Development of a high-throughput screen for inhibitors of replication protein A and its role in nucleotide excision repair

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Abstract

The heterotrimeric protein, replication protein A (RPA), is essential for DNA repair and replication. RPA is a viable target in the treatment of cancer as many chemotherapeutic agents act by blocking DNA replication. Furthermore, inhibition of RPA could prove useful in treating cancers that have acquired resistance to DNA damaging agents through enhanced DNA repair mechanisms as has been observed with certain platinum-resistant carcinomas. In an effort to identify inhibitors of RPA, we employed a novel fluorescent reporter and established a homogeneous high-throughput screening assay to measure RPA's DNA binding activity. Using this assay, we have screened a collection of small molecules and determined the effect they have on the RPA-DNA interaction. Of the 2000 compounds screened, 79 scored positive for inhibition of RPA binding activity. Secondary screenings were performed using an electrophoretic mobility shift assay; of the 79 compounds, 9 scored positive and were further characterized in titration experiments to determine the most potent inhibitor, resulting in several compounds showing an IC_{50} in the low micromolar range. Fluorescence polarization analyses were also performed to determine the mechanism of inhibition for each compound. Validation of the inhibitory activity of selected compounds was verified using *in vitro* nucleotide excision repair (NER) catalyzed excision of a single cisplatin lesion in a duplex DNA. The identification and use of RPA inhibitors may aid in inhibiting NER activity that could potentially circumvent resistance to certain chemotherapeutic agents as well as be useful in the characterization of RPA and its interaction with DNA. [Mol Cancer Ther. 2004;3(4):385–391]

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Introduction

Replication protein A (RPA) is a heterotrimeric protein consisting of 70, 34, and 14 kDa subunits and is known to bind with high affinity to single-stranded DNA (ssDNA) and with less affinity to double-stranded DNA (dsDNA; Ref. 1). RPA is the major eukaryotic ssDNA binding protein and is involved in many vital DNA metabolic pathways. Specifically, RPA is involved in the repair of damaged DNA via the nucleotide excision repair (NER) pathway and is essential for DNA replication and elongation (1). It is RPA's involvement in repair and replication that represents an attractive target in cancer treatment. Interfering with RPA's ability to bind DNA, ultimately interfering with DNA repair and/or replication, could potentially be useful in developing an effective chemotherapeutic treatment for established cancers. This treatment will also be beneficial in types of cancer that have become platinum resistant by means of enhanced DNA repair as has been observed in recurring ovarian tumors (2). The domains involved in RPA's DNA binding ability are sensitive to small changes, making these domains possible targets for prevention of the RPA-DNA interaction (3). This small change may be brought about by introducing a small molecule into the binding domain of RPA, which could ultimately curb RPA-dependent DNA repair of cisplatin lesions. Extensive characterization of RPA with respect to its DNA binding activity has been conducted (4–7), resulting in the development of a high-throughput screen (HTS) capable of monitoring the interaction of RPA and DNA and the effect small molecules have on that interaction. By using this screening process, we have been able to screen compounds with the intention of identifying a small molecule that has the ability to interact with RPA's DNA binding domains. The compounds being screened are from the structural diversity set provided by the National Cancer Institute (NCI) and are a collection of pure and synthetic products supplied from a variety of sources worldwide. Major features of the assay are as follows: it can be performed in 96-well plates, allowing a large number of compounds to be screened at one time; it can be performed in real time; and it does not require incubation, washing, or filtration. The system is a solution-based assay that makes use of purified RPA protein and a DNA substrate labeled with a fluorescent reporter. The fluorescein-labeled DNA was specifically chosen because it shows a change in fluorescence that is proportional to the change in the binding of RPA to DNA. By observing this change in fluorescence, we can determine the effect of each small molecule on RPA's DNA binding ability. Compounds showing no inhibition in binding were discarded, leaving a significantly smaller number of compounds to characterize in the next step of screening. The secondary screening uses an

electrophoretic mobility shift assay (EMSA), which further validates the inhibitory properties of the remaining compounds and provides additional criteria for eliminating the least effective compounds. The primary goal of the first two screening processes was to select the most effective compounds without regard to the compounds' method of inhibition (*i.e.*, through an interaction with the DNA or with RPA). The compounds that met the first two selection criteria were then tested using fluorescence polarization with the purpose of eliminating any compounds that exert their inhibitory activity through an interaction with the DNA substrate. Compounds showing an interaction with RPA were then assayed for their effect on NER. Current lead compounds were found to inhibit the repair of a cisplatin DNA lesion via NER and will serve as a model for selecting additional compounds for testing.

