Targeted Inhibition of Replication Protein A Reveals Cytotoxic Activity, Synergy with Chemotherapeutic DNA-Damaging Agents, and Insight into Cellular Function

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Abstract

Targeting uncontrolled cell proliferation and resistance to DNA-damaging chemotherapeutics with a single agent has significant potential in cancer treatment. Replication protein A (RPA), the eukaryotic ssDNA-binding protein, is essential for genomic maintenance and stability via roles in both DNA replication and repair. We have identified a novel small molecule that inhibits the in vitro and cellular ssDNA-binding activity of RPA, prevents cell cycle progression, induces cytotoxicity, and increases the efficacy of chemotherapeutic DNA-damaging agents. These results provide new insight into the mechanism of RPA-ssDNA interactions in chromosome maintenance and stability. This represents the first molecularly targeted eukaryotic DNA-binding inhibitor and reveals the utility of targeting a protein-DNA interaction as a therapeutic strategy for cancer treatment.

Introduction

Replication protein A (RPA) is a heterotrimeric ssDNA-binding protein made up of 70-, 34-, and 14-kDa subunits (1). The ssDNA-binding activity of RPA is required for several DNA metabolic pathways, including DNA replication, recombination, and repair. High-affinity interactions with DNA are sustained by the numerous oligosaccharide/oligonucleotide-binding (OB) folds present on each of the three subunits (2, 3). OB folds in DNA-binding domains A and B (DBD-A and DBD-B) in the central region of the p70 subunit contribute most of the binding energy for RPA-ssDNA interactions (2). Individual OB folds are compact modular domains populated with hydrophobic and basic amino acids. These structural features make the OB fold an attractive target for development of small-molecule inhibitors (SMI) of DNA-binding activity.

Inhibiting RPA-DNA interactions has the potential to affect numerous DNA metabolic pathways that are differentially activated in cancer cells. In DNA replication, RPA inhibition can be used to exploit the highly proliferative nature of cancer cells, which is characterized by a large population of cells in S phase. Consistent with this, a recent clinical trial correlated disease stage and metastasis in colon cancer with increased RPA p70 and p34 expression (4). RPA is also essential for several DNA repair pathways in the cell, including nucleotide excision repair (NER). Cisplatin, a common chemotherapeutic used in the treatment of various cancers, induces its cytotoxic effect by forming intrastrand covalent DNA adducts that are repaired primarily by the NER pathway (5). Consistent with the role of NER in the repair of cisplatin-induced DNA damage, resistance to this treatment has been observed to be influenced by DNA repair capacity (6, 7). Consequently, cisplatin treatment, in conjunction with decreased RPA ssDNA-binding activity, would be expected to result in decreased efficiency of cellular repair of cisplatin-DNA adducts and increased cytotoxicity. Thus, targeting RPA has the potential not only for single-agent activity but also to sensitize cancer cells to therapies that induce DNA damage and genetic instability, such as cisplatin, etoposide, and ionizing radiation.

We present the identification and development of the first small molecule that inhibits the ssDNA-binding activity of RPA. Cellular RPA inhibition results in the inability to enter S phase, cytotoxicity, and synergistic activity with the chemotherapeutic agents cisplatin and etoposide. This is the first characterization of a small molecule that is able to inhibit the ssDNA-binding activity of RPA and presents a novel chemotherapeutic target both as a single agent and in conjunction with commonly used chemotherapeutics.

Materials and Methods

In vitro protein analysis. SMIs were obtained from ChemDiv and resuspended in DMSO. Compound 505 was independently synthesized and structure was confirmed by mass spectrometry analysis. Human RPA was purified as previously described (8). Fluorescence anisotropy–based DNA-binding assays were performed with 40 nmol/L RPA and
20 nmol/L. 5'−fluorescein−labeled ss-dT12 DNA as previously described (9). Electrophoretic mobility shift assays (EMSA) were performed in 20 μL reactions containing 25 nmol/L RPA and 25 nmol/L 5'−32P-labeled 34-bp DNA as previously described (8).

