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An Optimized RAD51 Inhibitor That Disrupts Homologous Recombination without Requiring Michael Acceptor Reactivity

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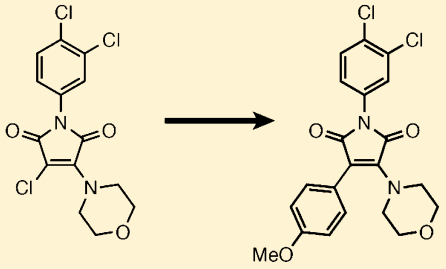
Supporting Information

ABSTRACT: Homologous recombination (HR) is an essential process in cells that provides repair of DNA double-strand breaks and lesions that block DNA replication. RAD51 is an evolutionarily conserved protein that is central to HR. Overexpression of RAD51 protein is common in cancer cells and represents a potential therapeutic target in oncology. We previously described a chemical inhibitor of RAD51, called RI-1 (referred to as compound **1** in this report). The chloromaleimide group of this compound is thought to act as a Michael acceptor and react with the thiol group on C319 of RAD51, using a conjugate addition−elimination mechanism. In order to reduce the likelihood of off-target effects and to improve compound stability in biological systems, we developed an analogue of compound **1** that lacks maleimide-based reactivity but retains RAD51 inhibitory activity. This compound, 1-(3,4-dichlorophenyl)-3-(4-methoxyphenyl)-4-morpholino-1*H*-pyrrole-2,5-dione, named RI-2 (referred to as compound **7a** in this report), appears to bind reversibly to the same site on the RAD51 protein as does compound **1**. Like compound **1**, compound **7a** specifically inhibits HR repair in human cells.



INTRODUCTION

Homologous recombination (HR) is an essential process in eukaryotic cells that provides repair of DNA double-strand breaks (DSBs) and lesions that block DNA replication. Faithful DSB repair by HR utilizes an undamaged sister chromatid as a template to guide the repair process. This distinguishes HR from the error-prone nonhomologous end joining (NHEJ) DNA repair pathway.[1] Since HR facilitates cellular recovery from replication-blocking lesions and collapsed replication forks, cells with impaired HR capacity are known to exhibit profound sensitivities to a class of chemotherapeutic drugs that generate interstrand DNA cross-links (ICLs).[2−4]

RAD51 is an evolutionarily conserved protein that is central to HR. The early steps involve 5′ to 3′ nuclease activity that generates a 3′ single-stranded DNA (ssDNA) tail at the site of damaged DNA, which becomes coated with replication protein A (RPA). RPA is subsequently replaced by RAD51 protein, wherein protomers of RAD51 oligomerize into a helical nucleoprotein filament at the site of damaged DNA. This RAD51 filament assembly utilizes conserved structural motifs to govern interactions between adjacent protein protomers.[5,6] The nucleoprotein filament subsequently searches for a homologous DNA sequence and invades it to form a joint molecule. With the assistance of other related HR proteins, accurate DNA synthesis is then performed using the undamaged sequence as a template.

Overexpression of RAD51 protein in cells has been shown to elevate HR efficiency and to generate resistance to ICL-forming drugs.[7−10] This has important implications for oncology research, since RAD51 is overexpressed in a wide range of human cancer cell types.[11,12] On the basis of these observations, we and others have suggested that malignant cells may develop "addiction" to abnormally high RAD51 levels.[13,14] Several groups have pursued the development of inhibitory small molecules[14−18] or BRCA2-derived peptides[19,20] that block RAD51 activities by mimicking the conserved structural motifs that govern interactions between adjacent RAD51 protomers.

Our drug development program has focused on RAD51 as an oncologic therapeutic target. This strategy was fueled by reports showing that HR inhibition can promote preferential sensitization of tumor cells relative to normal nontransformed cells,[21,22] suggesting that RAD51 inhibition may improve the therapeutic ratio of DNA-damaging chemotherapies and/or radiotherapy. A naive library of 10,000 small molecules was screened in search of compounds that modify the binding of RAD51 protein to ssDNA.[23] We previously reported on a RAD51-inhibitory compound referred to in this report as compound **1** (RI-1), which inactivates RAD51 by directly binding to a protein surface that serves as an interface between protein subunits in RAD51 filaments.[14] **1** was shown to

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specifically inhibit HR efficiency and to sensitize human cancer cells to the ICL-generating drug mitomycin C (MMC).

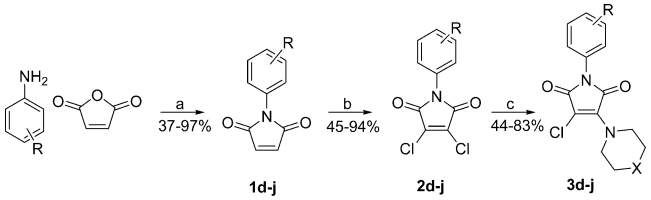
1 irreversibly binds to the C319 residue of human RAD51.[14] Specifically, the chloromaleimide group of **1** is thought to act as a Michael acceptor and react with the thiol group on C319, following a previously described conjugate addition−elimination mechanism.[24] The covalent binding of **1** facilitated mapping of the binding pocket within known crystal structures of RAD51. However, the reactivity of **1** may potentially limit its development in preclinical animal models. Here we report that **1** exhibits a relatively short half-life due to reactivity in thiol-containing solutions. To overcome this problem, we generated chemical analogues that maintain binding to RAD51 but lack the reactivity that promotes irreversible binding. This report describes the structure−activity relationship (SAR) optimization that yielded one such analogue of **1** called **7a** (RI-2).

