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Recent Developments Using Small Molecules to Target RAD51: How to Best Modulate RAD51 for Anticancer Therapy?

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Abstract

Homologous recombination (HR) is an evolutionarily conserved DNA repair process. Overexpression of the key HR protein RAD51 is a common feature of malignant cells. RAD51 plays two distinct genome-stabilizing roles, including HR-mediated repair of double-strand breaks (DSBs) and the promotion of replication fork stability during replication stress. Because upregulation of RAD51 in cancer cells can promote tumor resistance to DNA-damaging oncologic therapies, we and others have worked to develop cancer therapeutics that target various aspects of RAD51 protein function. Herein, we provide an overview of recent developments in this field, together with our perspectives on the challenges associated with these evolving anticancer strategies.

Keywords

cancer therapy; drug discovery; homologous recombination; medicinal chemistry; RAD51

RAD51 as a therapeutic target in oncology

Homologous recombination (HR) is a DNA repair process that is evolutionarily conserved in eukaryotic cells. It plays a critical role in the repair of the most harmful types of DNA damage, including DNA double-strand breaks (DSBs) and interstrand cross-links (ICLs).^[1] HR facilitates cellular recovery from several classes of DNA lesions, and cells with deficient HR are especially vulnerable to radiotherapy and chemotherapeutic agents that generate DSBs, ICLs, and other specific classes of DNA replication-blocking lesions.^[1,2] This makes HR an attractive target for inhibitory small-molecule development, with the central goal of sensitizing cancer cells to existing DNA-damaging therapies.^[3]

Homology-directed repair by gene conversion (GC) and synthesis-dependent strand annealing (SDSA) faithfully repairs the damaged DNA by using an undamaged homologous DNA template to guide error-free repair and thereby maintain genomic integrity.^[2b,4] Both

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of these high-fidelity processes are catalyzed by the RAD51 recombinase, together with other HR-related proteins. This distinguishes HR from more error-prone homology-directed repair pathways that do not use RAD51, such as single-strand annealing (SSA) and microhomology-mediated end joining (MMEJ).^[5] Furthermore, this distinguishes HR from non-homologous end joining (NHEJ), which ligates broken DNA ends together with little regard for sequence homology.^[6] A broad schematic overview of HR is shown in Figure 1. An initial step of HR involves nucleolytic resection of the DSB to generate a 3' single-stranded DNA (ssDNA) tail at the site of DNA damage. The single-stranded DNA binding protein replication protein A (RPA) is first loaded onto the resected ssDNA end (reviewed by Wold^[7]). RAD51 is then loaded onto the ssDNA end to form a helical nucleoprotein filament, displacing RPA with the help of BRCA2 and other accessory factors.^[8] This nucleoprotein filament is capable of identifying a homologous sequence in dsDNA and invading the duplex at sites of homology to form a joint molecule, consisting of a heteroduplex and a displaced strand of ssDNA (D-loop). Once a stable D-loop has been formed, RAD51 is cleared from the heteroduplex by RAD54L/B, allowing polymerases to synthesize DNA across the break using the homologous strand as a template.^[9]

In addition to its central role in HR, RAD51 plays an important role in allowing cells to tolerate lesions that interrupt DNA replication. During replication stress, RAD51 binds to areas of ssDNA at perturbed replication forks to prevent nucleolytic degradation of nascent DNA.^[10] Interestingly, this important function requires RAD51; however, the research on the key RAD51 accessory protein BRCA2 strongly suggests that at least some of RAD51's role in replication may not require homology-dependent recombination with a second DNA molecule. Thus, drugs that permit RAD51 filament assembly but prevent HR may sensitize cells to DSB-inducing treatments without inducing general cellular toxicity stemming from errant DNA replication.

