclei per experiment. Rad51 foci were counted in CENP-F-positive nuclei only because they are not detected in G₁ cells. Statistical significance was determined using Student's two-tailed *t* test with data from at least three independent experiments.

Analysis of Cellular Protein by SDS-PAGE and Immunoblotting

Whole cellular protein samples in SDS loading buffer were resolved by electrophoresis using 10% or 12% acrylamide or 8% low bis-acrylamide gels for analysis of Hsp70, Rad51, and BRCA2 protein levels, respectively. Proteins were transferred onto nitrocellulose membranes (Hybond ECL membranes, GE Healthcare) using wet transfer [containing 10% (w/v) SDS for BRCA2 protein analysis]. Membranes were blocked with 5% (w/v) nonfat milk, then incubated with primary antibodies against Hsp70 (1:1,000; goat polyclonal), Rad51 (1:1,000; as before), BRCA2 (1:200; mouse monoclonal OP95, Calbiochem), and loading controls β -actin (1:4,000; mouse monoclonal AC74, Sigma) or α -tubulin (1:5,000; mouse mono-

clonal B512, Sigma). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; DAKO) to enable chemiluminescent detection using ECL or ECL plus and Hyperfilm (GE Healthcare).

Results

17-AAG Inhibits HR

The effect of 17-AAG on HR was assessed by three separate methods. Figure 1A shows that pretreatment with 17-AAG ablated induction by ionizing radiation of Rad51 foci in G₂-phase T98G and U87-MG glioma cells. This effect was dose dependent and enhanced by exposure to 17-AAG for 24 hours before radiation. Representative images are shown in Supplementary Fig. S1A. HR activity in fibroblasts measured by the I-Sce1 assay was also down-regulated in a dose-dependent manner, with 500 nmol/L 17-AAG suppressing recombination to a similar degree to caffeine (Fig. 1B). Consistent with the mechanism proposed by Noguchi et al. (11), 17-AAG treatment caused dose-dependent down-regulation of Rad51 and BRCA2 protein levels, as shown by immunoblot in Fig. 1C. Up-regulation of Hsp70 occurs in response to Hsp90 inhibition and has been widely used as a marker of this (21). Immunoblot experiments confirmed that 17-AAG treatment was associated with dose-dependent up-regulation of Hsp70 in T98G and NF-DRGFP cells (Fig. 1D).

Continuous Exposure to 17-AAG Is Cytotoxic and Exacerbated by PARP Inhibition

To explore the cellular consequences of 17-AAG treatment, clonogenic survival assays were done using T98G glioma cells. Twenty-four-hour exposure to 17-AAG caused minimal cytotoxicity at doses of 100 and 500 nmol/L, which had been shown to abrogate HR function. Concomitant 24-hour treatment with the PARP inhibitor olaparib had no effect on survival (Fig. 2A, *left*). Continuous, prolonged exposure to 17-AAG was associated with significant toxicity at these doses, which was exacerbated by cotreatment with 1 μ mol/L olaparib (Fig. 2A, *right*), a dose that has previously been shown to be nontoxic as a single agent (5). The cytotoxic effect of 17-AAG is likely to reflect inhibition of multiple pathways, but the additional toxicity associated with continuous exposure to olaparib is consistent with defective HR.

100 nmol/L 17-AAG Does Not Affect Cell Cycle Checkpoint Responses

Because previous studies had indicated that Hsp90 inhibitors might inhibit cell cycle checkpoint proteins, we examined the effect of 100 nmol/L 17-AAG on the cell cycle response of T98G cells to radiation and/or PARP inhibition. In the absence of exogenous DNA damage, 17-AAG had no effect on cell cycle distribution, alone or in combination with olaparib (Fig. 2B). Olaparib enhanced transient, radiation-induced accumulation of cells in S phase (Fig. 2C, *right*, 6–12 hours) and G₂-M phase (*bottom*, 14–20 hours), as has been reported previously, whereas the only apparent effect of 17-AAG was a very brief delay in G₂-M