**Molecular modeling.** Molecular modeling of compound 505 with the central DBD of RPA p70 (1FGU) was performed using AutoDock 4.2 (10). Three independent grids established 60 Å in each dimension to encompass either the interdomain region, DBD-A, or DBD-B. Semiflexible automated ligand docking was performed using the Monte Carlo−based simulated annealing and locality search algorithms. The most stable complexes were selected based on binding energies from multiple analyses. Coordinates of the final docked complexes were displayed with PyMOL.

**Flow cytometry.** H460 cells were analyzed for apoptosis using an Annexin V−FITC/podiphiloid (PI) Vybrant Apoptosis Assay kit (Invitrogen) according to the manufacturer's instructions. Cells were plated at a density of 1 × 10^5/cm² for 24 h and then treated with compound 505 for 48 h. Following plating and treatment of H460 cells, adherent and nonadherent cells were collected, processed, and analyzed on a BD FACScan flow cytometer. Data were analyzed using WinMDI software (The Scripps Research Institute, San Diego, CA). Cell cycle analysis was performed by PI staining. Briefly, cells were plated and treated with compound, collected, and then washed twice with 1% bovine serum albumin (BSA) in PBS-EDTA. Cells were fixed in 70% ethanol at −20°C followed by incubation on ice for 30 min. Cells were then collected and stained with 1 μg/mL PI and 25 μg/mL RNaseA for 1.5 h and analyzed on a Becton Dickinson FACScan flow cytometer. Cells were gated and analyzed on a histogram with events plotted against the FL2-A parameter. Cell cycle distribution was analyzed using ModFit software. G2 arrest was induced by treatment with 0.8 μg/mL nocodazole for 12 h (11). Cells were then washed with PBS and treated with either vehicle or compound 505 (100 μmol/L). Cells were harvested and analyzed for cell cycle distribution as described above. To analyze bromodeoxyuridine (BrdUrd) incorporation, cells were labeled with 10 μmol/L BrdUrd, after which cells were collected, washed, and fixed in 70% ethanol. Following fixation, DNA was denatured with 2 mol/L HCl for 20 min at room temperature, washed, and neutralized with 0.5 mol/L sodium borate for 2 min. Cells were then incubated with anti-BrdUrd antibody (1:500; Calbiochem) for 20 min at room temperature, washed, and then incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:500; Invitrogen) also for 20 min at room temperature. Following antibody incubation, cells were washed and incubated in 1 μg/mL PI in PBS-EDTA for at least 30 min at 4°C. Cells were then analyzed as described above for cell cycle and BrdUrd staining using FLA and FL1 parameters, respectively.

**Indirect immunofluorescence.** H460 cells were plated on chamber slides (LabTek) as described above. Cells were then treated for 3 h with either 50 μmol/L compound 505 or vehicle as indicated, and following treatment, cells were fixed in 4% paraformaldehyde at 25°C for 3 min followed by washing in 0.2% Triton X-100 for 2 min at 4°C. The slides were then blocked in 15% fetal bovine serum (FBS) in PBS for 1 h at 25°C and then incubated with anti-RPA p34 primary antibody (1:500; NeoMarkers) in 15% FBS for 1 h. Slides were then washed thrice for 10 min with 15% FBS and then incubated with Alexa Fluor 594 goat anti-mouse secondary antibody (1:300; Invitrogen) for 1 h. Slides were again washed and stained with 300 nmol/L 4′,6-diamidino-2-phenylindole (DAPI) diluted in PBS-EDTA for 5 min. Slides were then mounted and images were captured using a Zeiss fluorescent microscope. Images were captured using filters for Texas red to visualize RPA staining and DAPI for visualizing DNA. Slides were visualized and images were analyzed and quantified using ImageJ software.

**Western blot analysis.** H460 cells were plated and treated with either vehicle or increasing concentrations of compound 505 as indicated for 8 h and then processed for Western blot analysis using a radioimmunoprecipitation assay lysis and extraction procedure. Protein concentrations were determined using the Bio-Rad protein assay with BSA standard and equal amounts of total protein were loaded in each well. Following electrophoresis and transfer to polyvinylidene difluoride membranes, RPA was detected with an anti-RPA p34 (1:2,000) or anti-p70 antibody (1:1,000; NeoMarkers) and goat anti-mouse horseradish peroxidase secondary (1:5,000 and 1:2,500, respectively; Santa Cruz Biotechnology). Bands were visualized using chemiluminescence detection.