CHEMISTRY

The inherent reactivity of our lead compound, **1**, could present a potential problem for future therapeutic applications as compounds containing Michael acceptors are often toxic. Therefore, in order to improve the drug-like characteristics of our lead compound, we undertook the investigation of the SAR surrounding the dichlorophenyl portion of the molecule (ring 1) as well as the substituents attached to the 3- and 4-positions of the maleimide core. To accomplish this, we used three separate synthetic routes. The first allowed for ready access to compounds containing modifications to ring 1, and the other two allowed for the facile synthesis of compounds containing modifications to the maleimide core.

Compounds containing ring 1 modifications were either purchased from the Chembridge Corporation (**3a−c**) or prepared from maleic anhydride and commercially available substituted anilines according to Scheme 1 (**3d−i**).[25] To form

Scheme 1. Synthesis of Derivatives with Modifications to Ring 1[a]

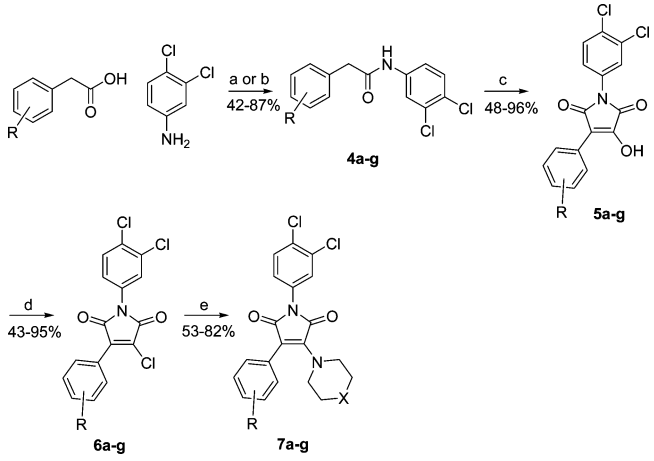


[a]Reagents and conditions: (a) (i) Et_2O, rt, 1.5 h; (ii) NaOAc, acetic anhydride, rt → 100 °C, 30 min; (b) $SOCl_2$, pyridine, 0 °C → reflux, 1.25 h; (c) morpholine or *N*-methylpiperazine, DCM, rt, 1.5 h.

the maleimide intermediates, maleic anhydride was treated with the appropriate substituted aniline in diethyl ether to yield the corresponding *N*-phenylmaleanic acids. These *N*-phenylmaleanic acids were then treated with acetic anhydride in the presence of sodium acetate to close the ring and yield maleimides **1d−i**. Conversion of these obtained maleimides to the respective dichloromaleimide intermediates **2d−i** was achieved through treatment with thionyl chloride and pyridine.[26] Lastly, the penultimate dichloromaleimides were converted to the final morpholino derivatives **3d−i** by treatment with the appropriate amine in dichloromethane.[27]

The second series comprising compounds with substitutions to the morpholino group on **1** were prepared from commercially available phenylacetic acids and substituted anilines according to Scheme 2. Standard coupling conditions were employed using either oxalyl chloride or EDCI to form

Scheme 2. Synthesis of Derivatives with Varying Substitutions at the 3- and 4-Positions of the Maleimide Core[a]

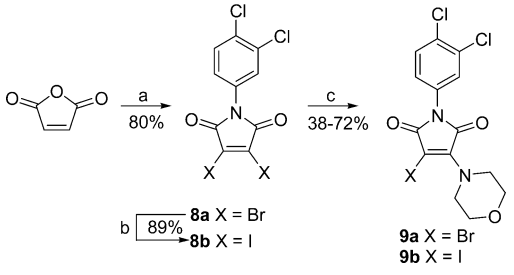


[a]Reagents and conditions: (a) oxalyl chloride, Et_3N, THF/DMF, rt → 80 °C, 2 h; (b) EDCI, DMAP, DCM, rt, 16 h; (c) diethyl oxalate, KO^{t}Bu, THF, 0 °C, 2 h; (d) oxalyl chloride, DCM/DMF, rt, 10 min; (e) morpholine, DMF, microwave, 150 °C, 10 min.

the acetamide intermediates **4a−g** from the appropriate aniline and substituted phenylacetic acid starting materials. These acetamide intermediates were then subjected to a condensation reaction with diethyl oxalate in the presence of potassium *tert*-butoxide to yield intermediate alcohols **5a−g**. Treatment of these alcohols with oxalyl chloride in the presence of a catalytic amount of DMF afforded chloro-substituted maleimides **6a−g**.[28] In addition, compound **3j** was synthesized from 3,4-dichloroaniline according to Scheme 1, substituting *N*-methylpiperazine for morpholine.

The third series consisted of compounds containing substitutions of the chlorine atom on the maleimide core. Compounds **6a−g** obtained from the penultimate step of Scheme 2 were treated with morpholine and subjected to microwave irradiation to yield maleimides **7a−g** (Scheme 2).[29] Also, to further evaluate the necessity of the Michael acceptor for activity, compounds with more reactive Michael acceptors were also synthesized according to Scheme 3. Treatment of maleic anhydride with bromine in the presence of

Scheme 3. Synthesis of Bromo- and Iodo-Substituted Derivatives[a]



[a]Reagents and conditions: (a) (i) $AlCl_3$, Br_2, 120 °C, 16 h; (ii) 3,4-dichloroaniline, acetic acid, 125 °C, 3 h; (b) NaI, acetic acid, 120 °C, 2 h; (c) morpholine, DCM, rt, 1.5 h.