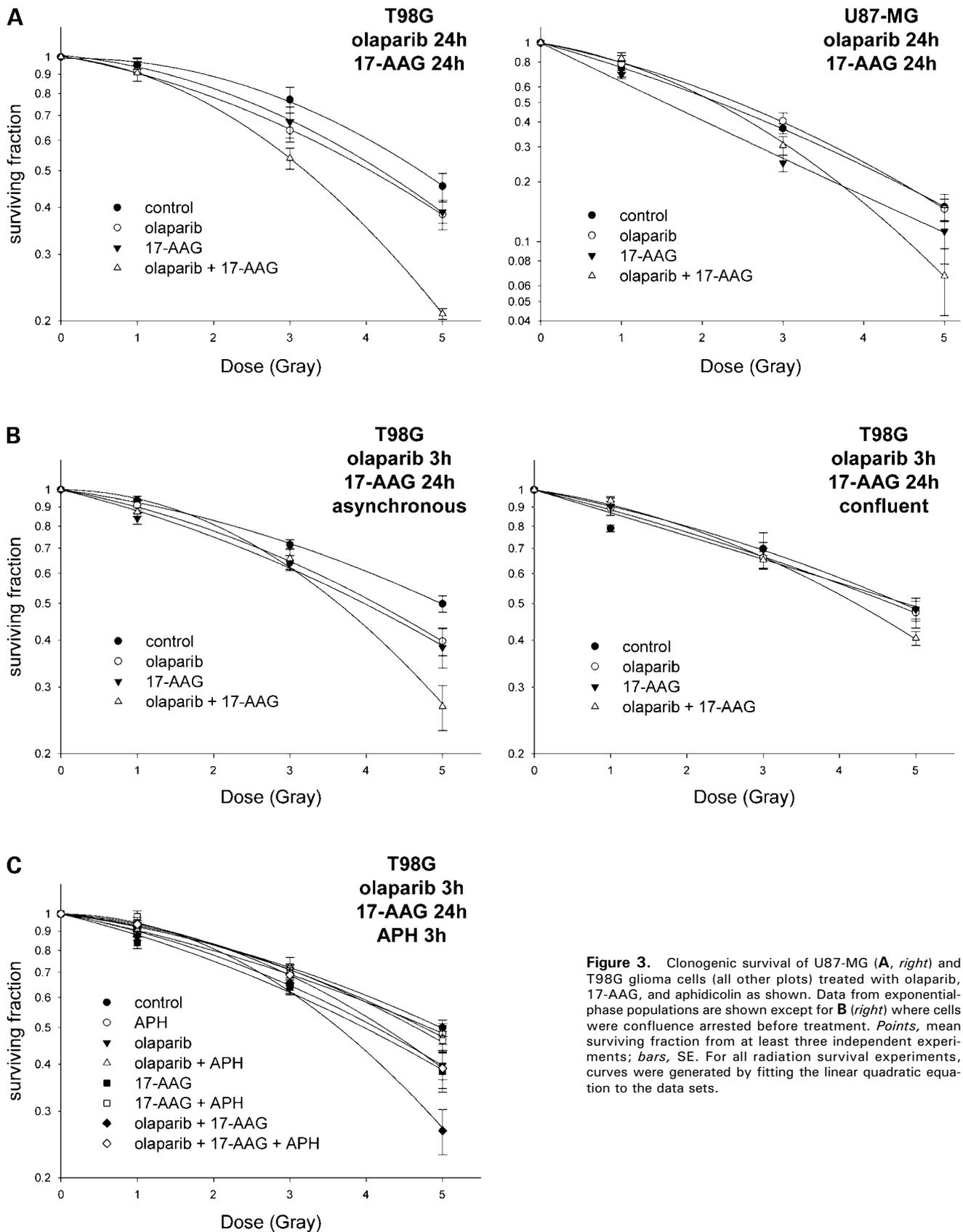


Figure 3. Clonogenic survival of U87-MG (**A**, right) and T98G glioma cells (all other plots) treated with olaparib, 17-AAG, and aphidicolin as shown. Data from exponential-phase populations are shown except for **B** (right) where cells were confluent arrested before treatment. *Points*, mean surviving fraction from at least three independent experiments; *bars*, SE. For all radiation survival experiments, curves were generated by fitting the linear quadratic equation to the data sets.

accumulation after radiation (Fig. 2C, *bottom*; $P < 0.05$ at 4 hours). 17-AAG did not influence the cell cycle effects of olaparib.

The Radiosensitizing Effects of 17-AAG and Olaparib Are Additive and Observed Only in Replicating Cells