**Results**

**Identification of a SMI of RPA.** Previous work from our laboratory has identified a series of small molecules from the National Cancer Institute library (9) and a ChemDiv library (12) that inhibit the DNA-binding activity of RPA. Hits obtained from the ChemDiv library were analyzed in a secondary assay using EMSAs to confirm inhibitory activity (Fig. 1A). Of those analyzed, two showed significant RPA inhibition in vitro (compounds 3 and 5). As in vivo inhibition of RPA using small interfering RNA (siRNA) is cytotoxic due to its critical role in DNA metabolic processes, we analyzed the effect of these two compounds on cellular viability (13). Although minimal cellular effects were observed with compounds 3 and 5, the analyses were somewhat hampered by poor solubility (data not shown). However, considering the in vitro activity of compound 3, we retained its core structure consisting of a substituted dihydropyrazole with a 4-oxobutanoic acid at N1 and a phenyl substituent at C3 to initiate analysis of structure-activity relationships (SAR) and identify compounds with improved cellular activity (Fig. 1C). Eighty-one analogues were identified and obtained from the ChemDiv library with differing substitutions off the phenyl ring (R2) and varying substituents at position C5 on the dihydropyrazole ring (R1) (Fig. 1C). These derivatives were analyzed for in vitro RPA-inhibitory activity using EMSAs (Fig. 1D; data not shown). A subset of these compounds that showed the ability to inhibit the DNA-binding activity of RPA was further characterized to determine how the structure of each contributes to this inhibition. Using EMSA analysis, titrations of each compound were performed and the IC₅₀ of...
each was determined. Among the compounds analyzed, compound 505 was the most potent inhibitor with an IC$_{50}$ of 13 μmol/L, whereas other compounds displayed varying capacities for RPA inhibition (Table 1; Fig. 2). To determine cellular activity of each of the compounds, cell viability was measured with Annexin V/PI analysis in the H460 non–small cell lung carcinoma (NSCLC) cell line and IC$_{50}$ values for each compound following a 48-hour exposure were determined. These data are presented in Table 1 and reveal a correlation between in vitro and cellular activity, consistent with cellular inhibition of RPA and indicating specificity for RPA inhibition. Interestingly, compound 523, which showed minimal inhibition of RPA in vitro, did display modest cellular activity, which could be attributed to metabolism or other cellular effects. As compound 505 displayed the lowest in vitro IC$_{50}$ and was the most potent compound of those examined in cells, we selected this compound to further investigate its in vitro and cellular mechanisms of action.

**In vitro inhibition of the DNA-binding activity of RPA targeting DBD-A and DBD-B in the 70-kDa subunit of RPA.** RPA binding to synthetic oligonucleotide substrates has been well characterized with respect to structural features and kinetics of binding (14–17). To determine the mechanism of action and the potential interactions of compound 505 with RPA, binding to oligonucleotides varying in length and sequence was examined. Compound 505 was first titrated with a constant amount of RPA in the presence of 5'-32P-labeled 34-bp purine-rich ssDNA. This length of DNA is capable of extending beyond the central OB folds of the RPA p70 subunit to allow interactions with other OB folds within the RPA heterotrimer. This analysis revealed a concentration-dependent decrease in binding with an IC$_{50}$ of 13 μmol/L (Fig. 2A). We then extended the analysis to a ssDNA 12 bases in length, which largely restricts binding to DBD-A and DBD-B, which has been shown in the cocrystal structure of RPA p70 (14). A fluorescence polarization assay was used to accommodate the dT12 substrate, and titration of compound 505 resulted in reduced RPA binding with an IC$_{50}$ value of 20.4 μmol/L (Fig. 2C). These data provide evidence for inhibition of RPA by blocking the interaction of these OB folds with DNA. In our initial screen of the ChemDiv library for RPA inhibitors, we counterscreened each compound against xeroderma pigmentosum group A protein (XPA), an essential DNA-binding protein in the NER pathway, and excluded any compound that inhibited both targets (12). To confirm this specificity for RPA, we examined the effect of 505 on XPA binding in an ELISA format, and no inhibitory activity was observed (data not shown).
**Table 1.** SARs of small-molecule RPA inhibitors