Materials and Methods

Materials

DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). T4 polynucleotide kinase and T4 DNA ligase were from New England Biolabs (Beverly, MA). Radiolabeled nucleotides were purchased from Perkin-Elmer Life Sciences (Boston, MA) and the unlabeled nucleotides were from Invitrogen (Carlsbad, CA).

Protein Purification

Recombinant human RPA was purified from *Escherichia coli* overexpressing the three subunits using the vector pdT11 provided by Marc Wold. Protein purification was carried out as described previously (5).

DNA Substrate Preparation

The 30-mer (containing a 5′ fluorescein label) depicted in Table 1 was purified on a 15% polyacrylamide/7 M urea preparative DNA sequencing gel. The sequence of the DNA is 5′-GGGGAAGTAAGGACGCGGAAAGGATAGGGG-3′. Duplex DNA substrates for EMSA analysis were purified as described previously (6). The fluorescein-labeled DNA used for fluorescence polarization was prepared and purified as described previously (4). The 120-bp DNA substrate for NER was prepared as described previously (8) with the following modifications. Components used to construct the 120-bp DNA varied from those used previously and the current component oligonucleotides appear in Table 1. SP5.3 was treated at a 10:1 molar ratio of cisplatin to d(GpXpG) site. SP5.3 was annealed to SP7.4 at a ratio of 1:3. Preannealed SP5.3/SP7.4, SP5.7b/SP5.8, and SP5.9b/SP6.0 were ligated at a ratio of 1:3:3 in the presence of 1 mM ATP.

Table 1. DNA oligonucleotides

DNA	Sequence
ILH2.2F	5′-GGGGAAGTAAGGACGCGGAAAGGATAGGGG-3′
SP7.4	5′-GAGAAGGGGGAGAGGAAAGGCGCAGGAAGGA AGGGGAAGGGA-3′
SP5.7b	5′-GCCCAAGGGTACCGGAATTCCCTGCTGGAAGTG CCAGGTCCTCTTC-3′
SP5.9b	5′-TTCCCTTGCTTGCATTACCGATTGCGCCCGGGTC CGATTCCGTCC-3′

High-Throughput Assay

RPA (10 pmol) in a volume of 100 μl was added to each well of a 96-well plate, and 250 μM of each compound were added followed by 10 pmol fluorescent reporter in a volume of 100 μl. Plates were then read in a fluorometer. Fluorescence excitation was at 498 nm with a slit width of 2.5 nm, and fluorescence emission was read at 518 nm with a slit width of 5 nm. To calculate the percentage inhibition, the difference between the fluorescence values of reactions containing DNA and RPA and reactions containing DNA, RPA, and the test compound was divided by the difference between the fluorescence values of reactions with DNA with RPA and the fluorescence of the DNA alone. This value was then multiplied by 100 to give percentage inhibition.

EMSAs

EMSAs were performed in 20 μl reactions containing 20 mM HEPES (pH 7.8), 2 mM DTT, 0.001% NP40, and 0.25% *myo*-inositol. Each reaction contained the following: 1 pmol RPA, 1 mM compound, and 50 fmol ^{32}P-labeled dsDNA. Reactions were mixed and loaded onto a 4% native polyacrylamide gel. Gels were subsequently dried and exposed to a PhosphorImager screen and quantified using ImageQuant software.

Fluorescence Polarization

Fluorescence polarization experiments were performed on a Cary Eclipse Fluorescence Spectrophotometer (Varian). Reactions were performed in a volume of 0.5 ml in buffer containing 20 mM HEPES (pH 7.8), 2 mM DTT, 50 mM NaCl, 0.001% NP40, and 2 mM $MgCl_2$. RPA was titrated into reactions containing 5 pmol fluorescein-labeled 30-base ssDNA in the absence or presence of 0.02 mM compound. Following equilibration, five data points were taken at 5-s intervals for each titration point. Fluorescence emission was monitored at 515 nm following excitation at 495 nm. The band widths for both excitation and emission monochrometers were set at 10 nm. The fluorescence for vertical and horizontal emission was corrected using the *G* factor, which was calculated using the fluorescein DNA substrate in the appropriate buffer as the depolarizing sample. The degree of RPA-DNA binding is represented as the *r* value and was calculated using the following equation, with fluorescence intensities measured with emission and excitation polarizers set either parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) to each other.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