RAD51 is an attractive cancer therapeutic target, in part, because it is overexpressed in a wide variety of human malignancies including breast, prostate, bladder, lung and soft tissue sarcomas.^[11] RAD51 overexpression in cancer cells elevates HR efficiency and contributes to resistance to certain DNA damaging chemotherapies and radiotherapy.^[12] Experimentally induced reduction of RAD51 expression by various techniques has been shown to overcome this effect, thereby elevating the sensitivity of cancer cells to DNA damaging therapies.^[13] This sensitization has been shown to occur preferentially in malignant cells, with relatively smaller effects in noncancerous human cell lines.^[13a,b] Given the genome-stabilizing role that RAD51 plays during DNA replication combined with the oncogene-driven growth stimulation in cancer cells, it is possible that RAD51 disruption may additionally induce single-agent toxicity that preferentially targets malignant cells.

Based on these encouraging data, we and others have developed small molecules that target RAD51. Published results with RAD51 inhibitory or stimulatory compounds are summarized in detail below.

Compounds that inhibit RAD51–ssDNA nucleoprotein filament formation

The ability of RAD51 to bind ssDNA is required for both its DSB repair and replication fork protection activities. Recently, a number of compounds capable of inhibiting the formation

of RAD51–ssDNA nucleoprotein filament have been identified, and their chemical structures are depicted in Figure 2.

4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS, **1**) and metatungstate (**2**) are two of the earliest compounds that were shown to inhibit the activity of RAD51. DIDS was originally used as an inhibitor of ionic channels and membrane transporters.^[14] Ishida et al. found that DIDS additionally inhibits RAD51-mediated D-loop formation with an IC₅₀ of 1–10 μM (at 6 μM RAD51).^[15] DIDS functions by inhibiting the DNA binding activity of RAD51. Surface plasmon resonance (SPR) analysis revealed that DIDS directly binds to RAD51 with $K_d = 2 \mu\text{M}$. Based on the observation that DIDS increases the DNA-independent ATPase activity of RAD51, DIDS might compete with ssDNA binding by binding at or near RAD51's ssDNA binding site. More recently, another group showed that DIDS is selectively cytotoxic to human chronic lymphocytic leukemias (CLL) expressing activation-induced cytidine deaminase (AID), which is aberrantly expressed in many CLL malignancies and normally generates DSBs during immunoglobulin class switching recombination. CLLs with increased AID activity are preferentially sensitized to DIDS, and they display a higher burden of unrepaired DNA damage upon treatment.^[16]

Metatungstate (**2**) was identified as a potent inhibitor of MvRadA, a homologue of RAD51 from the archaea *Methanococcus voltae*.^[17] Co-crystallization experiments revealed that metatungstate likely competes with DNA by binding to the L1 and L2 DNA binding loops of MvRadA, locking the protein into an inactive conformation. Metatungstate inhibits the ssDNA binding, dsDNA binding, strand exchange, and ATPase activities of MvRadA, with IC₅₀ values all in the low micromolar range. Despite these strong biochemical observations, the cellular effects on DNA repair and HR have not been reported for metatungstate. Of note, this compound is highly charged, suggesting that it may have difficulty penetrating cell membranes.

The compound B02 (**3**) was developed by Mazin and coworkers, and it is among the most thoroughly studied RAD51-inhibitory compounds. This compound was identified by high-throughput screening of the NIH Small Molecule Repository, in search of compounds that block homologous strand displacement by RAD51.^[18] B02 specifically inhibits D-loop formation by human RAD51 (IC₅₀ = 27.4 μM at 1 μM RAD51) but not by its *E. coli* homologue RecA (IC₅₀ > 250 μM). Subsequent mechanistic studies revealed that B02 acts by directly binding to RAD51 ($K_d = 5.6 \mu\text{M}$ based on SPR); this prevents RAD51 from forming nucleoprotein filaments on ssDNA and destabilizes pre-formed nucleoprotein filaments.^[19] An SAR study was also performed to identify more potent analogues of B02; while some of the resulting analogues showed significantly lower IC₅₀ values against the D-loop activity of RAD51, they also showed decreased selectivity for human RAD51 versus *E. coli* RecA. In cellular assays, B02 effectively inhibits DSB-induced HR in human cells. Importantly, B02 also suppresses ionizing radiation (IR)-induced RAD51 foci formation and increases cellular sensitivity to the DNA-crosslinking agents (cisplatin and MMC) and the topoisomerase II inhibitor doxorubicin.^[19,20] In a more recent in vivo study by the same group, B02 significantly enhanced the antitumor effects of cisplatin in a breast cancer xenograft model while being relatively well-tolerated by mice.^[21] These DNA damage-