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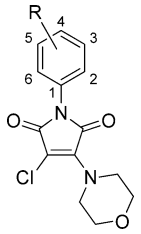
Table 1. Analogues with Substitutions on Ring 1[a]



analogue	R	DNA binding IC_{50} (μM)	LD_{50} (μM)	sensitization
1	3,4-diCl	6.82 ± 0.81	16.62 ± 1.10	+
3a	nonhalogenated	17.85 ± 1.17	40.21 ± 4.04	+
3b	4-Cl	38.17 ± 2.87	>80	N/A
3c	2-Cl	19.70 ± 0.99	>80	N/A
3d	2,3-diCl	14.88 ± 1.06	58.35 ± 5.31	+
3e	3,4-diF	13.16 ± 0.88	19.83 ± 0.79	+
3f	3-Cl, 4-F	8.32 ± 0.52	18.81 ± 0.34	+
3g	3-F, 4-Cl	7.91 ± 0.50	19.74 ± 1.93	−
3h	3-Br, 4-Cl	9.94 ± 2.37	11.65 ± 1.52	+
3i	3-Cl, 4-Br	3.10 ± 0.28	16.32 ± 2.21	+

[a]The effect of compounds on RAD51 binding to ssDNA was measured using the FP-based assay. The LD_{50} of each compound and its ability to sensitize cells to MMC were measured using the cell toxicity assays. Reporting of sensitization as "+" vs "−" is defined in the Experimental Section. N/A = compound not tested for sensitization because LD_{50} exceeded 80 μM.

aluminum(III) chloride resulted in the formation of 2,3-dibromomaleic anhydride which was subsequently reacted with 3,4-dichloroaniline in glacial acetic acid to yield the intermediate dibromomaleimide derivative **8a**. Treatment of this derivative with sodium iodide in glacial acetic acid resulted in the formation of the diiodo intermediate **8b**.[30] Lastly, **8a**,**b** were treated separately with morpholine as previously described to yield the respective halosubstituted maleimide final products **9a** and **9b**.[29]

RESULTS AND DISCUSSION

SAR for Compound 1 Derivatives. The newly synthesized compounds were tested for biological activity in a two-stage manner. They were first evaluated for their ability to inhibit the binding of human RAD51 protein to ssDNA. This primary assay utilizes a fluorescence polarization (FP) based method that involves incubation of purified RAD51 protein with oligonucleotides, which are end-labeled with a fluorescent tag. The binding of RAD51 to this substrate ssDNA is quantified as a function of polarization of light emitted by the fluorescent tag. Chemical analogues were then tested further using secondary cell-based assays. Since cell lethality from **1** is thought to be a function of cellular RAD51 inhibition, LD_{50} values were used as a surrogate measurement of in vivo activity against RAD51 in cells. This cell-based assay was also simultaneously utilized to evaluate for compound-induced sensitization of cells to the cross-linking chemotherapeutic drug MMC, thereby confirming that toxic effects are secondary to HR inhibition.

We investigated modifications to ring 1 in an effort to improve the activity of **1** (Table 1). **1** analogues that were nonhalogenated (**3a**) or monohalogenated (**3b**, **3c**) at ring 1 were considerably less active than **1** in both biochemical and cell-based assays. The 2,3-dichloroaniline derivative **3d** was also less active than **1**; therefore, we limited further changes to substitutions at the 3 and 4 positions of ring 1 with other halogens. Substitution of one or both ring 1 chlorine atoms with fluorine (**3e**, **3f**, **3g**) resulted in compounds that were less potent than **1**. Conversely, replacement of either chlorine atom with bromine (**3h**, **3i**) resulted in compounds that were slightly more active than **1** and still able to sensitize cells to MMC. Thus, incorporation of a bulkier halogen at either the 3- or 4-position of ring 1 appears to increase RAD51 inhibitory activity.

Next, we examined substitutions of the *N*-morpholino group at position 4 of the maleimide core of **1** with other cyclic groups (Table 2). We observed that compounds containing strong electron-donating groups attached to the phenyl ring (**6a**, **6b**, **6c**) and the *N*-methylpiperazine substituted compound (**3j**) exhibited comparable inhibition of RAD51 in biochemical assays and modestly increased effects in cell-based assays. However, compounds with weaker electron-donating groups (**6d** and **6e**) or strong electron-withdrawing groups (**6g**), or without any substituents (**6f**) attached to the phenyl ring did not sensitize cells to MMC, even though they were the most potent inhibitors of RAD51 in the DNA binding assay. This is likely due to activation of the Michael acceptor by electron-withdrawing groups. We surmise that mild to moderate activation of the Michael acceptor can enhance irreversible binding of chemical analogues to RAD51 in purified biochemical systems but that these activated analogues are more likely to react nonspecifically with cell culture media components and/or off-target proteins in cells.

Finally, we examined substitutions at position 3 of **1** with substituents that were predicted to be either more or less efficient leaving groups compared to chlorine (Table 3), in terms of the previously described conjugate addition−elimination mechanism. Bromine- and iodine-substituted compounds (**9a** and **9b**) had greatly increased biochemical activity relative to **1** but showed weakened activity in cell-based systems. This is consistent with what was previously observed for the compounds reported in Table 2 that had activated Michael acceptors. All compounds in which the chlorine leaving group was replaced with an aromatic group (**7a**−**g**) showed reduced or no activity in biochemical and cell-based assays. Although **7a** did exhibit reduced activity relative to **1**, it did retain enough activity to sensitize cells to MMC cell-based

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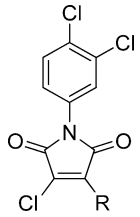
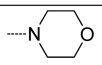
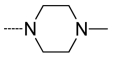
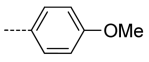
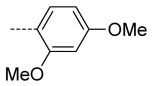
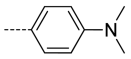
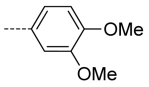
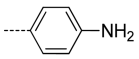
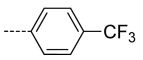
Table 2. Analogues with Substitutions to the Mopholino Group[a]