Our previous data showed that the radiosensitizing effects of PARP inhibition were replication dependent. To investigate the hypothesis that this is caused by excess DSB arising during replication that are repaired by HR, we measured the interactive effects of 1 $\mu\text{mol/L}$ olaparib and 100 nmol/L 17-AAG on clonogenic survival of T98G cells. Individually, both agents exerted a similar, modest radiosensitizing effect (Fig. 3A, *left*), which was significantly reduced by confluence arrest (Fig. 3B) and by inhibition of DNA replication by aphidicolin (Fig. 3C). The 17-AAG effect required a treatment duration of 24 hours (1 hour before and 23 hours after irradiation), whereas the olaparib effect was observed after 3 hours of treatment (1 hour before and 2 hours after radiation). Combining 17-AAG and olaparib yielded additive radiosensitization; this effect was most pronounced when cells were exposed to both agents for a total of 24 hours (Fig. 3A, *left*). Because exposure to aphidicolin for longer than 4 hours was cytotoxic, subsequent experiments were done using 3 hours of aphidicolin and olaparib and 24 hours of 17-AAG treatments. These showed that the additive radiosensitizing effect of olaparib and 17-AAG was abolished in confluence-arrested populations (Fig. 3B) and abrogated by aphidicolin treatment (Fig. 3C). These data are consistent with the hypothesis that 17-AAG inhibits HR repair and thereby exacerbates the DNA repair defect that arises when replicating cells are irradiated in the presence of a PARP inhibitor.

Our previous study showed that whereas olaparib had a significant radiosensitizing effect on three of the four glioma cell lines tested, its effect on U87-MG glioma cells was minimal (5). This was attributed to the relatively low proliferation rate of this cell line. Here, we investigated whether Hsp90 inhibition could overcome the lack of response to PARP inhibition in this cell line. Figure 3A (*right*) shows that the radiation sensitivity of exponential-phase U87-MG cells was unaffected by olaparib but enhanced by 100 nmol/L 17-AAG. Combining the two agents may therefore increase the probability of enhancing radiosensitivity across a range of GBM.

17-AAG and Olaparib Are Associated with an Additive, Replication-Dependent Increase in Unrepaired DSB

To investigate the DNA repair events underlying the effects of these agents on radiosensitivity, induction and repair of DSB were measured by quantitative immunofluorescent detection of phosphorylated H2AX (γH2AX) foci. To improve accuracy, cells were co-stained for CENP-F to enable identification of G_2 (CENP-F positive) and G_1 (CENP-F negative) nuclei. Phosphorylation of H2AX occurs predominantly in the vicinity of DNA DSB but may also be detected at replication forks and during apoptosis. To increase specificity for "true" DSB, S-phase nuclei were

identified by intermediate CENP-F and background γH2AX staining, whereas apoptotic nuclei were identified by 4',6-diamidino-2-phenylindole staining patterns. Both were excluded from the analysis.

As shown previously (5), treatment with 1 $\mu\text{mol/L}$ olaparib had no effect on induction or repair of γH2AX foci in G_1 -phase cells and was associated with a modest increase in foci at 4 hours in G_2 -phase T98G and HeLa cells (Fig. 4A and B). Similarly, pretreatment for 24 hours with 500 nmol/L 17-AAG did not affect DSB repair dynamics in G_1 cells and was associated with an increase in foci 4 hours after radiation in G_2 -phase HeLa and T98G cells ($P < 0.05$). In HeLa cells, a similar increase was observed in unirradiated G_2 cells exposed to 17-AAG, indicating that accumulation of DSB of endogenous origin made a major contribution to the excess damage in this cell line.

In T98G cells, combining the two agents yielded an increase in γH2AX foci at 4 hours ($P < 0.01$) and 24 hours ($P < 0.05$) after radiation that was at least additive (Fig. 4A, *right*). This increase was much more pronounced in G_2 than in G_1 cells, and in G_2 cells was abrogated by aphidicolin (Fig. 4C, *right*), indicating that DNA replication played an important part in the increase in DSB. A similar effect was observed in HeLa cells (Fig. 4B). With dual-drug treatment, a small increase in γH2AX foci in unirradiated G_2 cells was also observed [$P < 0.01$ for T98G at 24 hours (Fig. 4A); $P < 0.05$ for HeLa at 4 hours (Fig. 4B)], consistent with the theory that inhibition of PARP and HR promotes conversion of endogenous damage to DSB that are not efficiently repaired. Excess DSB thus arose from both radiation-induced and endogenous damage.

In both cell lines, combination drug treatment was associated with a small increase in radiation-induced foci in G_1 cells, which was not affected by aphidicolin (Fig. 4B and C, *left*). This may reflect the multiple targets of Hsp90 inhibitors, which include DNA repair proteins. The major effect, however, was observed in G_2 cells and was inhibited by aphidicolin.