In Vitro NER Assay

NER reactions were performed using 120-bp duplex DNA containing a single cisplatin DNA adduct as described previously (8) with the following modifications. Reactions (50 μl) contained 50 fmol 120-bp ^{32}P-labeled

dsDNA, 300 μg HeLa extract, and 200 μM compound. Reactions were carried out at 25°C for 1 h. Reactions were stopped with 1 mM EDTA. Proteinase K (40 μg) was added and allowed to incubate for 15 min at 56°C. DNA was phenol/chloroform extracted and ethanol precipitated. Samples were loaded onto a 10% polyacrylamide/7 M urea sequencing gel, exposed to a PhosphorImager screen, and quantified using ImageQuant software.

Results

Development of HTS

In an effort to investigate the effect small molecules have on the interaction of RPA with DNA, we have developed a HTS assay that relies on the use of a fluorescein-labeled ssDNA substrate. This purine-rich DNA substrate is 5′ fluorescein labeled and shows an increase in fluorescence that is proportional to its binding with RPA. When RPA was titrated into a reaction containing the fluorescein-labeled DNA, a plot of fluorescence *versus* RPA concentration reveals a hyperbolic curve that saturated at a 1:1 ratio of RPA to DNA (data not shown). In an effort to optimize the detection, fluorescent excitation and emission wavelengths were tested along with varying slit widths and voltages. The optimal parameters were excitation at 498 nm and emission at 518 nm. The optimal excitation and emission slit widths and voltage were 2.5 and 5 nm and 800 V, respectively (data not shown).

In an attempt to test a large number of compounds simultaneously, the assay was converted to a 96-well plate format. In this assay, the fluorescein-labeled DNA was diluted into assay buffer and 100 μl were dispensed into each well. The fluorescence of each well was then read. Each well then received 100 μl RPA and the fluorescence of each well was read again. Percentage increase of fluorescence was then calculated from these two readings and is represented in Fig. 1. This figure illustrates a significant increase in fluorescence as DNA and RPA bind, as well as demonstrates reproducibility across the 96-well plate.

To determine the effectiveness of this assay, a blind experiment was performed in which 3 of the 96 wells were added to 5, 25, or 50 pmol DNA substrate that has the exact sequence of the fluorescent DNA reporter without the fluorescein label. This DNA would compete for RPA, therefore acting as an inhibitor. RPA was added to each of the wells followed by the addition of the competitive inhibitor and finally the fluorescein-labeled DNA. The results of the assay are presented in Fig. 2 and are plotted as percentage inhibition. Clearly, three wells were identified as having a significant inhibition of RPA binding to the fluorescein-labeled DNA. We also assessed the effect of DMSO on reaction sensitivity and reproducibility and the results indicated no difference in assay sensitivity (data not shown).

Based on these results, we screened compounds from the NCI diversity set of pure and synthetic compounds. The compounds received from the NCI were prepared in 100% DMSO. Sets of 80 compounds were screened on a 96-well plate with remaining wells being used for controls. Data

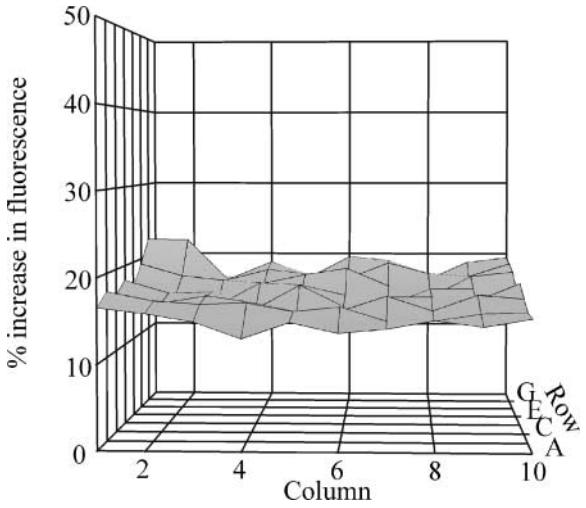

Figure 1. Fluorescence change due to RPA binding. The change in fluorescence of the reporter was tested as well as the accuracy of the dispenser. A fluorescent reporter (10 pmol) was added to a 96-well plate and read in a fluorometer. RPA (10 pmol) was then added to the same plate and again read on the fluorometer. *Z axis,* percentage increase of fluorescence; *X* and *Y axes,* columns and rows of the 96-well plate.

from a representative plate are presented in Fig. 3. The results clearly demonstrate that there are a series of compounds capable of inhibiting RPA's DNA binding. It should be noted that colored compounds were omitted from analysis regardless of their results because the color could interfere with the fluorescence reading and give incorrect results. In total, 2000 compounds were screened as described; of those screened, 79 were identified as inhibitors of RPA's DNA binding activity. Compounds were scored as positive if they resulted in a minimum of 50% decrease in the DNA binding activity of RPA.