Analog	R	DNA Binding IC_{50} (μM)	LD_{50} (μM)	Sensitization
1		6.82 ± 0.81	16.62 ± 1.10	+
3j		6.43 ± 0.98	14.58 ± 0.92	+
6a		5.29 ± 1.39	10.07 ± 2.36	-
6b		*	5.63 ± 0.92	-
6c		15.62 ± 2.30	6.62 ± 1.62	-
6d		2.14 ± 0.62	11.16 ± 2.85	-
6e		1.40 ± 0.20	2.18 ± 0.21	-
6f		0.93 ± 0.14	11.30 ± 2.71	-
6g		0.81 ± 0.02	28.29 ± 3.07	-

[a]The effect of compounds on RAD51 binding to ssDNA was measured using the FP-based assay. The LD_{50} of each compound and its ability to sensitize cells to MMC were measured using the cell toxicity assays. Reporting of sensitization as "+" vs "−" is defined in the Experimental Section. Compounds that could not be assayed by the FP method because of compound fluorescence are indicated with an asterisk.

assays. This was very encouraging, as we were able to achieve inhibitory activity with a compound that lacked the chloromaleimide core.

Reactivity of Compounds with Glutathione. Compound **1** contains a chloromaleimide core that functions as a Michael acceptor. One central goal of the SAR strategies was to develop active RAD51 inhibitors that are less susceptible to nucleophilic attack and are hence predicted to be more stable in biological systems. To quantify the potential for compounds to participate in such a reaction, several analogues were incubated at 37 °C in DMSO containing a 5-fold molar excess of glutathione, which is an ideal substrate for this conjugate addition−elimination mechanism. As expected, **1** reacted rapidly with glutathione with a half-life of approximately 2 h (Figure 1). Substitution of the chlorine on the maleimide ring with bromine or iodine (Table 3) was predicted to generate groups that act as more efficient leaving groups. One such analogue **9b** reacted very rapidly with glutathione, with no remaining substrate detectable at the earliest time points measured. Conversely, substitution of the chlorine on the maleimide ring with constituents that were less efficient leaving groups generated compounds with reduced reactivity. As shown in Figure 1, **7a** exhibited no reactivity toward glutathione after incubation for 24 h.

Compound 7a Is a Reversible Inhibitor of RAD51, and It Competes with 1 for Binding to RAD51. **1** has a high affinity toward the sulfhydryl side chains of cysteine residues. However, we previously demonstrated with mass spectrometry that it reacts preferentially with Cys319 on RAD51, despite the presence of four other cysteine residues on RAD51.[14] We hypothesize that this specificity was due to the ability of **1** to associate reversibly with a pocket on the protein surface via noncovalent interactions and that the proximity of **1** to Cys319 promotes a subsequent covalent attachment to this residue. Therefore, we sought a compound that exhibited little or no Michael acceptor reactivity but still maintained potency through noncovalent interactions. **7a** was selected as an appropriate candidate to satisfy these criteria. Physiochemical properties of this compound include a molecular weight of 433.28 g/mol and a CLogP of 4.25. It contains 6 H-bond acceptors and 0 H-bond donors. Therefore, **7a** satisfies four "Lipinski rules" for predicting druglikeness.[31]

We measured the reversibility of **7a** binding to RAD51 using several assays which are described schematically in Figure 2A.

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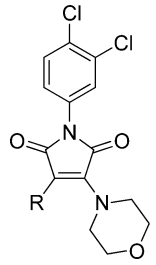
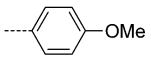
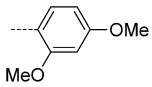
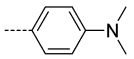
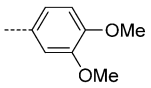
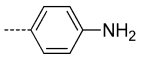
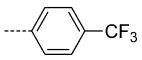
Table 3. Analogues with Substitutions of Chlorine on the Maleimide Core[a]



Analog	R	DNA Binding IC_{50} (μM)	LD_{50} (μM)	Sensitization
1	Cl	6.82 ± 0.81	16.62 ± 1.10	+
9a	Br	0.37 ± 0.04	45.88 ± 1.46	+
9b	I	0.25 ± 0.02	66.21 ± 3.33	-
7a		44.17 ± 6.75	70.16 ± 3.96	+
7b		*	>80	N/A
7c		>50	>80	N/A
7d		*	>80	N/A
7e		>50	>80	N/A
7f		*	>80	N/A
7g		*	>80	N/A

[a]The effect of compounds on RAD51 binding to ssDNA was measured using the FP-based assay. The LD_{50} of each compound and its ability to sensitize cells to MMC was measured using the cell toxicity assays. Reporting of sensitization as "+" vs "−" is defined in the Experimental Section. N/A = compound not tested for sensitization because LD_{50} exceeded 80 μM. Compounds that could not be assayed by the FP method because of compound fluorescence are indicated with an asterisk.

First, the inhibitory activity of **1** on the DNA binding activity of RAD51 was found to be time-dependent (Figure 2B), consistent with the requirement for a chemical reaction to achieve inhibitory activity. This time dependence was also observed with other **1** analogues that had intact Michael acceptor groups (data not shown). In contrast, we did not observe any time-dependent increase in the inhibitory activity induced by **7a** (Figure 2B). Second, we immobilized biotinylated RAD51 on streptavidin polyacrylamide beads. These RAD51-coated beads were incubated with compound and subsequently washed extensively to remove any compound that was not covalently attached. The treated protein was subsequently cleaved from the beads and tested for DNA binding activity using our standard FP-based assay. As expected, RAD51 protein that had been treated with 10 μM **1** prior to washing was significantly inactivated, consistent with **1**'s established role as an irreversible inhibitor (Figure 2C). By contrast, RAD51 protein that had been treated with **7a** (concentrations to 240 μM) prior to washing exhibited no measurable loss of DNA binding activity, even though **7a** is known to inhibit RAD51 with an IC_{50} of 44.17 μM in the standard DNA binding assay (Table 3). This indicates that RAD51 inhibition by **7a** is fully reversible. To determine if **7a** binds to the same site on RAD51 as does **1**, this incubation/wash assay was repeated such that both compounds were allowed to incubate with RAD51 simultaneously before washing beads. In this protocol, **7a** protected RAD51 from inhibition by **1** in a concentration-dependent manner, indicating that **7a** competes with **1** for binding to the same site on RAD51.

