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The RAD51-stimulatory compound RS-1 can exploit the RAD51 overexpression that exists in cancer cells and tumors

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Abstract

RAD51 is the central protein that catalyzes DNA repair via homologous recombination (HR), a process that ensures genomic stability. RAD51 protein is commonly expressed at high levels in cancer cells relative to their non-cancerous precursors. High levels of RAD51 expression can lead to the formation of genotoxic RAD51 protein complexes on undamaged chromatin. We developed a therapeutic approach that exploits this potentially toxic feature of malignancy, using compounds that stimulate the DNA binding activity of RAD51 to promote cancer cell death. A panel of immortalized cell lines was challenged with the RAD51-stimulatory compound RS-1. Resistance to RS-1 tended to occur in cells with higher levels of RAD54L and RAD54B, which are Swi2/Snf2-related translocases known to dissociate RAD51 filaments from double-stranded DNA. In PC3 prostate cancer cells, RS-1 induced lethality was accompanied by the formation of microscopically visible RAD51 nuclear protein foci occurring in the absence of any DNA-damaging treatment. Treatment with RS-1 promoted significant anti-tumor responses in a mouse model, providing proof of principle for this novel therapeutic strategy.

Keywords

DNA repair; Homologous recombination; RAD51; RS-1

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Introduction

Homologous recombination (HR) is an essential process that serves multiple roles including the repair of DNA double strand breaks (DSBs). HR utilizes an undamaged sister chromatid as a template to guide the repair of DSBs, thereby leading to error-free repair. HR also promotes cellular recovery from replication-blocking lesions or collapsed replication forks. Because of these repair activities, cells that harbor HR defects exhibit profound sensitivities to several classes of chemotherapeutics including PARP inhibitors and inter-strand DNA cross-linkers that interfere with DNA replication or replication-associated DNA repair(1–3).

RAD51 is a highly conserved protein that is central to HR. HR events involve 5' to 3' nuclease processing of DNA ends that generates 3' single-stranded DNA (ssDNA) tails at the sites of damaged DNA. These tracks of ssDNA rapidly become coated by single strand DNA-binding protein RPA. RPA is ultimately displaced from the ssDNA by oligomerization of RAD51 protein on ssDNA, wherein promoters of RAD51 oligomerize into a helical, right-handed nucleoprotein filament. The ability of RAD51 to displace RPA on ssDNA in cells requires several mediator proteins, which include BRCA2, RAD52, the RAD51 paralog complexes, and other proteins(4). Cells that harbor defects in mediator proteins exhibit low HR efficiency, and the overexpression of RAD51 protein can partially circumvent deficient mediator functions(3, 5–7).

Overexpression of RAD51 to modestly elevated levels can stimulate HR activity, at least in some systems(8–11). By contrast, RAD51 overexpression to high levels results in of lower HR efficiency and reduced viability(5, 12, 13). For example, RAD51 protein expression was experimentally increased by >10-fold using HT1080 cells that carry a repressible RAD51 transgene, and this resulted in slower growth rate, G2 arrest, and apoptosis(13). In another example, forced overexpression of RAD51 led to the formation of aberrant homology-mediated repair products and chromosomal translocations(14).

Under the normal conditions of proper HR repair, RAD51 is known to accumulate into sub-nuclear foci at sites of ssDNA that are undergoing repair(15, 16). However, some human cancer cell lines that overexpress RAD51 to very high levels exhibit nuclear foci of RAD51 in the absence of exogenous DNA damage, while such non-damage induced foci are far less prominent in nonmalignant cells(17). Therefore the toxicity associated with very high levels of RAD51 expression may be related to RAD51 complexes that accumulate on undamaged double-stranded DNA (dsDNA)(18). These damage-independent RAD51 complexes can be ameliorated, at least in part, by Swi2/Snf2-related translocases. For example, yeast Rad54 protein was shown to dissociate RAD51 nucleoprotein filaments formed on dsDNA in biochemical systems(19). Additional work in yeast has demonstrated that RAD51 accumulates spontaneously on chromatin when a set of three partially-redundant DNA translocases (Rad54, Rdh54, or Uls1) are absent. This cytologic observation coincides with slower cell growth and elevated genomic instability(18). Translocase depletion can also result in accumulation of non-damage-associated RAD51 complexes bound to DNA in human tumor cells(20). Therefore, the propensity for cancer cells to form toxic RAD51 complexes likely reflects an imbalance between RAD51 protein concentration and the combined activities of RAD54 family translocases.