Effect of Compounds on the DNA Binding Activity of RPA Using EMSA

Further validation of the inhibitory effect of the 79 compounds was done by means of EMSAs using ^{32}P-labeled dsDNA. Importantly, the interaction of RPA with dsDNA is likely to be required for NER. Therefore, while initial screenings were performed with ssDNA, a duplex DNA substrate was employed for subsequent validation. The results presented in Fig. 4 confirmed the inhibitory activity of a subset of the compounds tested (lanes 4, 7, 9, 11, and 13). The inability to validate all of the compounds in the EMSA is likely a result of different ratios of RPA to DNA used in the EMSA assay *versus* the HTS. In the EMSA analysis, the RPA/DNA ratio is 20:1; in the HTS analysis, the ratio is 1:1. The larger ratio required for binding dsDNA in the EMSA experiment was a result of the requirement to denature the DNA strands before high-affinity binding was observed (5). The results presented in Fig. 4 also provide evidence that a few compounds directly interact with the DNA. This is evident in lanes 6 and 8 where the free DNA runs as a dispersed band, while the free DNA migrates as a tight, homogenous band in lanes 1 and 4. This indicates that the compounds in lanes 6

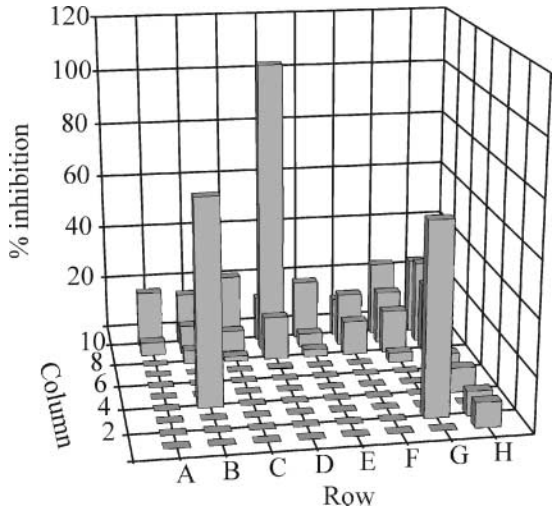

Figure 2. RPA inhibitor assay validation. A blind test was performed to determine the ability of the high-throughput assay to detect inhibitors. A fluorescent reporter (10 pmol) was added to the wells of a 96-well plate. The same reporter, without the fluorescent label, was added as an inhibitor to random wells in concentrations of 5, 25, and 50 pmol. Each well then received 10 pmol RPA. The plate was read in a fluorometer and wells containing the inhibitors were identified. Percentage inhibition was calculated as described in Materials and Methods.

and 8 are potentially interacting with the DNA, altering its electrophoretic mobility. The fact that with these compounds the RPA-DNA complex is not observed and that the compounds appear to interact with the DNA does not preclude the possibility that these compounds also interact with RPA. Of the 79 compounds screened using this method, 9 were identified as having a strong inhibitory effect on RPA's DNA binding ability. To characterize these nine compounds further, titration reactions were performed in which compounds were added at increasing concentrations ranging from 1 μM to 1 mM (data not shown). Results indicate that an increase in compound concentration causes an increase in unbound ^{32}P-labeled dsDNA, indicating an increase of free RPA. From these titration experiments, compounds were ranked in order of their potency, and lead compounds were further characterized. Interestingly, a few compounds that were identified as inhibitors of RPA's DNA binding activity in the HTS resulted in an increase in RPA-DNA complex formation in EMSA analysis. An example of this is compound 1.C44 shown in Fig. 4, lane 5, which resulted in greater dsDNA binding activity compared with RPA alone.