<table>
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<tr>
<th>Name</th>
<th>Structure</th>
<th>$IC_{50}$ (μmol/L)</th>
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<td>&gt;100‡</td>
<td>31.0 ± 5.2</td>
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NOTE: The *in vitro* $IC_{50}$ was determined by EMSA analysis as described in Fig. 1A. Cellular $IC_{50}$ was determined by treating H460 cells and analyzing Annexin V/PI staining as described in Materials and Methods. The *in vitro* and cellular data were analyzed using standard four-parameter logistic curve. The $IC_{50}$ values and SE of the fit were determined from this analysis.

Abbreviation: NA, not available.

*Inhibition at the highest concentration tested (100 μmol/L) was 9%.
†Maximum observed cytotoxicity was 80% of control.
‡Inhibition at the highest concentration tested (100 μmol/L) was 36%.
To further define the interaction between compound 505 and DBD-A and DBD-B, we undertook a molecular modeling approach. The crystal structure of RPA p70 181–422 was solved without DNA (18), and three-dimensional coordinates were obtained from the protein data bank (1FGU). Compound 505 was prepared as the ligand using AutoDock tools 1.5.2 as described in Materials and Methods. Three potential binding sites were identified based on the lowest interaction energies. These sites include each of the OB folds and the interdomain region between the two folds (Fig. 2D). The most stable interaction was with DBD-B, whereas DBD-A domain ranked second in binding energy, and the interdomain region showed the least stable interaction. The close-up of the interaction presented in the insets reveals that most of the stability is driven via hydrophobic interactions, although the conserved oxobutyric acid is stabilized via interactions with basic amino acids in each of the three positions. To determine the relative importance of the interaction of compound 505 with each domain, docking analysis of compound 518 was performed, which shows no inhibition of the DNA-binding activity of RPA in vitro. Significantly increased ΔG values were obtained for interactions of 518 with DBD-B and the interdomain region, whereas only modest increases were calculated for the interaction with DBD-A. These data suggest that the majority of the inhibitory effect of compound 505 is manifested through interaction with the DBD-B OB fold and potentially the interdomain region.

Compound 505 decreases cell viability and nuclear RPA staining without induction of apoptosis and degradation of RPA. Having determined the in vitro activity of compound 505, we sought to determine the mechanism of cellular activity and the ramifications of inhibiting the central p70 OB folds of RPA. To measure the induction of apoptosis, H460 NSCLC cells were treated with increasing concentrations of compound 505 for 48 hours, after which Annexin V/PI staining was measured using flow cytometry (Fig. 3). Minimal Annexin V staining was observed, suggesting that classic apoptosis was not initiated (19). Interestingly, a concentration-dependent increase in PI staining was observed, indicating a general loss of membrane integrity, suggestive of necrotic cell death (Fig. 3A; ref. 20). Quantification of the viable cells (Annexin V−/PI−) resulted in an IC50 of 30.8 μmol/L (Fig. 3B).
As an independent measure of the effect of compound 505 on cell viability, we used a crystal violet staining assay and obtained an IC50 of 64 μmol/L (data not shown). A similar result was also observed in treatment of the A549 NSCLC cell line with compound 505, whereas analysis using freshly isolated peripheral blood mononuclear cells revealed minimal cytotoxic activity (data not shown). Therefore, compound 505 has shown significant cytotoxic effects in NSCLC cell lines while showing only modest activity in noncancerous cells, supporting the possibility that a therapeutic treatment window may be achievable.