The Cytotoxic and Radiosensitizing Effects of 17-AAG Are Not Mediated Exclusively through Effects on HR

To investigate whether the effects of 17-AAG were mediated via inhibition of HR repair, clonogenic survival assays were conducted in two HR-defective backgrounds: T98G cells depleted of Rad51 by transient siRNA transfection (immunoblot shown in Supplementary Fig. S1B) and CHO cells deficient in the HR protein XRCC3. Figure 5A shows clonogenic survival of T98G (*left*) and irs1SF ($\text{XRCC3}^{-/-}$) and CXR3 (XRCC3 corrected) CHO cells (*right*) exposed to olaparib or 17-AAG for 24 hours. Sensitivity of T98G cells to olaparib was greatly enhanced by Rad51 depletion, consistent with defective HR repair. The modest sensitivity of T98G cells to 17-AAG was slightly increased by Rad51 depletion, indicating either that these treatments caused partial and additive HR inhibition or that 17-AAG was cytotoxic to Rad51-depleted cells by inhibiting alternative repair or survival pathways. Neither irs1SF nor CXR3 cells were sensitive to 17-AAG, consistent with the reported tumor-specific activity of this agent. Olaparib was highly toxic to

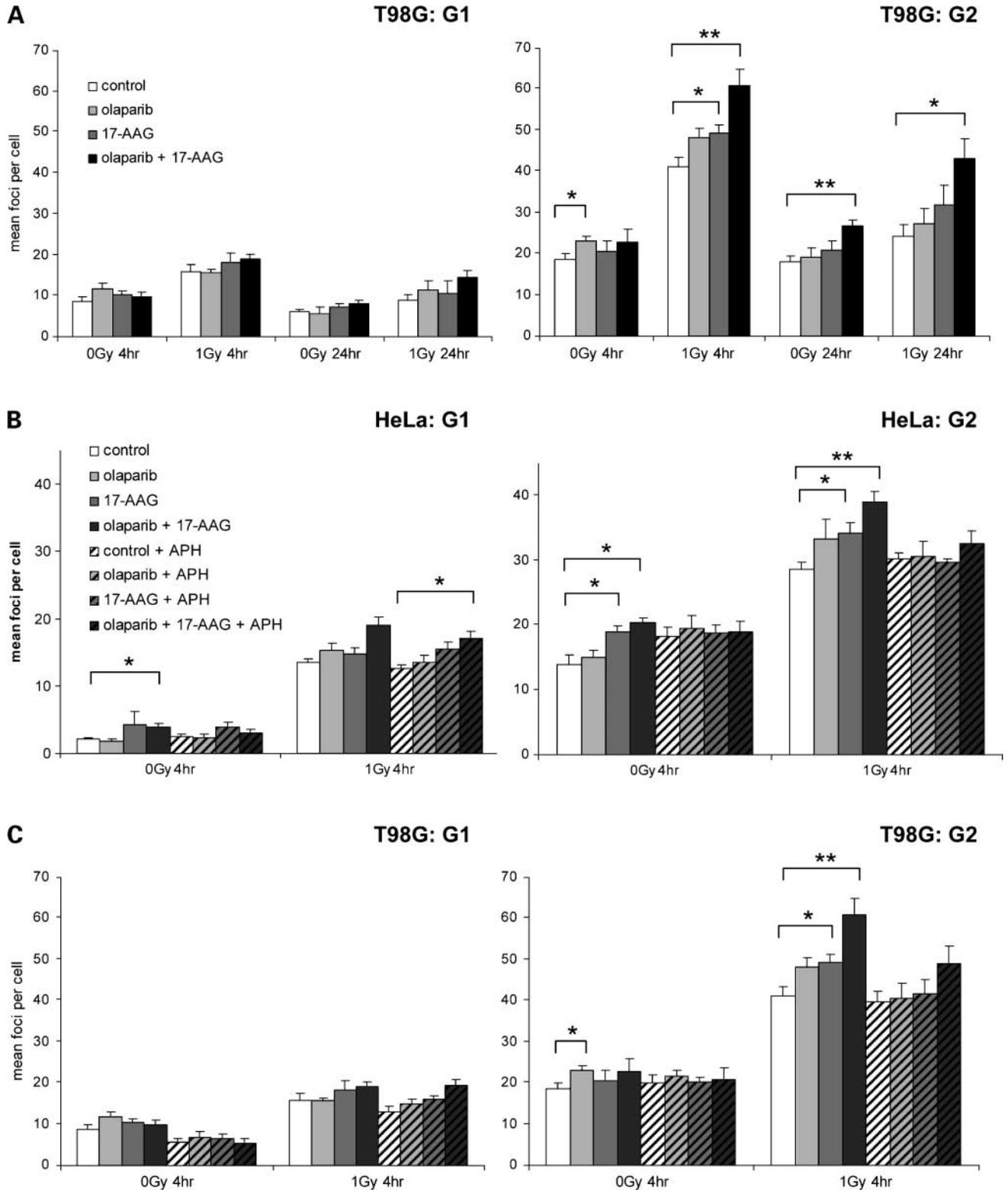


Figure 4. A, mean number of γ H2AX foci per G₁ (CENP-F negative) or G₂ (CENP-F positive) T98G nucleus 4 and 24 h after treatment with 500 nmol/L 17-AAG \pm 1 μ mol/L olaparib 1 h before X-irradiation (1 Gy). B and C, mean number of γ H2AX foci per G₁- or G₂-phase HeLa or T98G nucleus 4 h after X-irradiation (1 Gy). Cells were treated with 500 nmol/L 17-AAG \pm 1 μ mol/L olaparib \pm 2 mmol/L aphidicolin 1 h before radiation. Columns, mean of at least four independent experiments of at least 30 G₁ and G₂ cells per experiment; bars, SE. *, $P < 0.05$; **, $P < 0.01$, drug-treated versus control cells.