These findings have important implications to human malignancies, since RAD51 protein is commonly overexpressed in human cancers(21). This overexpression seems largely due to transcriptional up-regulation, given that the RAD51 promoter is activated an average of 840-fold (with a maximum difference of 12,500-fold) in a wide range of cancer cell lines, relative to normal human fibroblasts(22). Human tumors with the highest levels of RAD51 overexpression tend to exhibit aggressive pathologic features(23, 24), and patients accordingly experience relatively poor outcomes(25–27). Taken together, RAD51 overexpression may be a common mechanism leading to genomic instability, which in turn fuels malignant progression of human cancers. Analysis of tumor cells containing high levels of non-damage-associated RAD51 complexes indicates that defects in chromosome segregation underlie this instability (20).

We have explored a novel therapeutic strategy that exploits the high levels of RAD51 overexpression that occur in human cancers. Since malignant cells are already susceptible to forming RAD51 complexes on undamaged chromatin, our strategy makes use of agents that can further increase this toxic effect. We made use of the RAD51-stimulatory compound RS-1, which increases the DNA binding activity of RAD51(28). We show here that RS-1 can be used to kill human cancer cells, and that its toxicity is modulated by both RAD51 and RAD54 translocase expression levels.

Methods

Compound preparation

RS-1 (3-(N-benzylsulfamoyl)-4-bromo-N-(4-bromophenyl)-benzamide) was synthesized in our labs, using methods described in the supplementary materials.

Knockdown of RAD51, RAD54L, and RAD54B

The RAD51 siRNA and the All-Stars negative control siRNA (NS) were ordered from Qiagen. RAD54B siRNAs were ordered from Invitrogen (Stealth). The RAD54L siRNA cocktail was ordered from Santa Cruz (sc-36362). All siRNAs were transfected using RNAiMax as per manufacturer's instructions (Invitrogen). Briefly, 2.0×10^5 cells were plated in 6 well dishes containing siRNA complexes to achieve the desired final concentration of siRNAs. The RAD54B and RAD54L siRNAs consisted of a cocktail of three independent siRNAs. The concentration of siRNAs transfected were 25 nM for RAD54L and 50 nM RAD54B. Co-depletion of RAD54L and RAD54B was performed by transfecting cells simultaneously with both siRNAs. At 48 hours post transfection, cells were harvested for cell survival assays and western blotting as described.

The target sequences for siRNA depletion are as follows:

RAD51 5' AAGCTGAAGCGAGTTCGCCA

RAD54B-1 5' CCTCATTAGCCTTTCTTGTGAGAAA

RAD54B-2 5' GCTAGGAAGTGAAAGGATCAAGATA

RAD54B-3 5' GACATTGGAAGAGGCATTGGTTATA

RAD54L-A 5' GATCTGCTTGAGTATTTCA

RAD54L-B 5' CCGTAGCAGTGACAAAGTA

RAD54L-C 5' GAACCCAGCCAATGATGAA

Western Blotting

Whole cell protein extracts were separated via SDS PAGE and subjected to western blotting. Primary antibodies included protein A purified rabbit anti HsRAD51 (1:1000 dilution, gift of Akira Shinohara), RAD54L antibody (1:1000 dilution, 4E3/1 from Abcam), RAD54B antibody (1:1000 dilution, PA529881 from Thermo Scientific), mouse anti α tubulin (1:5000 dilution, Ab-2 from Fitzgerald). Secondary antibodies consisted of HRP-conjugated anti-rabbit IgG (1:1000 dilution, GE healthcare) and HRP-conjugated anti-mouse IgG (1:2000 dilution, GE healthcare).