sensitizing effects of B02 have been further demonstrated by other groups using human lung cancer and multiple myeloma-derived cell lines.^[22]

Chicago Sky Blue (CSB, **4**) was also reported to be a potent inhibitor of RAD51, with an IC₅₀ of 0.4 μ M in a homologous strand exchange assay using 0.5 μ M RAD51.^[23] SAR was also performed on CSB in the same study, with no improvements found relative to the starting compound. As with the previously described compounds in this class, CSB prevents RAD51 from forming stable filaments on ssDNA, thereby preventing subsequent steps of homologous strand exchange to proceed.

Our group is also dedicated to developing therapeutic compounds that specifically inhibit RAD51's ssDNA binding. We initiated our research by high-throughput screening of a naïve library of 10 000 compounds (ChemBridge DIVERSet™) using a fluorescence anisotropy-based assay that measures the binding of RAD51 to ssDNA.^[24] Our screening efforts led to the discovery of RI-1 (**5**), which effectively blocks RAD51's filament formation and D-loop activities. These biochemical findings were further corroborated by cell-based assays showing sensitization of multiple tumor-derived human cell lines to the DNA crosslinking agent mitomycin C. RI-1 also inhibits cytological RAD51 focus formation following DNA damage. RI-1 contains a chloromaleimide group that functions chemically as a Michael acceptor, thereby inhibiting RAD51 by covalently binding to the Cys319 residue. Cys319 resides on a surface of RAD51 protein used as an interface between subunits of a RAD51 filament; thus RI-1 is thought to disrupt RAD51–ssDNA binding by preventing oligomerization of RAD51 into filaments on DNA. Cysteine 319 is conserved among eukaryotic RAD51 homologs, but not among the bacterial homolog RecA. While human and yeast RAD51 proteins are equally sensitive to inhibition of ssDNA binding by RI-1, *E. coli* RecA and a C319S mutant of human RAD51 are refractory to inhibition by RI-1.

The chloromaleimide group of RI-1 promotes stable, irreversible inhibition of RAD51. As a result of this, inhibitory activity is observed even when bead-immobilized RAD51 is treated with RI-1, extensively washed, and then subsequently eluted and tested.^[25] While this irreversible mode of binding has some potential advantages, the reactive nature of the chloromaleimide group raises potential concerns of in vivo off-target toxic effects and/or sequestration of the compound by irreversible binding to serum proteins like albumin. Furthermore, RI-1 can bind to glutathione and be inactivated by thiol-containing reducing agents, which might limit RI-1's effectiveness within the reducing microenvironments of cells.^[24,25] To overcome this challenge, we conducted an extensive SAR study to identify analogues of RI-1 that could retain inhibitory activity without requiring Michael acceptor activity. These efforts yielded RI-2, which possesses RAD51–ssDNA inhibitory activity in cell-based and purified biochemical systems, despite its lack of any covalent binding to RAD51 or reactivity toward other sulfhydryl-containing compounds. Although RI-2 requires two- to six-fold higher concentrations to achieve the biochemical and cellular effects observed with RI-1, the improved chemical stability of RI-2 makes it a favorable candidate for further development as a therapeutic agent.