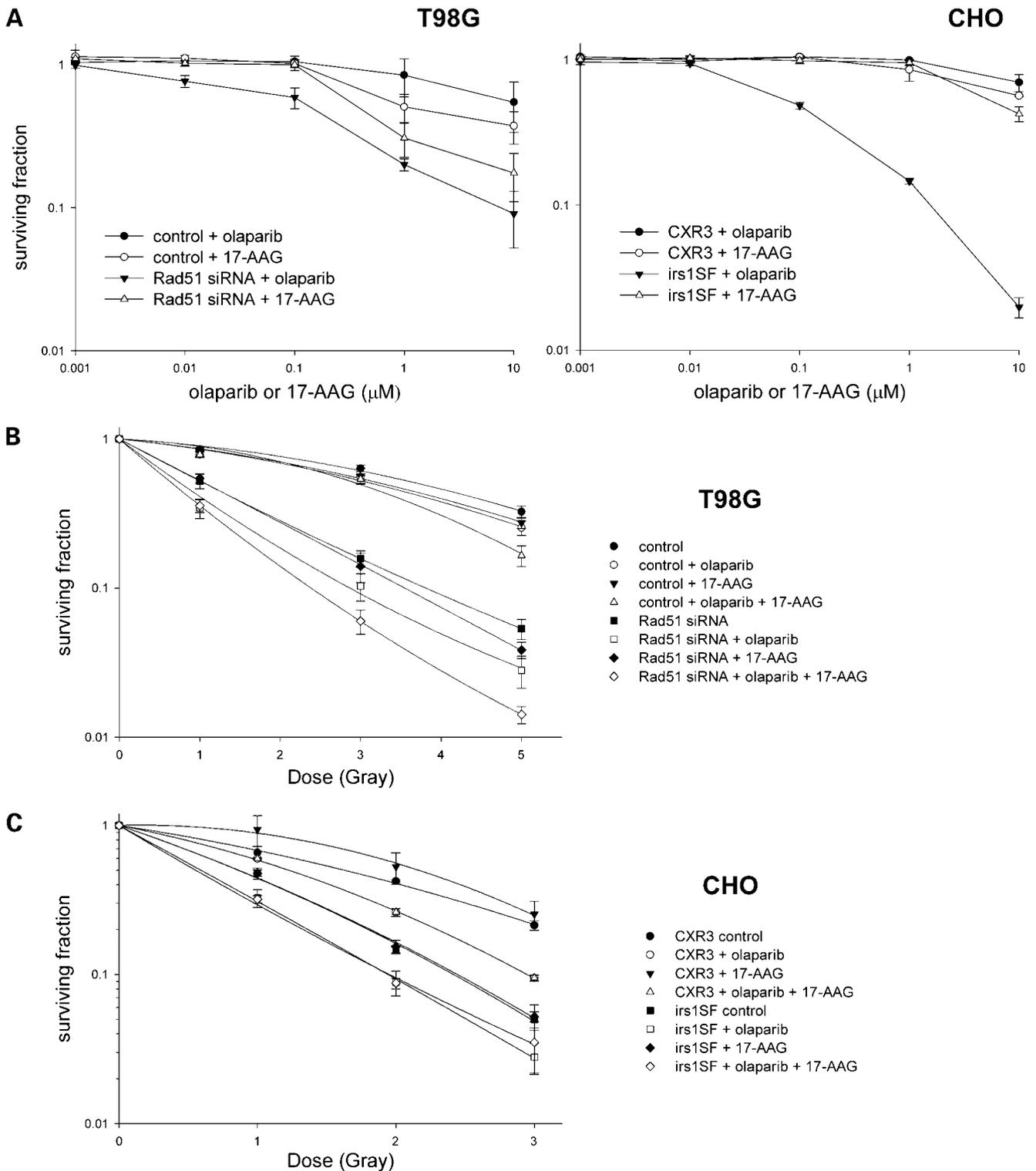


Figure 5. **A, left,** clonogenic survival of T98G cells plated 24 h after transfection by electroporation with siRNA targeting Rad51 or luciferase (control) and then exposed for 24 h to 17-AAG or olaparib at the concentrations shown. **A, right,** clonogenic survival of irs1SF (*XRCC3*^{-/-}) or CXR3 (*XRCC3* corrected) CHO cells exposed for 24 h to 17-AAG or olaparib at the concentrations shown. **B,** clonogenic survival of T98G cells plated 24 h after transfection with siRNA targeting Rad51 or luciferase (control) and then exposed to 100 nmol/L 17-AAG \pm 1 $\mu\text{mol/L}$ olaparib for 1 h before and 23 h after irradiation. **C,** clonogenic survival of irs1SF or CXR3 CHO cells exposed to 17-AAG \pm 1 $\mu\text{mol/L}$ olaparib for 1 h before and 23 h after irradiation. Points, mean surviving fraction from at least three independent experiments; bars, SE.

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irs1SF cells because of their profound HR repair deficit, as described in a recent publication using alternative PARP inhibitors (22).

As expected, Rad51 depletion caused a significant increase in the radiosensitivity of T98G cells (Fig. 5B); this effect was of similar magnitude to the radiosensitivity observed in irs1SF cells (Fig. 5C). The effects of 17-AAG on radiosensitivity of T98G cells were dose dependent (Supplementary Fig. S1C) but were not affected by Rad51 status (Fig. 5B; SER₃₇ values shown in Fig. 6A). This indicates that they were not predominantly mediated via inhibition of HR. 17-AAG had no effect on the radiosensitivity of CHO cells, regardless of dose (Supplementary Fig. S1C) or XRCC3 status (Fig. 5C). This observation further supported the concept that 17-AAG has minimal activity in nonmalignant cells.