Cell Lines

PC3, LNCap, DU 145, COLO 205, MCF-7, HEK-293, U2OS, and MDA-MB-231 cells are routinely grown in our labs. HT1080 cells carrying a doxycycline-repressible RAD51 transgene were received from Jennifer Flygare. All of these cell lines were recently authenticated by short tandem repeat (STR) profiling at the Genetic Resources Core Facility (GRCF) at Johns Hopkins School of Medicine.

Cell Survival Assays

Cells were plated into 96-well tissue culture plates at a density of 300 cells per well in the presence or absence of RS-1 for 24 hours at 37°C, 5% CO₂. RS-1 was then removed, and cultures were allowed to grow for approximately one week to a 50–70% confluence. Average survival from six replicates was measured using CellGlo reagent (Promega), and error bars represent the standard error.

Microscopy to detect protein localization

Cells were grown on coverslips and treated with RS-1 or radiation as indicated. They were subsequently fixed with 3% paraformaldehyde/3.4% sucrose, and permeabilized with a standard buffer (20mM HEPES pH 7.4, 0.5% TritonX-100, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose). For PCNA staining, cells were treated with ice cold Methanol for 10 minutes at –20°C following fixation. The slides were immunostained for the indicated proteins using the following antibodies: a rabbit polyclonal HsRAD51 antibody (1:2500 dilution), a mouse monoclonal RPA antibody (1:1000 dilution, Ab-2 from CalBioChem), a mouse monoclonal PCNA antibody (1:1000 dilution, 1G7 from Abnova), or γ H2AX ser139 (1:1000 dilution, JBW301 from Millipore) followed by Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse secondary antibodies (Invitrogen, both 1:2000 dilution). Slides were viewed using a Zeiss Axio Imager.M1 microscope that allows high-resolution detection of foci throughout the entire nuclear volume. Images were recorded at a single representative focal plane using a CCD camera. For each experimental condition, 50 randomly selected nuclei were quantified using NIH Image software. For the purpose of RPA quantification, cells with diffuse RPA staining patterns, including S-phase cells, were excluded from the analysis as it is difficult to obtain reliable focus counts in these cells.

Cell cycle analysis

PC3 cells were treated with 60 μ M RS-1 for the indicated times. Cells were collected and fixed in ice cold 70% ethanol for at least 4 hours. Cells were rinsed and stained with 50 μ g/ml PI, 50 μ g/ml RNase A in PBS for 20 minutes. Cell cycle distributions were analyzed by flow cytometry using a LSR-II (BD biosciences) cytometer. The percentage of cells in G1, S, and G2/M phases of the cell cycle was determined using FlowJo software.

Annexin V staining

PC3 cells treated with 60 μ M RS-1 for the indicated times were harvested and stained with FITC-Annexin V for 10 minutes at room temperature as per manufacturer's instructions (BMS500FI, Ebioscience). Cells were washed and resuspended in PBS containing PI. Samples were analyzed by flow cytometry using a LSR-II (BD biosciences) cytometer. The percentage of cell positive for both Annexin V and PI was determined using FlowJo software.

Mouse tumor experiments

Xenograft tumors were induced in the hind limbs of athymic nude mice by subcutaneous injection of 1×10^6 PC3 or 1×10^7 HEK-293 cells, and tumors were allowed to grow to an average volume of about 50 mm³. Mice were then randomized into treatment groups, each consisting of 7–8 mice. Peritoneal administrations of RS-1 were delivered in 200 μ l of a vehicle solutions, which consisted of 30% DMSO, 35% PEG-400, 35% PBS. Tumor measurements were taken 3 times per week with a caliper and expressed as tumor volume, which was approximated from the product of width $\times$ length $\times$ height $\times$ 0.5. Displayed points denote the median fractional tumor volume, and error bars denote standard error.