The cellular effects seen following treatment with compound 505 are consistent with an inability of RPA to interact with DNA, which could then result in numerous possibilities, including degradation or redistribution of RPA within the cell. We therefore used indirect immunofluorescence to assess how inhibition of RPA binding influences cellular localization. After 3 hours of treatment with compound, cells showed a decrease in the intensity of RPA staining compared with vehicle-treated control without a change in overall subcellular localization (Fig. 3C). Interestingly, Western blot analysis revealed that both RPA p70 and p34 levels remain constant following treatment with increasing concentrations of compound 505 (Fig. 3D). These results show that treatment with compound 505 decreases the association of RPA with DNA, whereas the overall level of RPA is not dramatically affected.

**Compound 505 affects cell cycle progression and DNA replication.** The ability of compound 505 to reduce RPA nuclear staining and cell viability led us to assess the effect of treatment on cell cycle progression. siRNA knockdown of RPA has been shown to induce a G1 cell cycle arrest, consistent with the essential role of RPA in the initiation of S-phase DNA replication (21). Therefore, we assessed the effect of compound 505 treatment on H460 cell cycle progression, and an increase in the proportion of cells in G1 phase was observed in response to treatment (Fig. 4A). To determine
Figure 4. Synchronized H460 cells show an inability to reenter S phase with compound 505. A, H460 NSCLC cells were treated with vehicle or compound 505 for 48 h and then analyzed for cell cycle distribution. B, H460 cells were treated with 0.8 μg/mL nocodazole for 12 h, washed, and then treated with either vehicle or 100 μmol/L compound 505 for 4, 8, and 12 h. BrdUrd (10 μmol/L) was added during the final 2 h of treatment. Cells were then harvested and analyzed for cell cycle distribution and BrdUrd incorporation. C, asynchronous cells were treated for 3 h with either vehicle or compound 505. BrdUrd (10 μmol/L) was added during the final hour of treatment, and representative dot plots of the flow cytometry data are presented.
if entry into S phase is inhibited in compound 505–treated cells, cells were synchronized in G2-M with nocodazole and then released from G2 arrest and refed complete medium supplemented with either vehicle or compound 505. Both control and treated cells rapidly progressed through mitosis into G1 after removal of nocodazole (Fig. 4B). Cells that were treated with vehicle alone entered into G1, as seen at the 4-hour time point, and progression into S phase is apparent after 8 hours, with progression into G2 evident at 12 hours (Fig. 4B). Cells that were treated with compound 505 after release from nocodazole progressed into G1 but did not enter S phase after 12 hours after release. To increase the resolution of the biochemical steps in the transition from G1 to S phase, we assessed BrdUrd incorporation using the same treatment protocol. Following release of nocodazole-arrested cells, 10 μmol/L BrdUrd was added 2 hours before collection, and cells were then analyzed by flow cytometry for BrdUrd incorporation. In the presence of compound 505, no BrdUrd incorporation was observed, whereas vehicle controls showed incorporation and progression through the cell cycle (Fig. 4B). To determine the acute effect of compound 505 on DNA replication, asynchronous cells were treated with compound 505 for a short period of time (3 hours), and small but distinct differences in BrdUrd incorporation were observed (Fig. 4C). Cells treated with TDRL-505 seem to display a lengthening of S phase and fewer cells that have incorporated BrdUrd progressing through the cell cycle into G2 (Fig. 4C).

The effect of RPA inhibition on DNA repair and the response to DNA damage. In addition to its essential role in DNA replication, RPA is involved in the DNA damage response and is required for the repair of bulky DNA adducts as well as DNA breaks induced by various types of exogenous and endogenous agents. The association of RPA with ssDNA is a critical feature of all of these pathways, indicating that inhibition of this activity would increase the cytotoxic effects induced by DNA damage. To determine the effect of RPA inhibition on cellular sensitivity to cisplatin, we evaluated the combination index (CI) using a concurrent treatment protocol (22). When cisplatin and compound 505 were used in combination, cell viability was decreased to a level that was greater than that induced by either agent alone, resulting in synergy between the two compounds and CI of 0.4 at the highest fraction of cells affected (Fig. 5). The interaction became additive and then antagonistic (revealed from CI values of >1) at lower fractions of cells affected (Fig. 5). These results show that compound 505 is able to potentiate the effect of cisplatin in H460 cells and is consistent with inhibition of the cellular activity of RPA in NER. The ability of compound 505 to synergize with etoposide was also examined. Etoposide induces replication fork arrest and DNA damage response, both cellular processes that require RPA (23). Using the same analysis as described above for cisplatin, compound 505 showed synergistic activity with etoposide at all fractions of cells affected (Fig. 5). Interestingly, when the formation of etoposide-induced RPA foci (23) or phosphorylation of DNA-PKcs at Ser2056 was measured (24), the cotreatment with TDRL-505 resulted in a slight increase in the number of cells with foci and DNA-PKcs phosphorylation (Supplementary Figs. S1 and S2). These data show that TDRL-505 can influence the cellular response to etoposide, consistent with the synergy observed between the two agents in the CI analyses (Fig. 5).