The radiosensitizing effects of olaparib on T98G cells were enhanced by Rad51 knockdown (Fig. 5B; SER₃₇ values of 1.14 and 1.42 for control siRNA and Rad51 siRNA, Fig. 6A). This is consistent with the hypothesis that PARP inhibition increases the burden of radiation-induced DSB that require HR for repair. Cotreatment with 17-AAG yielded a further, dose-dependent increase in radiosensitivity

in both Rad51-depleted and control cells, indicating that combined treatment inhibits a number of DNA repair and/or survival pathways in malignant glioma cells. By contrast, 17-AAG did not enhance the radiosensitizing effects of olaparib in irs1SF or CXR3 cells.

The cytotoxic and radiosensitizing effects of 17-AAG on T98G glioma cells were dose dependent (Supplementary Fig. S1D). At 100 nmol/L, cytotoxicity was negligible, even in combination with olaparib, but radiosensitization was observed and was enhanced by PARP inhibition. In contrast, 500 nmol/L 17-AAG was associated with marked cytotoxic and radiosensitizing effects, both of which were enhanced by olaparib. These data indicate that careful dosing will be required *in vivo* to achieve a beneficial therapeutic ratio.

Discussion

We have previously shown that the PARP inhibitor olaparib enhances the radiation sensitivity of human glioma cells in a replication-dependent manner, indicating that this nontoxic agent may have a useful role in the treatment of GBM. Here we show that adding the Hsp90 inhibitor 17-AAG to