Measurements of RAD51 binding to DNA

Experiments were performed as previously described with some modifications(29). Briefly, 75 nM purified human RAD51 protein was incubated with various concentrations of RS-1 or RS-4 in FP reaction buffer at 37 °C for 40 minutes. FP reaction buffer consisted of 20 mM Hepes pH 7.5, 10 mM MgCl₂, 0.25 μ M BSA, 2% glycerol, 30 mM NaCl, 4% DMSO, 0.1 mM tris(2-carboxyethyl)phosphine (TCEP), and 2 mM ATP. Fluorescently tagged DNA substrate was then added to a final concentration of 100 nM (nucleotide concentration for ssDNA or base pair concentration for dsDNA) and incubated at 37°C for another 40 minutes. DNA substrates consisted of either an Alexa Fluor 488-labeled oligo-dT 45-mer, a fluorescein-labeled ssDNA oligonucleotide (DHD162-CD-CF), or a fluorescein-labeled dsDNA double hairpin (DHD162) which were previously described(30). Fluorescence polarization measurements were obtained as previously described(29). The indicated concentrations of RAD51 and compounds reflect their concentrations in the final 50 μ l reaction mixture.

Results

Low levels of RAD54B and RAD54L expression are associated with sensitivity to RS-1 in immortalized human cells

High levels of RAD51 overexpression render cells susceptible to the formation of toxic RAD51 complexes, particularly in cell types that harbor inadequate translocase activity(18). Therefore, we predicted that malignant human cells with low/limiting levels of RAD54 translocase proteins would be hypersensitive to RS-1, a compound that increases the DNA binding activity of RAD51(28). We examined this hypothesis using a panel of immortalized human cell lines. Whole cell levels for RAD51, RAD54L, and RAD54B proteins were measured by western blot, and the quantification for each cell line normalized to levels in PC3 cells (Figure 1). These relative protein levels were directly compared against RS-1 sensitivity (LD₉₀ values) by linear regression analysis. The factor most strongly associated with RS-1 LD₉₀ was RAD54B protein level ($R^2=0.33$). By contrast, the association RS-1 LD₉₀ was considerably weaker for RAD51 and RAD54L protein levels ($R^2 = 0.04$ and 0.19 , respectively). A combined translocase protein expression level score was generated, which represents the sum of the RAD54B and RAD54L levels. Using this score, a significant correlation ($R^2 = 0.53$, $p=0.039$) was observed between low translocase expression level and RS-1 sensitivity.

Sensitivity to RS-1 is dependent on RAD51 and RAD54B/RAD54L translocases

To confirm that RS-1 toxicity is directly related to RAD51 and translocases protein levels, these proteins were differentially expressed in cells. First, RAD51 was overexpressed in human fibrosarcoma HT1080 cells carrying a doxycycline-repressible RAD51 transgene. Consistent with published data(13) the removal of doxycycline from media generated high levels of RAD51 doxycycline (Figure 2A, see quantifications of western blots in Supplementary Figure 1). Cells with the highest RAD51 expression levels were significantly more sensitive to RS-1. Next, we asked if knocking down RAD51 levels with RNAi would ameliorate RS-1 toxicity. The prostate cancer cell line PC3 was selected for these experiments, because the low LD₉₀ to RS-1 suggests a particular susceptibility of PC3 to forming toxic RAD51 complexes. When RAD51 siRNA was combined with RS-1 treatment, the RAD51 depletion generated significant protection from RS-1 induced toxicity (Figure 2B). These results suggest that the level of RAD51 in PC3 cells limits survival, a likely consequence of toxic RAD51 complexes. Correspondingly, stimulation of RAD51 complex formation by RS-1 reduces survival.

The ability of translocase proteins to ameliorate RS-1 induced toxicity was tested by knocking down RAD54B and RAD54L with RNAi (Figure 2C) in PC3 cells. The knockdown of either translocase significantly sensitized PC3 cells to RS-1 toxicity, though the impact of RAD54B was larger than that of RAD54L. Combined knockdown of both RAD54 translocases did not generate more RS-1 sensitization than RAD54B siRNA alone, suggesting RAD54B has more activity in ameliorating RAD51-dependent toxicity, at least in the context of RS-1 treatment.