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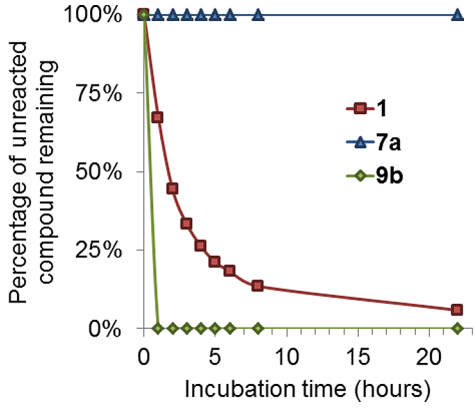

Figure 1. Reactivity with glutathione is influenced by modifications to the chloromaleimide core that modulate the efficiency of the Michael acceptor. Compounds (10 mg) and L-glutathione (5 mol equiv) were allowed to react at 37 °C in 1 mL of DMSO. Aliquots of the reaction mixture were analyzed at different time points by HPLC to quantify the amount of starting material that remained.

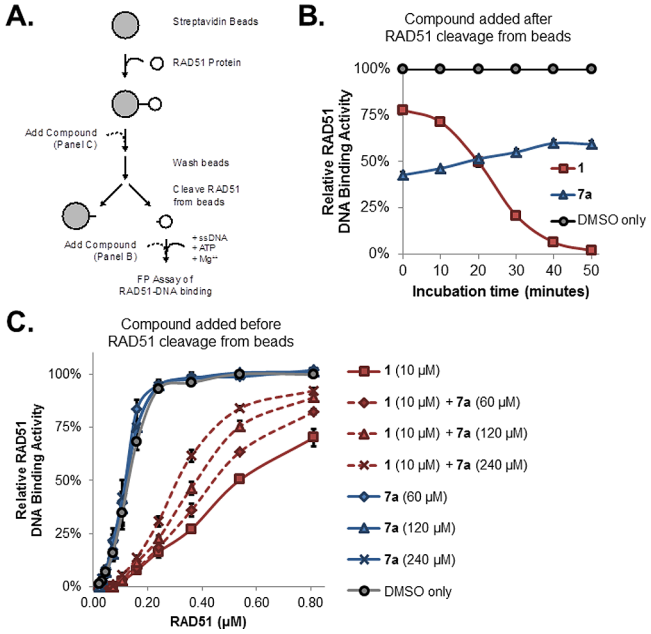

Figure 2. Compound **7a** inhibits RAD51 in a manner that does not require Michael reactivity, and it competes for the same binding site on RAD51 as **1**. (A) Schematic representation of the FP experiments. DNA binding activity was measured using the FP technique described in the Experimental Section. Reversible vs irreversible inhibition of RAD51 by compound was tested by incubating compounds with RAD51 while still attached to beads and prior to washing. Alternatively, inhibition of RAD51 by compound can be tested by adding the compound after the protein has been released from the beads. (B) Compounds (both at 59 μM) were incubated separately with 0.23 μM RAD51. Relative RAD51 DNA binding activity was normalized at each time point to DNA binding activity obtained from 0.23 μM RAD51 in the DMSO vehicle alone. (C) While immobilized on beads, RAD51 protein was treated with indicated compounds or DMSO vehicle. RAD51 was subsequently washed, released from beads, and assayed for DNA binding activity. Reported values were normalized to the FP measurements observed with RAD51 at 0.8 μM in the presence of DMSO vehicle alone. Error bars indicate standard deviations.

Compound 7a Inhibits HR DNA Repair and Sensitizes Cells to Cross-Linking Chemotherapy. To demonstrate that **7a** is capable of gaining intracellular access and specifically inhibiting RAD51 in cells, the compound was tested in human cells that contain integrated reporter constructs that quantify the efficiency of specific DNA repair pathways. The reporter construct that measures HR repair consists of two nonfunctional copies of green fluorescence protein (GFP), one of which is interrupted by an I-SceI endonuclease site. Induction of a DNA break at the I-SceI site can lead to repair by homologous gene conversion that generates a functional copy of GFP.[32] A second parallel assay makes use of cells that contain a different reporter that measures single-strand annealing (SSA), which is an alternative method of religating DNA when HR cannot be completed. SSA efficiency is known to be elevated in situations where RAD51 function has been disrupted.[33−35] Consistent with our prior report,[14] incubation of cells with 20 μM **1** led to an inhibition of HR and a stimulation of SSA (Figure 3). **7a** at

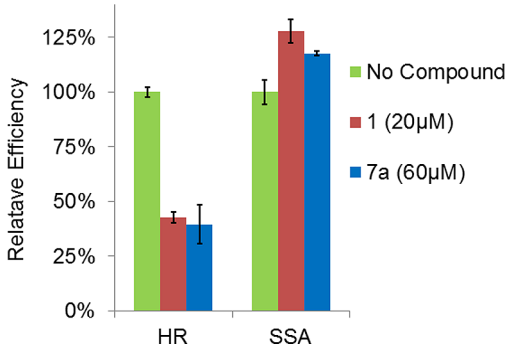

Figure 3. Compound **7a** modulates the efficiency of DNA repair in human cells, containing either an HR or SSA reporter. Reporter-containing HEK293 cells were electroporated with an I-SceI endonuclease-expressing plasmid, incubated with compounds as indicated for 24 h, and subjected to FACS analysis 24 h thereafter. Relative repair efficiencies were measured as described in the Experimental Section.