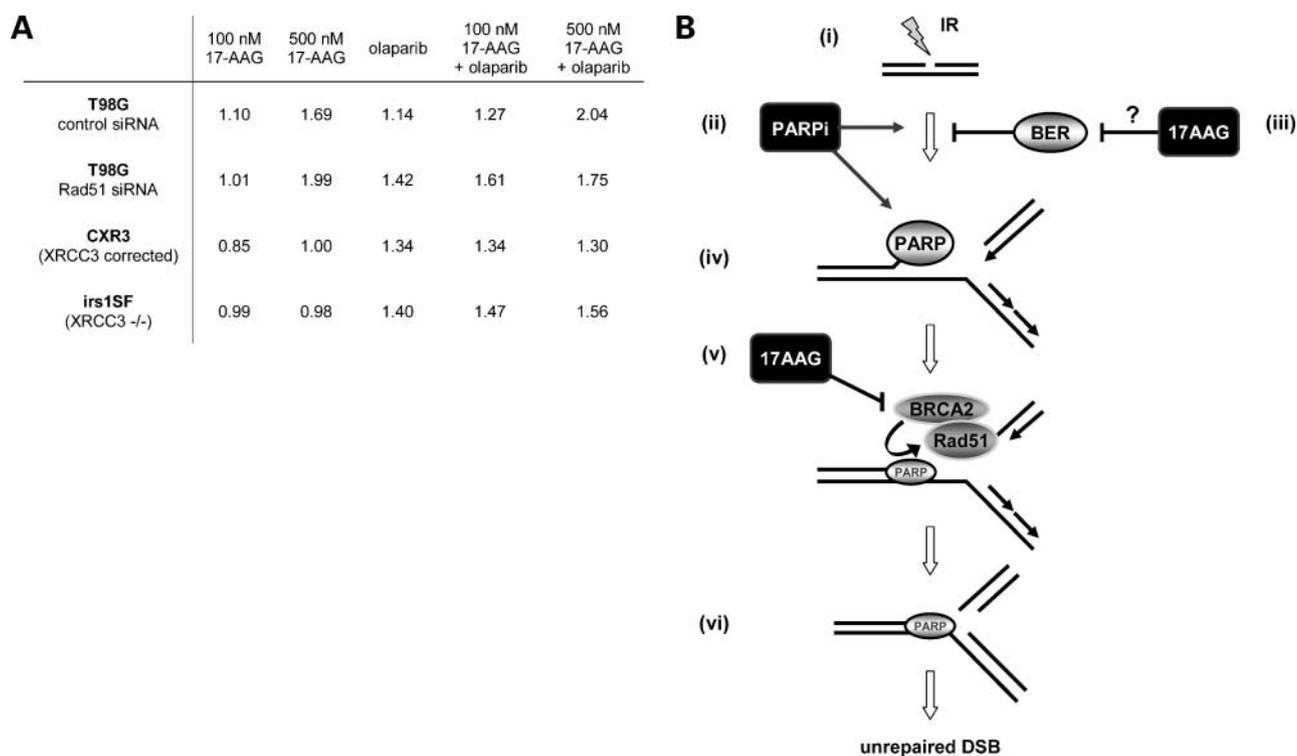


Figure 6. **A**, SER values calculated at 37% surviving fraction (SER₃₇) for 17-AAG and olaparib in T98G cells transfected with siRNA targeting Rad51 or luciferase (control) and in CXR3 or irs1SF CHO cells. **B**, proposed model for the interactive, replication-dependent radiosensitizing effects of PARP inhibition and Hsp90 inhibition. *i*, ionizing radiation (IR) induces predominantly ssDNA breaks, the majority of which are normally repaired by base excision repair (BER). *ii*, PARP inhibition (PARPi) delays repair of ssDNA breaks and promotes binding of inhibited PARP to damaged DNA. *iii*, 17-AAG may exacerbate ssDNA break repair defect by down-regulation of base excision repair components such as apurinic/aprimidinic endonuclease-1 and DNA polymerase β . *iv*, in replicating cells, unrepaired ssDNA breaks generate collapsed replication forks, which require HR for repair. *v*, BRCA2 promotes loading of Rad51 onto ssDNA at collapsed forks to initiate strand invasion and facilitate HR repair. Inhibition of Hsp90 destabilizes BRCA2 and, consequently, Rad51, inhibiting this process. Persistence of bound PARP may further impede repair. *vi*, the result is an increase in unrepaired and potentially cytotoxic DSB.

olaparib increases the radiosensitizing effect while maintaining its replication dependence. Based on these data and the intrinsic tumor specificity of 17-AAG, we propose that the combination of radiation, olaparib, and 17-AAG (or similar Hsp90 inhibitor) merits further preclinical testing in the treatment of GBM.

It is well established that continuous exposure of cells to PARP inhibition generates collapsed replication forks that are repaired by the HR pathway. Our previous study also showed that brief treatment with a PARP inhibitor was associated with a replication-dependent increase in radiation-induced HR substrates. We therefore hypothesized that inhibition of HR would exacerbate the radiosensitizing effects of PARP inhibition. In the absence of a specific inhibitor of HR repair, we tested the Hsp90 inhibitor 17-AAG, which has been reported to inhibit HR repair of radiation-induced DNA damage by down-regulating the key HR proteins Rad51 and BRCA2.

Our data show that 17-AAG inhibited HR in human glioma cells in a dose-dependent manner and enhanced the radiosensitizing activity of olaparib. Consistent with our hypothesis, the radiosensitizing effect of olaparib was also more marked in T98G cells depleted of Rad51. Hence, PARP inhibition increases the dependence on HR for repair of DNA damage arising endogenously over multiple cell cycles (Fig. 2A and previous published reports) or arising acutely in replicating cells exposed to ionizing radiation.