To further support our proposed mechanism action, the RAD51 knockdown experiment was repeated using ionizing radiation in the place of RS-1. Unlike the results with RS-1, PC3 cells did not exhibit radiation resistance following RAD51 siRNA (Supplemental Figure 2). Instead, RAD51 siRNA promoted modest radiation sensitization. This implies that RS-1 kills cells by catalyzing the formation of toxic RAD51 complexes, rather than generating non-specific effects on DNA damage response and repair.

RS-1 treatment results in the accumulation of RAD51 complexes on undamaged chromatin in PC3 cells

Some cancer cells that strongly overexpress RAD51 are known have increased spontaneous RAD51 nuclear complexes compared to non-cancerous cells(17). Therefore we hypothesized that such cells would be especially susceptible to RS-1 mediated RAD51 complexes on undamaged dsDNA. This is especially likely, since RS-1 stimulates the binding of RAD51 to both ssDNA and dsDNA (Figure 3A). To study this further, PC3 prostate cancer cells and normal primary human fibroblasts (MRC-5) were treated with RS-1 and examined by immunofluorescence microscopy. To determine whether RAD51-staining structures represented sites of DNA repair vs. non-damage-associated sites, nuclei were counterstained for RPA which forms punctate sub-nuclear foci specifically in response to DNA damage at sites that colocalize with damage-induced RAD51 foci(31).

At baseline with no treatment, PC3 cells exhibited 1.4 ± 3.4 RAD51 foci/nucleus, whereas the non-cancerous control cells (MRC-5) exhibited 0.8 ± 1.5 RAD51 foci/nucleus (Figure 3B–C). After treatment with RS-1, this difference became markedly more obvious. Specifically, PC3 cells exhibited 21.1 ± 28.9 RAD51 foci/nucleus after RS-1 treatment, while MRC5 nuclei exhibited only 0.8 ± 1.5 RAD51 foci/nucleus ($p < 0.005$). Treatment with RS-1 did not significantly induce RPA focus formation in either PC3 cells (1.4 ± 2.4 RS-1 treated vs. 2 ± 3 in controls) or MRC5 cells (0.2 ± 0.5 foci/nucleus RS-1 treated vs. 0.1 ± 0.5 foci/nucleus in controls). As a control, both cell types were also examined after ionizing radiation, and as expected both PC3 and MRC5 cells exhibited significant induction of both RAD51 (11.6 ± 15.8 and 7.2 ± 14.6 foci/nucleus, respectively) and RPA staining (12.4 ± 15.8 and 8 ± 15.4 foci/nucleus, respectively). This indicates that RAD51 levels are not limiting for RAD51 focus formation in MRC5 cells. These results suggest that RS-1 treatment specifically leads to the accumulation of RAD51 foci in PC3 and not MRC-5 cells, via a mechanism that is independent of DNA damage. This interpretation is further supported by a high degree of RPA/RAD51 focus co-localization after radiation ($75 \pm 18\%$ in PC3 and $78 \pm 19\%$ in MRC5), but not after RS-1 treatment ($2 \pm 3\%$ in PC3 and 0% in MRC5).

Toxic effects of RS-1 occur independently of DSB formation and cell cycle

To further ensure that RS-1 was generating RAD51 complexes on undamaged chromatin, PC3 cells were continuously treated with RS-1 and examined microscopically at different time points out to 48 hours (Figure 4A–B). Consistent with earlier experiments, RAD51 foci accumulated 6 hours after addition of RS-1 (25.2 ± 22 foci/nucleus), and thereafter the number of foci slowly decreased down to 16.5 ± 12.7 foci/nucleus at 48 hours. This slow decline over time is likely due to cell death, which leads to an attrition of adherent measurable cells. To determine if RAD51 foci in RS-1 treated cells were DNA damage