Analysis of Compound Binding via Fluorescence Polarization

The previous two procedures, HTS and EMSA, were performed to select the most effective inhibitors of RPA. EMSA analysis suggested that some of the compounds interact with DNA. To more precisely determine if the method of inhibition was via an interaction with DNA or RPA, fluorescence polarization experiments were conducted. Fluorescence polarization is a measure of the rate at which an object tumbles or rotates in three-dimensional space. In this experiment, we employ a fluorescein-labeled dT_{30} substrate DNA, which results in a baseline polarization that is increased upon RPA binding to the DNA and slowing its rotation. Using this method, we were able to determine if the interaction of the compound was with DNA or RPA by constructing binding isotherms varying RPA at a fixed concentration of DNA with and without the addition of specific compounds. Using fluorescence polarization, we were able to identify compounds that exert their inhibitory effect through an interaction with the DNA. Fluorescence polarization was performed by titrating RPA into a reaction containing fluorescein-labeled dT_{30} DNA in the presence and absence of 20 μM compound (Fig. 5). The results presented in Fig. 5 demonstrate that in the control reactions performed in the absence of compounds, RPA binds the DNA as measured by the increase in r value with increasing RPA (Fig. 5, open circles). The analyses of RPA-DNA binding in reactions containing compound E51 yield significantly different results (filled circles). The maximum r value obtained in reactions containing compound E51 (r = 0.13) was less than that observed for reactions performed without the addition of compound E51 (r = 0.23), suggesting that the compound was decreasing the effective concentration of the DNA. This interpretation was supported by the fact that saturation was achieved at 8 pmol less RPA in the presence of compound E51 than that observed in the control experiment (15 pmol RPA). These data alone suggest that the compound was binding the DNA directly and slowing the rotation of the fluorescein-labeled DNA. This conclusion is further supported by experiments performed measuring the effect of compound E51 on polarization of the DNA in the absence of RPA. In these reactions, the r value of the DNA plus compound was slightly greater than the r value of the DNA without

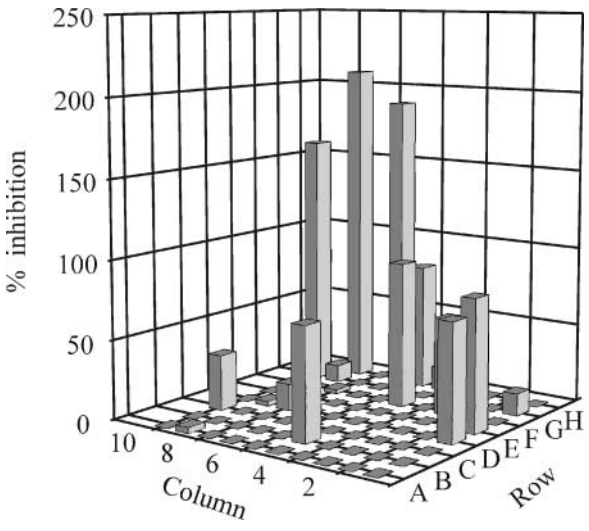

Figure 3. Testing compounds with HTS. An HTS was established to identify compounds that decreased the fluorescence of the reporter. RPA (10 pmol) was dispensed into a 96-well plate followed by 250 μM of each compound. A fluorescent reporter (10 pmol) was then added to each well, and the plate was read in a fluorometer. Percentage inhibition was calculated as described in Materials and Methods.