Because Hsp90 inhibitors affect the expression and function of multiple cellular proteins, we then explored the mechanisms underlying our observations. 17-AAG at 100 nmol/L did not affect activation of cell cycle checkpoints by radiation, in contrast with previous studies using the 17-AAG derivative 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (23, 24). In our experiments, a lower dose of 17-AAG was chosen to avoid direct cytotoxicity; higher doses may be required for checkpoint effects. In terms of DNA repair, 17-AAG had a modest inhibitory effect on resolution of radiation-induced γ H2AX foci in G₂-phase cells, which was similar to the PARP inhibitor effect. Treatment with both agents was associated with a further increase in foci that was at least additive and was abrogated by cotreatment with aphidicolin. These findings are consistent with the hypothesis that a proportion of DSB induced by radiation during S and G₂ phases is repaired by HR, and that PARP inhibition during DNA replication increases this proportion. The failure of aphidicolin to completely abolish the effect may be due to the population of G₂-phase cells identified by CENP-F staining at the time of fixation, consisting of cells that were in either G₂ or S phase at the time of radiation.

Additional effects of combined treatment with olaparib and 17-AAG were observed. Unirradiated T98G and HeLa cells showed a small but significant increase in γ H2AX foci in G₂ cells exposed to both agents, which is probably explained by DSB arising from endogenous single-stranded or base damage. A small, nonsignificant increase in γ H2AX foci in irradiated G₁ cells was also seen with combination treatment. This was not affected by aphidicolin and may represent down-regulation by Hsp90 inhibition of alterna-

tive DNA repair proteins such as DNA-dependent protein kinase catalytic subunit, as was observed by Dote and colleagues (24). This and other studies have described inhibition of a range of DNA damage responses including ataxia telangiectasia mutated (ATM) activation (24, 25) and base excision repair function (25). Our study showed that the effects of 17-AAG on DNA repair and radiosensitivity were more apparent on a background of PARP inhibition and in replicating cells. These observations are consistent with HR inhibition as a mechanism, but do not rule out effects on other repair pathways because PARP inhibition has been shown to increase the radiosensitivity of cells deficient in ATM (26, 27) and base excision repair proteins (28).

HR-deficient and HR-proficient tumor cells were similarly affected by 17-AAG in terms of cytotoxicity and radiosensitivity. It is likely that the HR defect associated with Rad51 knockdown was incomplete, and thus this observation does not exclude HR inhibition as an important mechanism of action for 17-AAG. However, it is also consistent with the likely scenario in which 17-AAG down-regulates proteins involved in multiple pathways regulating proliferation and inhibiting apoptosis, many of which are known to be up-regulated in GBM. These will not be discussed in detail here, but one likely candidate is the phosphoinositide 3-kinase signaling protein Akt, the expression and phosphorylation of which is associated with radioresistance (29), and which is down-regulated by 17-AAG (30, 31). Akt is a key target in the treatment of GBM because its activity is normally inhibited by the tumor suppressor protein phosphatase and tensin homologue, which is mutated in a large proportion of GBM (32).

One final piece of evidence that supports the use of HR inhibitors in GBM comes from the studies by Short and colleagues, which showed up-regulation of Rad51 protein levels and elevated induction of Rad51 foci by ionizing radiation in a panel of glioma cell lines as compared with normal astrocytes (33). These data indicate that GBM may be more dependent on HR for repair of radiation-induced DNA damage than the normal brain, and support the hypothesis that Hsp90 inhibition will achieve tumor-specific radiosensitization that may be further enhanced by PARP inhibition.

Clinically, radiation is delivered in 30 daily 2-Gy fractions. The radiosensitizing effects observed in our *in vitro* studies will only translate into clinical benefit if they are sustained over the course of treatment. We previously showed that the radiosensitizing effect of olaparib was maintained and enhanced by a fractionated course of 2-Gy treatments and that the proportion of replicating cells was not depleted (5). Based on this finding and our observation here that the S-phase delay associated with radiation combined with olaparib and 17-AAG had resolved by 24 hours, we predict that the cumulative tumor-specific radiosensitizing effects of these agents over a fractionated course of radiotherapy will be clinically significant.

In summary, combination therapy with chemical inhibitors of PARP and Hsp90 exerts a significant radiosensitizing effect on human glioma cells. The replication dependence of the effect and the innate tumor selectivity of Hsp90

inhibitors indicate the potential tumor specificity of this combination. These factors indicate that systemic toxicity may be acceptable, although obviously, this must be confirmed *in vivo*. Additional effects of Hsp90 inhibition on proliferation and survival pathways that are up-regulated in many GBM provide further opportunities for overcoming resistance of these tumors to existing therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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