independent, cells were counterstained for phosphorylated histone H2AX, γ H2AX, as a surrogate marker of DSBs. In contrast to the kinetics of RAD51 foci, γ H2AX foci did not significantly increase until 48 hours after addition of RS-1 (8 ± 7 γ H2AX at 0 hours to 22 ± 14 γ H2AX at 48 hours). Furthermore, RAD51 exhibited minimal co-localization with γ H2AX in cells treated with RS-1. For example, only $31 \pm 18\%$ of RAD51 co-localized with γ H2AX at 48 hours. In stark contrast, $93 \pm 15\%$ of irradiation-induced RAD51 foci co-localized with γ H2AX. The timing of γ H2AX focus formation also coincided with a loss of cell viability, as measured in cell survival assays (Figure 4C) and the induction of apoptosis, as measured by Annexin V and propidium iodide (PI) staining (Figure 4D). Taken together, these results indicate that RS-1 first catalyzes the accumulation of toxic RAD51 complexes on undamaged DNA, which is subsequently followed by hallmarks of cell death.

Since RAD51 foci associated with DNA damage form during the S and G2 phases of the cell cycle, we investigated whether or not RS-1 mediated RAD51 accumulation is restricted to these phases of the cell cycle. To test this, RAD51 localization was examined in PC3 cells treated continuously with RS-1 using proliferating cell nuclear antigen (PCNA) staining as a surrogate for S phase (Figure 5A–B). As expected, spontaneous RAD51 foci were observed in 98–100% PCNA-positive cells regardless of treatment, which is consistent with the normal roles of RAD51 in DNA replication and HR-mediated repair. Interestingly, RS-1 treatment significantly increased the number of RAD51 foci in both PCNA-positive and PCNA-negative nuclei. At 12 hours for example, RS-1 generated a 2-fold and 3.2-fold increase in RAD51 foci in PCNA-positive and PCNA-negative cells, respectively. Additionally, cell cycle profiles were examined by flow cytometry. After treatment with RS-1 for 6 hours (when RAD51 accumulation was first observed), cell cycle distribution did not differ between RS-1-treated and control cells. RS-1 treatment induced only a small enrichment of cells in S phase, and this effect did not reach statistical significance until 48 hours into RS-1 treatment (Figure 5C). Taken together, these data demonstrate that the toxic effects of RS-1 are not limited only to cells undergoing replication.

RS-1 generates anti-tumor responses in an animal model

An in-vivo tumor model was used to further test the concept of RAD51 stimulation as a cancer treatment. Treatment consisted of 5 daily peritoneal injections of RS-1, using a daily dose of 110 mg/kg. This was the maximum RS-1 concentration that could be delivered in 100 μ l of our buffer vehicle (30% DMSO, 35% PEG-400, 35% PBS), due to limited solubility of RS-1 in aqueous buffers. With this dose and delivery schedule, mice experienced a transient weight loss of about 2–3% during the week of treatment; however, they completely regained this weight in the post-treatment period and demonstrated no other overt signs of drug toxicity.

Subcutaneous xenografted PC3 tumors were established in the hind limbs of athymic nude mice, and the mice were subsequently treated with RS-1 or vehicle control. Treatment with RS-1 generated significant anti-tumor responses, relative to the vehicle-alone control mice whose tumors all progressively grew (Figure 6A). 43% of tumors (3 of 7) in the RS-1 group completely disappeared after treatment and never regrew during a two month observation period. The remaining tumors in the RS-1 treated group did eventually regrow, however

treatment generated a >2 week delay in tumor regrowth relative to the vehicle-alone control. RS-1 treatment was well-tolerated, with no toxic deaths observed. This PC3-based tumor experiment was repeated, and the result reproduced. A similar experiment was then performed using tumors derived from HEK-293 cells, which are faster growing and more resistant to RS-1 than are PC3 cells. As expected, the degree of anti-tumor response was smaller in these tumors (Figure 6B). Tumor regrowth was significantly delayed by RS-1 treatment; however, the magnitude of delay was only 2 days.