Compounds that stimulate RAD51–ssDNA binding

In contrast to the various reported RAD51–ssDNA binding inhibitors, we have discovered a compound (RS-1, **7**) that is capable of stimulating the binding of RAD51 to DNA.^[26] RS-1 was identified in the same high-throughput screen that yielded RI-1. It enhances the formation and stability of RAD51–ssDNA nucleoprotein filaments. RS-1 allows RAD51 to remain tightly bound to ssDNA even after RAD51 hydrolyzes ATP, thereby locking the RAD51 filament in an active conformation. RS-1 also promotes cellular survival of untransformed human dermal fibroblasts following treatment with cisplatin,^[26] and it improves gene targeting after site-specific DSB formation by CRISPR-Cas9 based techniques.^[27]

RAD51 is commonly overexpressed in various types of human cancer cells relative to normal cells.^[28] High levels of RAD51 expression in cancer cells promote DNA repair and resistance to DNA-damaging chemotherapy and radiotherapy. Paradoxically, however, these high levels of RAD51 in cancer cells can lead to genome instability and cell death due to the accumulation of toxic complexes of RAD51 with undamaged chromatin.^[29] Under these circumstances, the RAD54L and RAD54B chromatin remodeling factors are especially important for cellular survival, as these key proteins promote removal of RAD51 from dsDNA. Malignant cells must therefore maintain a delicate balance, whereby RAD51 activity is elevated enough to drive malignant growth while simultaneously using RAD51-antagonizing factors to cope with RAD51 binding to undamaged dsDNA. We believe that this malignant state presents a unique opportunity to target cancer cells, using agents that disrupt this complex balance that permits tolerance of RAD51 overexpression (Figure 1).

Consistent with this hypothesis, we found that RS-1 promotes the accumulation of genotoxic RAD51 complexes on undamaged chromatin in tumor-derived cells but not in normal non-immortalized cells. Furthermore, we observed that RS-1 is especially toxic in malignant cells that express low levels of the RAD54L and B translocase proteins, and in cells with especially high levels of RAD51 overexpression. Finally, RS-1 possesses single-agent antitumor activity in a mouse xenograft model, while being well-tolerated by mice at doses up to 110 mg kg⁻¹.^[30]

Compounds that inhibit RAD51 D-loop formation

In addition to its role in DSB repair, RAD51 also plays a key role in stabilizing stalled replication forks.^[10,31] Thus, generalized inhibitors of RAD51–ssDNA binding are expected to interfere with both roles. For this reason we and others have proposed a second class of RAD51 inhibitors, which prevent RAD51 from carrying out homologous strand exchange while preserving its ability to bind ssDNA. Such inhibitors are predicted to sensitize malignant cells to DNA-damaging treatments, while sparing its function in replication. This property is predicted to decrease normal tissue toxicity in highly proliferative normal compartments like bone marrow.

In 1992, Lee et al. reported antitumor effects using fractionated extracts from *Xestospongia*. This demonstrated that several sponge-derived polyketide compounds and their synthetic analogues can inhibit the activity of the proto-oncogene c-Src tyrosine kinase at low

micromolar concentrations.^[32] Among the most potent of these inhibitors was halenaquinone (**8**), which inhibits c-Src at 1.5 μM and preferentially decreases the proliferation of immortalized cell lines bearing the v-src oncogene. Like RI-1, halenaquinone is predicted to be a strong Michael acceptor, as its IC_{50} value increases several-fold if thiol-containing reducing agents are added to the reaction mix. Halenaquinone has since been shown to also act as a phosphatidylinositol 3-kinase inhibitor,^[33] and more recently as a RAD51 inhibitor.^[34] Halenaquinone binds directly to RAD51, thereby inhibiting its activity in the D-loop assay and a dsDNA capture assay (concentration range of 30–40 μM). However, halenaquinone does not produce any inhibition of ssDNA binding by RAD51, even at concentrations up to 60 μM . Furthermore, while 10 μM halenaquinone fully permits the formation of RAD51–ssDNA complexes on 50-nt ssDNA, the ability of these RAD51–ssDNA complexes to form a ternary complex with supercoiled dsDNA is greatly diminished. As such, halenaquinone appears to inhibit RAD51's D-loop activity by rendering the pre-synaptic RAD51–ssDNA filament catalytically inactive. Curiously, halenaquinone prevents the appearance of visible IR-induced RAD1 complexes in cytological assays. The authors propose that halenaquinone blocks the ability of RAD51 nucleoprotein filaments to form ternary complexes, and that this results in a faster turnover of RAD51–ssDNA complexes at sites of damage (i.e., absent RAD51 foci). An alternative possibility, however, is that halenaquinone simply prevents RAD51 nucleoprotein filament formation altogether in cells.