60 μM was capable of generating very similar effects. This suggests that **7a** can indeed generate specific inhibition of RAD51 in cells, even though this compound lacks the Michael acceptor reactivity of **1**.

The long-term goal of this project is to develop an anticancer agent that is capable of sensitizing tumor cells to cross-linking chemotherapeutic drugs. We previously reported that **1** can sensitize a broad range of immortalized human cells to MMC, which is a chemotherapeutic drug that induces ICLs.[14] To test for this activity with **7a**, HEK293 cells were treated sequentially for 24 h with MMC followed by 24 h with a RAD51 inhibitory compound. Like **1**, **7a** induced a significant sensitization of cells (Figure 4). These data suggest that **7a** can specifically interfere with RAD51 functions in human cancer cells, even though it lacks the Michael acceptor reactivity of **1**.

CONCLUSION

Compound **1** was previously identified as an attractive candidate for drug development because it specifically inhibits RAD51 within tumor cells.[14] However, the Michael acceptor group on **1** poses challenges that potentially limit its development in preclinical animal models. Specifically, **1** exhibits a relatively short half-life in tissue culture media and aqueous buffers containing sulfhydryl components.

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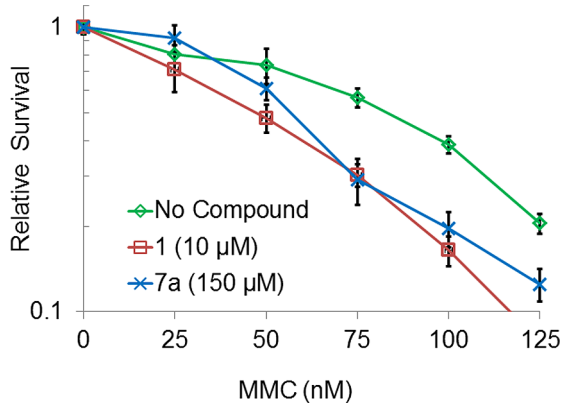

Figure 4. Compound **7a** sensitizes human cells to cross-linking chemotherapy. HEK293 cells were sequentially incubated for 24 h in medium containing varying concentrations of MMC, followed by 24 h in medium containing **1** or **7a**. Cells were then allowed to grow in drug-free medium for an additional 5−7 days. Average survival for each condition is normalized to the MMC-free control of that condition.

SAR analyses were performed using targeted analogues of the **1** scaffold, and these revealed several classes of effects. First, substitution of chlorine groups within the phenyl ring to other halogens (Table 1) affected compound function, such that bulkier halogens induced modestly improved inhibitory activity. Second, replacement of the chlorine atom on the maleimide ring with better leaving groups (e.g., **9a** or **9b**, Table 3) or substitution of the morpolino ring by groups that are stronger electron-withdrawing groups (e.g., **6a**, Table 2) resulted in elevated Michael reactivity. Finally, substitution of the chlorine atom on the maleimide ring by substituents that are unable to act as leaving groups (e.g., methoxyphenyl in **7a**, Table 3) generated compounds with reduced Michael reactivity. Further work is required to determine how these different classes of SAR effects will affect RAD51-inhibitory activity, when two or more of them are combined together into third generation analogues.

Other laboratories have reported several other compounds that inhibit RAD51 in purified biochemical systems.[15−18] Two of these compounds, halenaquinone and B02, also exhibit some activity against HR in human cells. However, halenaquinone contains Michael acceptors and was not capable of sensitizing cells to ionizing radiation, which raises doubts about its specificity to RAD51 protein.[16] B02, on the other hand, does successfully sensitize cells to cross-linking chemotherapeutic drugs; however, this activity requires a long (>10 h) preincubation of cells with compound,[18] which poses major practical challenges from a pharmacologic perspective. Furthermore, B02 is composed of a relatively flat, hydrophobic structure that raises potential concerns for nonspecific protein binding and problematic pharmacokinetics.

Even though **7a** has a higher IC_{50} than the starting **1** compound, we believe that it represents an important advance. Specifically, we have shown that **7a** is a reversible binder of RAD51. Even though it lacks the maleimide-based reactivity, **7a** retains HR-inhibitory activity in multiple assays in human cells. This suggests that **7a** will be more stable in biological systems than **1**, and thus, it represents a favorable candidate for further development. Importantly, we have shown that **7a** competes with **1** for binding to RAD51, presumably because it binds to the same pocket on the protein surface. This previously mapped binding site offers important opportunities, since computational modeling of compounds together with known RAD51 crystal structures may empower us with additional insights to guide further optimization.

EXPERIMENTAL SECTION

DNA Binding Assay. Human RAD51 protein was prepared as previously described.[14] RAD51 was incubated with 100 nM (nucleotide concentration) of a 45-mer oligo-dT 5′ Alexa 488 end-labeled substrate. Binding was measured as a function of fluorescence polarization (FP) of the Alexa 488 tag. 50 μL reactions were performed in 20 mM HEPES, pH 7.5, 2 mM ATP, 10 mM $MgCl_2$, 30 mM NaCl, 2% glycerol, 250 μM BSA, and 4% DMSO. Black polystyrene flat-bottom 384-well reaction plates were read using a Tecan Infinite F200 Pro plate reader equipped with 485(20)/535(25) FP filters. For experiments to determine IC_{50} values, RAD51 concentrations (typically 190−290 nM) were selected to generate FP signal of 50−80% maximum in the absence of any inhibitory compound. Reported IC_{50} values represent the mean result of at least three replicate wells, and reported errors connote standard deviation.