Discussion

We have developed a novel therapeutic approach for oncology using compounds that stimulate the DNA binding activity of RAD51. This exploits the propensity of human cancers to express high levels of RAD51 protein. Since malignant cells are prone to forming aberrant RAD51 complexes on undamaged chromatin, they are predisposed to killing by RAD51 stimulators which further enhance this toxic phenotype. Our results demonstrate that the toxicity of RS-1 depends on both RAD51 and RAD54 family translocase expression levels. Furthermore, xenograft mouse experiments demonstrate that this RAD51-stimulatory compound generates anti-tumor responses in-vivo, thereby providing proof in principle for this therapeutic strategy.

Cellular resistance to RAD51 stimulation depends on RAD54B and RAD54L protein levels, consistent with the ability of Swi2/Snf2-related translocases to remove aberrant RAD51 complexes from undamaged chromatin(18, 20). We found, however, that RAD54B depletion results in greater RS-1 sensitization than RAD54L depletion. Therefore, RAD54B appears to be the more relevant translocase for this function, at least in the context of RS-1 treatment. This is consistent with published results on Rdh54, the yeast homolog of human RAD54B, which is most important of these Swi2/Snf2-related translocases for preventing spontaneous RAD51 focus formation(18). Therefore, human tumors harboring an imbalance of RAD51 and RAD54B are predicted to be sensitive to treatment with RAD51-stimulators. Newer diagnostic methods may help to better quantify the degree of RAD51-to-RAD54B imbalance in clinical tumor specimens, in order to better predict RS-1 sensitivity.

While a wide variety of immortalized human cells (e.g. carcinoma, sarcomas, and virally transformed cells) overexpress RAD51 protein(22), we found that the sensitivity of immortalized cell lines to RS-1 is quite variable. Furthermore, the degree of RS-1 sensitivities among different cell lines could not be predicted based on measurements of RAD51 protein levels. While cellular resistance to RS-1 does depend on RAD54B and RAD54L protein levels, the vulnerability of different cancer types to killing by RAD51 stimulators probably depends on additional factors. We also observed that RAD51-stimulatory compounds can generate a reduction in total cellular RAD51 protein levels in some cell types (data not shown). Further work is underway to define the role of RAD51-stimulatory compounds in these processes, since they may contribute to the differential susceptibility of cancers.

In conclusion, we have developed a novel therapeutic strategy that exploits the propensity of human cancers to form toxic RAD51 complexes on undamaged chromatin. We are now

developing second-generation chemical analogs with greater RAD51 stimulatory potency and improved pharmacological characteristics(32). Additional work is underway to better identify tumor characteristics that influence the susceptibility of different cancers to this therapeutic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Low expression levels of the RAD54 translocase proteins are significantly associated with RS-1 sensitivity in cell lines

A) Relative protein levels are listed for a panel of human cell lines. Whole cell extracts (100 µg protein/well) were subjected to western blots for RAD51, RAD54L, RAD54B. Protein levels quantified were controlled based on tubulin loading control, and they were then normalized to the levels of the PC3 cell line. **B)** Protein levels are plotted as a function of RS-1 sensitivity. Translocase protein expression level is defined as RAD54B + RAD54L. The displayed trend line is the result of linear regression analysis.

Figure 2. Protein levels of RAD51, RAD54L, and RAD54B affect sensitivity to RS-1

Western blot shows are shown in the top of each panel, and quantifications thereof are displayed Supplemental Figure 1. **A)** Forced overexpression of RAD51 expression levels sensitizes cells to RS-1. HT1080 cells carrying a doxycycline-repressible RAD51 transgene were pre-treated with varying levels of doxycycline for 24 hours. Cells were subsequently incubated for 24 hours in media containing varying concentrations of RS-1. Cells were then allowed to grow in drug-free media for an additional 6 days, and indicated doxycycline concentrations were maintained throughout the entire experiment. Average survival for each condition is normalized to the 0 μM RS-1 control of that condition. **B)** Knockdown of RAD51 in PC3 protects cells from the toxicity of RS-1. PC3 cells were treated with various concentrations of RAD51 siRNA or a non-silencing (NS) control for 48 hours. Following RNAi, cells were incubated for 24 hours in media containing varying concentrations of RS-1. Cells were then allowed to grow in drug-free media for an additional 6 days. **C)** Knockdown of RAD54L and RAD54B sensitizes cancer cells to the toxicity of RS-1. PC3 cells were treated with siRNA against RAD54L, RAD54B, both, or a non-silencing (NS) control for 48 hours and processed as above. Statistical significance was determined using ANOVA followed by student t-tests. The p-values are reported in Supplemental Table 1.