To develop compounds that specifically block the D-loop activity of RAD51 without affecting its ssDNA binding activity, we designed two parallel screens. The first component of the screen made use of our previous fluorescence polarization-based assay for detecting RAD51–ssDNA binding,^[26] and the second screen used a newly described assay that measures RAD51-dependent D-loop formation in high throughput.^[35] This parallel screen was applied to a 6800-compound ASDI library of drug-like compounds and the 1280-compound LOPAC library of existing drugs. The lead compound from this screen, **9** (Figure 2), effectively inhibits the ability of RAD51 to form D-loops ($\text{IC}_{50} = 21.3 \mu\text{M}$) with minimal effects on RAD51–ssDNA filament formation (only 33 % inhibition at 100 μM).^[36] Furthermore, compound **9** inhibits cellular HR as measured by a chromosomal reporter assay using the DR-GFP construct (50 % inhibition at 13.1 μM).^[37]

This screening hit (compound **9**) is commercially available and originally reported to represent 4-(7-methoxy-4,5-dihydropyrrolo[1,2-*a*]-quinoxalin-4-yl)-*N,N*-dimethylaniline (structure shown as “reduced form” in Figure 3). To confirm its structure and activity, we synthesized a pure sample of the reduced form. Surprisingly, the newly synthesized compound showed a much lower D-loop inhibitory activity than the original compound from the screening library, indicating that they are different substances. We also observed that the reduced form was unstable and readily oxidized to **9** in solutions. Therefore, a pure sample of **9** was synthesized and it displayed similar D-loop inhibitory activity ($\text{IC}_{50} = 13.0 \mu\text{M}$). The NMR and mass spectra of newly synthesized compound **9** were also consistent with that of the compound from the screening library. These results strongly indicate that **9** is the active constituent in the screening library compound that inhibits RAD51's D-loop activity.

Therefore, we have named compound **9** as “RI(dl)-1”, for D-loop activity of RAD51 inhibitor number 1.

Subsequently, a SAR study was performed aimed to optimize the potency of **9**. Our SAR study was focused on incorporating different substituents to rings A and B of **9** (Figure 3). Our efforts led to the discovery of a series of analogues with improved D-loop inhibitory activity, and the best compound **10** (named RI(dl)-2) displayed improved D-loop inhibitory activity (up to 91 % inhibition of total D-loop activity at 100 μ M, IC_{50} = 11.1 μ M) without any detectable effects on RAD51–ssDNA binding. Compound **10** also potently inhibits HR in the cellular DR-GFP assay with an IC_{50} of 3.0 μ M, which is over four-fold lower than the lead compound **9**. As expected for an inhibitor of HR compound **10** sensitizes multiple tumor-derived cell lines to IR.^[36] We also found that compound **10** can inhibit single-strand annealing (SSA), an alternative method of DSB repair that is used by cells when HR cannot be completed.^[38] In contrast to halenaquinone, compound **10** permits the timely assembly of RAD51 into cytologically visible sub-nuclear foci in response to IR-induced DNA damage. While **10** and halenaquinone display similar properties with respect to their effects on RAD51's biochemical activities, the different cytological observations suggest that they affect different cellular processes. It is possible that RAD51 is assembled onto damaged DNA in halenaquinone-treated cells and that these RAD51-DNA complexes are too small or too rapidly disassembled to be observed cytologically. Alternatively, the absence of cytologically visible IR-induced RAD51 foci in cells treated with halenaquinone could be due to off-target effects, as halenaquinone is a known inhibitor of other key proteins at low micromolar concentrations.^[32,33,39]