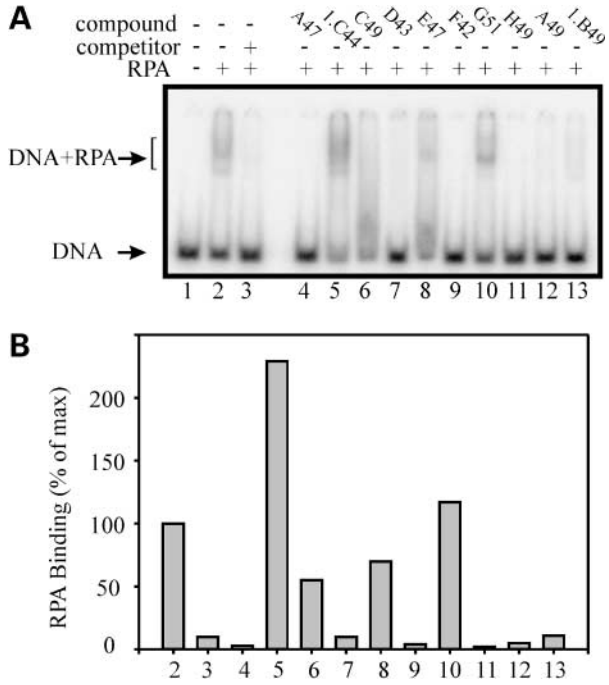

Figure 4. Secondary screening of compounds with EMSA. Compounds showing a decrease in fluorescence in the initial screen were further characterized using EMSA. RPA (1 pmol) was mixed with 1 mM compound. ^{32}P-labeled dsDNA (50 fmol) was then added to the mixture. Controls for the experiment included ^{32}P-labeled DNA (*lane 1*), ^{32}P-labeled DNA with RPA and DMSO (*lane 2*), and ^{32}P-labeled DNA with RPA and unlabeled competitor DNA (*lane 3*). Samples were analyzed on a 4% native polyacrylamide gel, dried, and exposed to a PhosphorImager screen and quantified using ImageQuant software.

compound (data not shown). In comparison, the analysis of compound D42 yielded results different from both the control and compound E51. Results with compound D42 (triangles) revealed a titration curve with a reduced initial slope, while the maximum *r* values were similar to the control reaction. The higher RPA concentration necessary to reach saturation suggests that the compound is interacting with RPA, therefore inhibiting the binding of RPA to the DNA substrate. In essence, the compound is removing the RPA from the reaction by binding it, resulting in the need for additional RPA to reach saturation.

In Vitro NER Assay

The removal of damaged DNA by means such as NER is essential for cell survival (9), and the ability to inhibit this removal would be expected to result in a more effective cancer treatment. RPA's early involvement in the NER pathway makes it a viable target. Therefore, we next used a NER assay to examine whether the previously identified compounds could in fact inhibit NER via an interaction with RPA. The experiment was performed by mixing the indicated compounds with whole cell HeLa extracts along with a 120-bp duplex DNA that contained a single cisplatin 1,3-d(GpXpG) adduct. Following recognition and incision, a segment of the DNA, ~25 base long and containing the cisplatin adduct, was observed (Fig. 6, lane 2). The nine remaining compounds were introduced into separate NER reactions and results are shown in Fig. 6. The results demonstrate that the compounds have a variety of effects on NER catalyzed removal of a cisplatin DNA lesion. Specifically, some compounds significantly reduce repair activity by as much as 70% (lanes 3 and 6), while other compounds have less of an inhibitory affect, a ~35% decrease in NER activity (lanes 4, 5, and 7). The remaining compounds show no significant inhibition of NER catalyzed incision (lanes 9 and 11); in some cases, a slight increase in activity was observed (lanes 8 and 10). These results demonstrate that inhibiting the DNA binding activity of RPA with a small molecule influences its essential role in NER and potentially other DNA metabolic pathways. As expected, compounds that were found to interact directly with DNA inhibited *in vitro* NER-catalyzed incision of cisplatin-damaged DNA. More importantly, several of the compounds that were found to interact with RPA also resulted in inhibition of NER.

Discussion

Targeted therapies in treating cancer are becoming more popular as advances in our understanding of the pathways and processes in cancer cell development and progression continue. Despite many of the advances in the treatment of ovarian cancer, ~14,000 women will die of the disease this year and ~23,000 new cases will be diagnosed. Of the women diagnosed, only 25% are identified early enough to give them a 95% chance of surviving 5 years and women diagnosed in later stages have only a 50% chance of surviving 5 years. Of the women that do survive, ~50% will have multiple recurrences (10). Clearly, there is a need for better treatment options for those diagnosed with resistant ovarian cancer.

Recurrent ovarian cancer is often associated with resistance, which is usually multifactorial in nature (11).

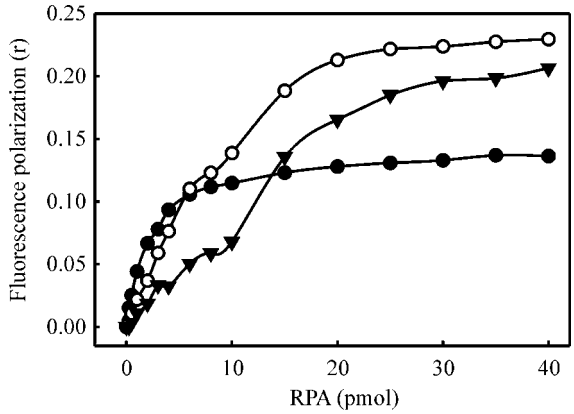

Figure 5. Fluorescence polarization. Fluorescence polarization was performed to determine if compounds were inhibiting through an interaction with DNA or RPA. Increasing concentrations of RPA were titrated into a reaction containing 5 pmol fluorescein-labeled ssDNA and 0.02 mM E51 (*filled circles*) or D42 (*triangles*). Control experiments were performed in the absence of compounds (*open circles*). Anisotropy values were calculated and plotted *versus* RPA concentrations.