DNA Binding Competition Assays. By use of a previously described method, human RAD51 protein was prepared with an N-terminal biotin tag.[23] The tag is linked to the protein by a peptide sequence recognized by the tobacco etch virus (TEV) protease. Tagged RAD51 was immobilized on streptavidin-conjugated polyacrylamide beads (Ultralink, Pierce) and incubated for 30 min at 37 °C with candidate compounds in 20 mM HEPES, pH 7.5, 30 mM NaCl, 2% glycerol, 4% DMSO. The polyacrylamide beads were then washed twice with ice-cold wash buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl) containing 4% DMSO and then once with ice-cold wash buffer without DMSO. RAD51 protein was subsequently eluted from beads with TEV protease (gift from Phoebe Rice) and analyzed for DNA binding activity using the above FP-based DNA binding assay.

Cell Toxicity Assays. HEK293 cells were plated into 96-well tissue culture plates at a density of 300 cells per well in the presence or absence of 50 nM MMC (Ben Venue Laboratories) for 24 h at 37 °C, 5% CO_2. Medium was subsequently replaced with fresh medium containing 0.5% DMSO plus a candidate compound for an additional 24 h. Compounds were then removed, and cultures were allowed to grow to a 50−70% confluence. Average survival from at least three replicates was measured using CellGlo reagent (Promega) or via a previously described sulforhodamine B method.[23] Compounds were deemed successful in sensitizing cells to MMC if they generated significantly greater toxicity in the presence of MMC relative to the absence of MMC. Specifically, sensitization was scored as a "+" when nonoverlapping standard errors were observed for at least two pairs of compound doses.

Quantification of HR and SSA Efficiencies in Cells. HEK293 cells stably transfected with the DR-GFP or SA-GFP reporters were provided by Jeremy Stark.[35] (0.5−1.0) × 10^7 cells grown to 80% confluence were electroporated with 30−60 μg of pCβASce (or pCAGGS) in 4 mm cuvettes, using the following settings: 240−350 V, 975 μF. Cells were transferred into the appropriate complete growth medium and allowed to grow for 24 h in the presence or absence of a candidate compound. Compounds were removed, and cells were incubated for an additional 24 h in normal medium, following which they were analyzed with a Becton-Dickinson FACScan. Live cells were collected based on size/complexity and 7-aminoactinomycyin D (7-AAD) exclusion. The fraction of live cells exhibiting GFP positivity is displayed, and error bars denote standard error.

Glutathione Stability Experiments. Compound (10 mg) and L-glutathione (5 mol equiv) were placed in a round-bottomed flask under argon and dissolved in DMSO (1 mL). The mixture was heated to 37 °C and stirred. Aliquots of the reaction mixture were removed every hour, including one at t = 0 min, and analyzed by HPLC. Ratio of substrate and glutathione adduct was recorded, and the expected glutathione adduct was then identified using mass spectrometry.

General Information for Synthetic Procedures. ^1H NMR and ^{13}C NMR spectra were obtained using a Bruker spectrometer with TMS as an internal standard. The following standard abbreviations

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indicating multiplicity were used: s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. HRMS experiments were carried out using a Shimadzu IT-TOF instrument with MeCN and H_2O spiked with 0.1% formic acid as the mobile phase. Reaction progress was monitored by TLC using precoated silica gel plates (Merck silica gel 60 F254, 250 μm thickness). Automated column chromatography was performed using the CombiFlash Rf apparatus available from Teledyne ISCO and prepacked cartridges (50 g) loaded with Merck silica gel (40−60 mesh) along with the following conditions: gradient, 100% hexane, 5 min; 0−50% EtOAc/hexane, 25 min; 50% EtOAc/hexane, 5 min; flow rate = 40 mL/min with wavelength monitoring at 254 and 280 nm. Preparative HPLC was carried out using a Shimadzu preparative liquid chromatograph with the following specifications: column, ACE 5 AQ (150 mm × 21.2 mm) with 5 μm particle size; gradient, 25−100% MeOH/H_2O, 30 min; 100% MeOH, 5 min; 100−25% MeOH/H_2O, 4 min; 25% MeOH/H_2O, 1 min; flow rate = 17 mL/min with wavelength monitoring at 254 and 280 nm. Both solvents were spiked with 0.05% TFA. Resin bound bicarbonate was used to neutralize residual trifluoroacetic acid remaining from preparatory HPLC purification. Analytical HPLC was carried out using an Agilent 1100 series instrument with the following specifications: column, Luna 5 μm C18(2) 100 Å (150 mm × 4.60 mm) with 5 μm particle size; flow rate = 1.4 mL/min with wavelength monitoring at 254 nm; gradient, 10−100% MeOH/H_2O, 18 min; 100% MeOH, 3 min; 100−10% MeOH/H_2O, 3 min; 10% MeOH/H_2O, 5 min. Both solvents were spiked with 0.05% TFA. The purity of all tested compounds was ≥95%. Biotage initiator microwave system was used for synthesis.