Discussion

Advances in high-throughput screening of chemical libraries have resulted in an explosion of putative cancer targets and their inhibitors. To date, the majority of these target enzymatic activity associated with a specific protein. Our results targeting the nonenzymatic DNA-binding activity of RPA open up an entire new class of putative interactions for therapeutic development. One such compound, 505, inhibits RPA-DNA interactions in vitro, blocks cell entry into S phase, and results in a cytotoxic/cytostatic response. Each of these responses is consistent with inhibiting the role of RPA in the initiation of DNA replication, which involves a complex series of interactions, one of which is the loading of RPA at replication origins in an S-phase cyclin-dependent kinase–dependent process (21, 25). Data presented show that TDRL-505 also abrogates BrdUrd incorporation into DNA in cells released from G2 arrest without an overall decrease in asynchronous cells, consistent with the importance of RPA in replication fork firing in the initial stages of DNA replication. Interestingly, treatment of asynchronous cells with TDRL-505 did display a prolongation of S phase evidenced by fewer cells completing S phase and progressing into G2 in the pulse-labeling experiments. Therefore, replication forks that are established before the addition of TDRL-505 seem not to be disrupted; however, the initiation from additional late-firing replication origins is decreased in the presence of TDRL-505. The potential exists that cells that have already begun replicating their DNA are not affected by the inhibition of RPA binding to DNA, which correlates with observations that TDRL-505 is unable to compete RPA away from DNA.
from DNA once it is already bound (data not shown). Therefore, cells dependent on entry into S phase for continuous cell proliferation would be expected to be negatively affected by treatment with TDRL-505. The inability of cells to enter S phase is a potential mechanism contributing to cytotoxicity. This allows for the possibility of a therapeutic window for specifically targeting actively dividing cells in the context of cancer treatment using SMIs to block the cellular activity of RPA. This rationale is further bolstered by the clinical observation that high levels of RPA expression correlate with disease stage in colon cancers (4).

The role of RPA in DNA repair also allows for inhibition of its activity to increase the efficacy of current chemotherapy that induce DNA damage in the context of combination therapy. The inhibition of DNA repair is anticipated to result in persistent DNA damage, which would increase cytotoxicity. The indispensable role of RPA in the recognition and verification steps of NER is well characterized, and in addition, RPA participates in the resynthesis step following excision of the damaged oligonucleotide (26). Previous studies have shown that cells with decreased levels of NER proteins show increased sensitivity to cisplatin treatment (6). Consistent with this, our data reveal a synergistic interaction between compound 505 and cisplatin at high fractions of cells affected. Interestingly, at low fractions of cells affected, an antagonistic interaction is observed with CIs >1. This is likely the result of interactions not at the level of repair but at the level of signaling. As cisplatin leads to activation of a G2 checkpoint and induces apoptosis from an extended G2 arrest, the finding that compound 505 blocks cells in G1 indicates that fewer cells would be subjected to cisplatin-induced G2 arrest. Likewise, if compound 505 toxicity stems from an extended G1 arrest, the G2 checkpoint induced by cisplatin would result in less cell death as a result of treatment. At high concentrations, this effect is mitigated by the interaction at the level of DNA repair, with RPA inhibition increasing cisplatin toxicity and overcoming the antagonistic signaling interaction.