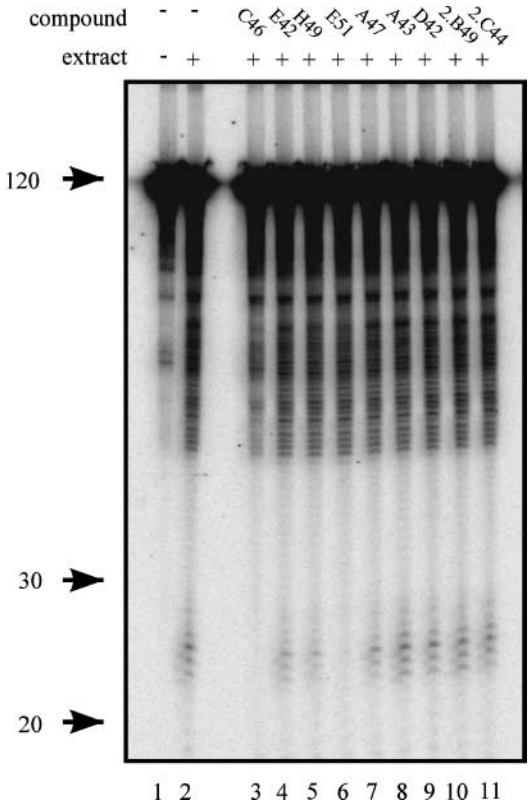

Figure 6. NER. Compounds showing inhibition of RPA-DNA binding activity in the EMSA were assessed for their effect in an *in vitro* NER assay. Control experiments were performed with 50 fmol ^{32}P-labeled 120-bp dsDNA containing a single cisplatin DNA adduct without HeLa cell-free extract (*lane 1*) or with extract (*lane 2*) both in the absence of inhibitor. Each compound (0.2 mM) was mixed with the extract from HeLa cells (0.3 mg) and incision of the 120-bp cisplatin-damaged DNA was analyzed on a 10% sequencing gel, dried, and exposed to a PhosphorImager screen and quantified using ImageQuant software.

Acquired resistance to DNA damaging agents, such as cisplatin, through enhanced DNA repair has been observed for certain ovarian carcinomas (12). One mechanism for increasing the sensitivity of resistant cancers to cisplatin is the inhibition of the repair systems responsible for removal of cisplatin DNA lesions. One protein, RPA, is required for this process (13). Our focus is on identifying and developing small molecule inhibitors that will block or disrupt the interaction of RPA with DNA, therefore blocking repair of DNA lesions. The extensive characterization of RPA with respect to its DNA binding activity has aided in our development of a HTS capable of identifying the small molecule inhibitors. This assay makes use of a fluorescein-labeled DNA reporter that allows the assessment of the interaction of RPA with DNA and therefore is useful for detecting inhibition of this interaction. One major benefit of this assay is that it is performed in real time and requires no incubation, washing, or filtration; in essence, it is a mix and read assay. This is relevant because it requires less hands on time, resulting in less human error as well as allowing ~500 compounds to be analyzed in 1 day. This approximation is based on assays using 96-well plates, and with slight modification, 384-well plates could be used resulting in a 4-fold increase in throughput. Not only has this assay proven to have high-throughput capabilities, it has also proven to be very sensitive. In the process of screening a library of small pure and synthetic compounds, inhibitors as well as activators of RPA's DNA binding activity were identified. The identification of activators shows the potential for an increase in the dynamic range of the assay. This increase can come from the addition of higher concentrations of RPA resulting in a higher maximum value and thus a wider range of fluorescence values in which to detect inhibitors.

One limitation that became apparent was that ~20% of the compounds screened had absorption characteristics that appeared to interfere with detection of fluorescence from the labeled DNA. This is an issue because independent of the effect on RPA binding DNA, spectral characteristics of the compounds alone resulted in inaccurate readings. For this reason, these compounds were omitted from the analysis.