General Procedure A. Maleic anhydride was placed in a round-bottomed flask, and to it was added anhydrous Et_2O (2 mL/mmol) at room temperature. Upon complete dissolution of the maleic anhydride, the appropriate substituted aniline (1.1 mol equiv) dissolved in anhydrous Et_2O (1 mL/mmol) was added dropwise at room temperature. The mixture was stirred for approximately 2 h, resulting in the formation of a white precipitate. The mixture was then concentrated in vacuo, cooled in an ice bath, and the *N*-substituted maleanic acid precipitate was isolated by filtration. The filter cake was then added to a round-bottomed flask charged with sodium acetate (1 mol equiv) and acetic anhydride (0.5 mL/mmol). The mixture was heated to 100 °C and stirred for 20−30 min, after which it was cooled to room temperature and poured into ice−water (50 mL). The organic products were extracted with EtOAc (3 × 15 mL), washed with water (3 × 15 mL) and brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The resulting product was then purified using automated column chromatography.

General Procedure B. The appropriate maleimide was placed in a round-bottomed flask and dissolved in thionyl chloride (1 mL/mmol) at room temperature. The mixture was cooled to 0 °C in an ice bath, and then pyridine (2.1 mol equiv) was added dropwise. The mixture was stirred at 0 °C for 15 min, after which it was removed from the ice bath and heated at reflux for 1 h. The mixture was then cooled to room temperature, and excess thionyl chloride was removed in vacuo. The resulting residue was taken up in $CHCl_3$ (30 mL), washed with 1 N HCl (3 × 10 mL) and brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The resulting 3,4-dichloromaleimide was then purified by automated column chromatography.

General Procedure C. The appropriate 3,4-dihalomaleimide was placed in a round-bottomed flask and dissolved in DCM (3 mL/mmol). To it was added either morpholine or *N*-methylpiperazine (2 mol equiv) at room temperature, and the mixture was stirred for approximately 2 h. Upon addition of the amine, the mixture turned from clear to yellow and a yellow precipitate formed. When the reaction was complete as evidenced by TLC, the mixture was poured into water (15 mL) and the organic products were extracted with DCM (3 × 15 mL). The combined organic extracts were washed with brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude product was then dissolved in DMF and purified by preparative HPLC.

General Procedure D. The appropriate phenylacetic acid was placed in a two-necked round-bottomed flask fitted with a condenser under argon. Anhydrous THF (2 mL/mmol) was added followed by three drops of anhydrous DMF, after which the mixture was cooled to 0 °C in an ice bath. Oxalyl chloride (2 mol equiv) was added dropwise to the mixture, which was then removed from the ice bath and heated to 80 °C for 15 min. Upon completion as evidenced by TLC, the mixture was cooled to room temperature and the volatiles were removed in vacuo. The freshly obtained acid chloride was then placed under argon and dissolved in anhydrous THF (2 mL/mmol). The appropriate aniline (1.2 mol equiv) dissolved in anhydrous THF (1 mL/mmol) was added followed by triethylamine (1 mol equiv) dropwise at room temperature. The mixture was then stirred at room temperature for 2 h, after which water (15 mL) was added and the organic products were extracted with EtOAc (3 × 15 mL). The combined organic fractions were washed with brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The desired amide product was purified by automated column chromatography.

General Procedure E. The appropriate phenylacetic acid, aniline (1 mol equiv), EDCI (1.2 molar equiv), and DMAP (0.1 mol equiv) were placed in a round-bottomed flask under argon and dissolved in anhydrous DCM (5 mL/mmol) at room temperature. The mixture was then stirred at room temperature overnight (approximately 16 h), after which water (15 mL) was added and the organic layer was isolated. The aqueous layer was further extracted with DCM (2 × 15 mL), and the combined organic fractions were washed with water (2 × 20 mL) and brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The desired amide product was then purified by automated column chromatography.

General Procedure F. The respective amide was placed in a round-bottomed flask under argon and dissolved in anhydrous THF (5 mL/mmol). The mixture was cooled to 0 °C with an ice bath, and then diethyl oxalate (2 mol equiv) was added after which potassium *tert*-butoxide (2.5 mol equiv) dissolved/suspended in anhydrous THF (3 mL/mmol) was added dropwise. The mixture was allowed to warm to room temperature and then stirred for 2 h. After 2 h, the mixture was poured into ice−water (50 mL) and the pH was adjusted to approximately 3 with 1 N HCl. The organic products were extracted with EtOAc (3 × 20 mL), and the combined organic extracts were washed with brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The resulting maleimide product was purified by washing with MeOH.

General Procedure G. The appropriate alcohol was dissolved in DCM (2 mL/mmol) and DMF (2 mL/mmol) at room temperature. Then oxalyl chloride (1.1 mol equiv) dissolved in DCM (2 mL/mmol) was added dropwise to the mixture at room temperature. The mixture was stirred for approximately 10 min and then poured into ice−water (30 mL). The organic products were extracted with DCM (3 × 15 mL), washed with water (3 × 10 mL) and brine (15 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The desired chloromaleimide product was purified by washing with MeOH.

General Procedure H. The appropriate chloromaleimide and either morpholine or *N*-methylpiperazine (2 mol equiv) were dissolved in anhydrous DMF (3 mL/mmol) and sealed inside a microwave reactor vessel. The mixture was then heated to 150 °C for 10 min, after which the mixture was cooled to room temperature and poured into ice−water (15 mL). The organic products were extracted with EtOAc (3 × 15 mL), washed with water (10 mL) and brine (10 mL), dried with Na_2SO_4, filtered, and concentrated in vacuo. The desired final product was then purified by preparative HPLC.

Synthetic Procedures for Individual Compounds. See details in Supporting Information.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of chemical syntheses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

HR, homologous recombination; DSB, double stranded break; NHEJ, nonhomologous end joining; ICL, interstrand DNA cross-link; ssDNA, single-stranded DNA; RPA, replication protein A; MMC, mitomycin C; RI-1, RAD51-inhibitory compound **1**; RI-2, RAD51-inhibitory compound **2**; SAR, structure−activity relationship; SSA, single-strand annealing

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