The role of RPA in DNA replication restart and processing of collapsed replication forks also presents opportunities for combination therapy (27, 28). Interestingly, CI analysis of the activity of etoposide with compound 505 showed synergistic activity at all fractions of cells affected. Etoposide inhibits the enzymatic activity of topoisomerase II (topo II), resulting in persistent covalent-cleavage complexes on DNA, which lead to replication fork arrest and both single- and double-strand breaks (29). RPA has been shown to respond to and repair these types of lesions, and reduction of the levels of RPA p70 using siRNA results in increased formation of DNA double-strand breaks in response to etoposide treatment (23). These data are consistent with our observation that inhibiting the DNA-binding activity of RPA potentiates the effects seen by inhibiting topo II. Additional data show that the formation of etoposide-induced RPA foci measured at 4 hours of treatment was not inhibited by concurrent treatment with TDRL-505, although increased cytotoxicity was observed. Clearly, it will be of interest to assess foci dynamics as a function of the timing of RPA inhibition with respect to the induction of DNA damage. In addition, in asynchronous cultures, at any given time, a cell undergoing replication would be expected to be in various stages of origin firing. RPA is required in early replication firing, whereas topo II is required for later-stage replication events (30). Therefore, inhibition of both stages of replication progression would be expected to show a greater effect than inhibiting either one of the steps individually, which is consistent with the synergistic relationship between compound 505 and etoposide. Inhibition of RPA activity and abrogation of pathway function has the potential for widespread utility in cancer treatment. Although we focused on a subset of pathways, the role of RPA in several other repair pathways opens up other opportunities for combination therapy. Specifically, combining molecularly targeted RPA inhibition with radiation therapy could lead to increased cytotoxicity in tumor cells via inhibition of DNA double-strand break repair via nonhomologous DNA end joining or homologous recombination, both of which have been shown to require RPA (31–33).

As in the case of any novel SMI, specificity for the proposed target is of concern. We have shown specificity of TDRL-505 for RPA in in vitro analysis and have shown a correlation between the in vitro IC50 and cellular IC50 for all compounds analyzed except one. TDRL-523 did show robust cellular activity with modest in vitro RPA inhibition. The potential exists that TDRL-523 undergoes extracellular and/or intracellular modification that results in a higher affinity for RPA in a cellular model compared with in vitro conditions. It is also possible that TDRL-523 does not show a high specificity for RPA and is interacting with other proteins that contain structurally similar OB fold regions, such as telomere end protection proteins (34). Although OB folds adopt a similar global structure, the lack of sequence similarity between these domains suggests that specific interactions between amino acids and nucleic acids allow for differential interactions with nucleic acids (35). We are currently elucidating the interaction between TDRL-505 and specific amino acids in DBD-A and DBD-B of RPA p70 to determine if TDRL-505 can potentially interact with other OB fold–containing proteins. It is also likely that compounds specific for a given OB fold within RPA will have different effects on DNA metabolism dependent on the role each OB fold plays in a given pathway.

Although targeting the enzymatic activity of proteins with small molecules is well accepted, the research presented in this article shows the feasibility and utility of targeting a nonenzymatic protein-DNA interaction. These compounds therefore represent the first SMIs of RPA that display both in vitro and cellular activity. The approach of targeting RPA for cancer chemotherapy has several unique advantages, including the lack of redundancy resulting from no backup systems to counteract a loss of RPA activity. In addition, inhibition of RPA is expected to have broad-spectrum utility, as the reliance on RPA for increased cell proliferation and repair of chemotherapeutic DNA-damaging agents is not unique to any single cancer. Our targeting of the DNA-binding activity of RPA with a small drug-like molecule sets the precedent for targeting this class of proteins and thus alters the current drug discovery paradigm to open up an entire new class of targets with potential broad-spectrum utility.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank John Montgomery for excellent technical support and all members of the Turchi lab for their helpful discussions and critical reading of this manuscript.

References


Grant Support

NIH grant CA92741 (J.J. Turchi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/15/2009; revised 01/25/2010; accepted 01/27/2010.

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Cancer Res; 70(8) April 15, 2010

Cancer Research

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Cancer Res 2010;70:3189-3198.

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