The use of different DNA substrates in each step of the screening processes was both necessary and beneficial. For the HTS, optimization of the assay focused mainly on sensitivity and simplicity, resulting in the selection of a fluorescence-based assay where binding was measured by an increase in fluorescence intensity. The selection of the EMSA for secondary screening enabled us to assess the effect of each compound on RPA's ability to bind dsDNA. However, the DNA substrate used for the EMSA could not be used for fluorescence polarization because of the high RPA/DNA ratio needed to observe binding and the potential for DNA denaturation (5, 6). The DNA substrate used in the HTS was not particularly well suited for fluorescence polarization studies because of the dramatic increase of yield on RPA binding to the DNA. The fluorescent-labeled dT_{30} circumvented this problem as no increase in yield was observed on RPA binding. The use of the different DNA substrates enabled us to assess the inhibitory activity of the compounds under the variety of conditions required for each assay. In addition, the differing affinity of RPA for the different DNA substrates undoubtedly contributed to the degree of inhibition exhibited by each compound. RPA exhibits the highest affinity for the dT_{30} substrate, and the degrees of inhibition observed in the fluorescence polarization assay using this substrate are lower for each of the compounds tested.

The selection criteria employed for positive identification was relatively stringent, such that 4% of compounds screened were identified as inhibitors in the initial screen. Secondary screening resulted in 10% of those identified in the initial screen being validated while 30% of these validated compounds were shown to inhibit a tertiary reconstitution NER assay.

In this report, we have established a HTS capable of identifying inhibitors of RPA's DNA binding ability, thus interfering in the repair of a cisplatin DNA lesion. Obviously, further characterization of these compounds will include assessment of anticancer activity and the

determination of how the compounds interact with cisplatin. Undoubtedly, the utility of these compounds will have to ultimately be judged by determining their effectiveness in treating cisplatin-resistant ovarian cancers.

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References

1. Wold MS. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Biochem, 1997;66:61 – 92.

2. Johnson SW, Ozols RF, Hamilton TC. Mechanisms of drug resistance in ovarian cancer. Cancer, 1993;71:644 – 9.

3. Bochkarev A, Pfuetzner RA, Edwards AM, Frappier L. Structure of the single-stranded-DNA-binding domain of replication protein A bound to DNA. Nature, 1997;385:176 – 81.

4. Patrick SM, Turchi JJ. Stopped-flow kinetic analysis of replication protein A-binding DNA—damage recognition and affinity for single-stranded DNA reveal differential contributions of $k_{(on)}$ and $k_{(off)}$ rate constants. J Biol Chem, 2001;276:22630 – 7.

5. Patrick SM, Turchi JJ. Replication protein A (RPA) binding to duplex cisplatin-damaged DNA is mediated through the generation of single-stranded DNA. J Biol Chem, 1999;274:14972 – 8.

6. Patrick SM, Turchi JJ. Human replication protein A preferentially binds duplex DNA damaged with cisplatin. Biochemistry, 1998;37:8808 – 15.

7. Patrick SM, Turchi JJ. Xeroderma pigmentosum complementation group A protein (XPA) modulates RPA-DNA interactions via enhanced complex stability and inhibition of strand separation activity. J Biol Chem, 2002;277:16096 – 101.

8. Hermanson IL, Turchi JJ. Overexpression and purification of human XPA using a Baculovirus expression system. Protein Expr Purif, 2000;19: 1 – 11.

9. Heim MM, Eberhardt W, Seeber S, Muller MR. Differential modulation of chemosensitivity to alkylating agents and platinum compounds by DNA repair modulators in human lung cancer cell lines. J Cancer Res Clin Oncol, 2000;126:198 – 204.

10. Surveillance, Epindemiology, and End Results (SEER) Program, National Cancer Institute, DCCPS Surveillance Research Program, Cancer Statistics Branch (1973 – 2000).

11. Perez RP, Hamilton TC, Ozols RF, Young RC. Mechanisms and modulation of resistance to chemotherapy in ovarian cancer. Cancer, 1993;71:1571 – 80.

12. Hamilton TC, Lai GM, Rothenberg ML, Fojo AT, Young RC, Ozols RF. Mechanisms of resistance to cisplatin and alkylating agents. Cancer Treat Res, 1989;48:151 – 69.

13. Coverley D, Kenny MK, Lane DP, Wood RD. A role for the human single-stranded DNA binding protein HSSB/RPA in an early stage of nucleotide excision repair. Nucleic Acids Res, 1992;20: 3873 – 80.