Figure 3. RS-1 stimulates the binding of RAD51 to DNA

A) RS-1 stimulates the binding both ssDNA and dsDNA. Various concentrations of RS-1 were incubated with purified hRAD51 protein and a fluorescently tagged DNA substrate, consisting of either a ssDNA oligonucleotide (DHD162-CD-CF) or a dsDNA double hairpin (DHD162). Binding of RAD51 to DNA was measured as a function of fluorescence polarization of the tag, as described in the methods section. **B)** RS-1 generates microscopically visible RAD51 complexes in undamaged PC3 nuclei, but not in non-immortalized MRC-5 nuclei. Cells were grown on cover slips, incubated for 6 hours in media containing 60 μ M RS-1. In the 8 Gy radiation control condition, cells were irradiated 6 hours before harvest. Cells were subsequently indirectly immunostained. Representative images of key conditions are displayed with RAD51 displayed in green and RPA displayed in red. **C)** Fifty randomly selected nuclei per treatment group were examined and discrete foci were quantified. The reported p values were calculated using the Wilcoxon Rank Sum test: n.s.= not significant, * p -value<0.05, ** p -value<0.005.

Figure 4. Toxic RAD51 complexes accumulate independent of DSB formation in RS-1 treated PC3 cells

PC3 cells were incubated in 60 μ M RS-1 for the indicated times prior to fixation and immunostaining for RAD51 (green) and γ H2AX (red). **A)** Representative nuclei of RS-1 treated nuclei are shown. **B)** Dot plots quantify RAD51 and γ H2AX foci in 50 random nuclei. Statistical significance was determined using the Wilcoxon Rank Sum Test. **C)** Cell survival is shown after treatment with RS-1 for the indicated times. Error bars = standard error **D)** The percentage of cells undergoing apoptosis was determined by staining cells with Annexin V and propidium iodide (PI) and analysis by flow cytometry. Error bars = standard deviation. Statistical significance was determined using a Student's t-test. n.s. = not significant, * p -value < 0.05, ** p -value < 0.005.

Figure 5. RS-1 mediated RAD51 accumulation is independent of cell cycle

PC3 cells were incubated in 60 μ M RS-1 for the indicated times prior to fixation and immunostaining for RAD51 (green) and PCNA (red). **A)** Representative nuclei depicting RAD51 accumulation after treatment with RS-1 in PCNA-positive (S phase) and PCNA-negative (non-S phase) nuclei. **B)** Dot blot depicting RAD51 focus counts in PCNA-negative and PCNA-positive nuclei after the indicated treatments. The reported p values were calculated using the Wilcoxon Rank Sum test: n.s.= not significant, * p -value<0.05, ** p -value<0.005. **C)** Quantitation of cell cycle distributions of PC-3 cells after the indicated treatments. Statistical significance was determined using the Student's t-test.

Figure 6. RS-1 generates anti-tumor responses in a mouse xenograft tumor model

Tumors were induced in the hind limbs of athymic nude mice, using either PC3 (A) or HEK-293 (B) cells. Mice were then randomized into two treatment groups. Starting on day 0, mice then received 5 daily intra-peritoneal injections with either RS-1 (110 mg/kg) or vehicle alone control. Median tumor volume is plotted, normalized to the starting tumor volume on day 0. The results were tested using the Wilcoxon Rank Sum test, and significant (p -value<0.05) differences are denoted with an asterisk.