Ultimately, our long-term goal for a D-loop inhibitors is to block HR while preserving the protective function of RAD51 at stalled replication forks. Therefore, it will be interesting to see whether these compounds are able to inhibit HR (e.g., DR-GFP assays) while protecting nascent DNA from Mre11-dependent degradation under conditions of replication stress (e.g., DNA fiber assays).

Summary and Perspectives

Overexpression of the RAD51 recombinase protein is a common feature of malignant cells. Numerous reports suggest that malignant cells are especially dependent on these elevated RAD51 levels for survival and growth.^[13a,b,28] RAD51 likely plays two distinct physiological roles, including HR-mediated repair of DSBs and the promotion of replication fork stability during replication stress. Upregulation of RAD51-mediated repair in cancer cells can promote tumor resistance to DSB-inducing oncologic therapies. Likewise, upregulation of RAD51's replication fork stabilization activity likely enables more rapid tumor cell growth and tolerance of replication-blocking chemotherapeutics.^[31]

Several small molecules have been developed to modulate the biological activity of RAD51. A majority of those compounds act by preventing the formation of RAD51–ssDNA nucleoprotein filaments, either by disrupting RAD51–ssDNA binding or inhibiting RAD51 polymerization. At least some of these compounds have been shown to sensitize tumors in

animal models to radiotherapy and certain DNA damaging chemotherapy agents, however, none have yet progressed to testing in clinical trials.

RS-1 represents a novel strategy for modulating RAD51 by stimulating RAD51–ssDNA binding and promoting the formation of RAD51–ssDNA nucleoprotein filament. This strategy is based on the observation that tumor cells typically express a higher level of RAD51 than normal cells, which makes them vulnerable to the formation of genotoxic RAD51 protein complexes on undamaged chromatin. A co-crystal structure of RS-1–RAD51 complex may improve our understanding of its mechanism of action and also guide further structure-based optimization of this series of compounds. We are also actively developing other compounds of this class, based on a newer unpublished screening campaign.

The third series compounds include the RAD51 D-loop inhibitors, which block RAD51's ability to perform homologous strand exchange while sparing its ability to bind ssDNA. These compounds behave like other RAD51 inhibitors that block ssDNA binding with respect to inhibition of cellular HR and IR sensitization, but they do not prevent RAD51 from loading onto DNA at sites of IR-induced damage. As such, they are predicted to permit RAD51's role in replication stress tolerance. RI(dl)-2 represents one of the best optimized RAD51 D-loop inhibitors to date. Further research efforts on RI(dl)-2 and third-generation analogues are underway to evaluate their ability to effectively treat tumors in animal models.

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Abbreviations

HR	homologous recombination
DSBs	double-strand breaks
ICLs	interstrand cross-links
IR	ionizing radiation
ssDNA	single-strand DNA
dsDNA	double-strand DNA
SPR	surface plasmon resonance
SAR	structure-activity relationship

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Figure 1. Schematic representation of the process of DNA double-strand break repair by homologous recombination (left), and summary of therapeutic approaches by targeting various roles of RAD51 (right).

Figure 2.

Chemical structures are shown for compounds that specifically modulate RAD51–ssDNA binding (**1–7**) or that block RAD51 D-loop formation (**8, 9**).

Figure 3.
Development of RI(dl)-1 and RI(dl)-2 as RAD51 D-loop formation inhibitors.