Therapeutic Discovery

Novel Irreversible Small Molecule Inhibitors of Replication Protein A Display Single-Agent Activity and Synergize with Cisplatin

Tracy M. Neher¹, Diane Bodenmiller¹, Richard W. Fitch³, Shadia I. Jalal¹, and John J. Turchi¹,²

Abstract

Replication protein A (RPA) is a single-strand DNA-binding protein with essential roles in DNA replication, recombination, and repair. It is necessary for the formation of the preincision complex that is required for proper incision of damaged DNA nucleotides during DNA repair. We have previously identified small molecule inhibitors (SMI) with the ability to disrupt RPA-binding activity to ssDNA. Further characterization of these RPA inhibitors was done using both lung and ovarian cancer cell lines. Lung cancer cell lines showed increased apoptotic cell death following treatment with the SMI MCI13E, with IC₅₀ values of approximately 5 μmol/L. The ovarian cancer cell line A2780 and the p53-null lung cancer cell line H1299 were particularly sensitive to MCI13E treatment, with IC₅₀ values less than 3 μmol/L. Furthermore, a cell-cycle effect was observed in lung cancer cell lines that resulted in a lengthening of either G₁ or S-phases of the cell cycle following single-agent treatment. Sequential treatment with MCI13E and cisplatin resulted in synergism. Overall, these data suggest that decreasing DNA-binding activity of RPA via a SMI may disrupt the role of RPA in cell-cycle regulation. Thus, SMIs of RPA hold the potential to be used as single-agent chemotherapeutics or in combination with current chemotherapeutic regimens to increase efficacy.

Introduction

The nucleotide excision repair (NER) pathway is a highly versatile DNA repair pathway present in a number of organisms from bacteria to mammals that requires the contribution of more than 30 proteins (1). The NER pathway repairs a wide array of bulky DNA damage from a variety of sources such as reactive chemicals and exposure to UV light (1, 2). Numerous nonenzymatic protein–DNA interactions are essential for the proper functioning of the NER machinery and play important roles in nearly every reaction in the pathway including lesion recognition (3, 4). Damaged DNA is recognized by the trimeric complex consisting of xeroderma pigmentosum group C (XPC), Rad23B, and centrin-2 during global genomic nucleotide excision repair, whereas the stalling of RNA polymerase during transcription is the method of damage recognition during transcription-coupled nucleotide excision repair (5, 6). Following damage recognition, the preincision NER complex is completed with the subsequent recruitment of xeroderma pigmentosum group A (XPA) protein, transcription factor II H protein, and the human ssDNA-binding protein replication protein A (RPA) to the site of DNA damage. RPA is one of the first proteins that functions in both the global genomic and transcription-coupled NER subpathways (2, 7, 8). It is a heterotrimeric DNA-binding protein containing 3 subunits p70, p34, and p14 (kDa) and plays an important role in DNA replication and recombination in addition to repair (9, 10). The p70 RPA subunit contains DNA-binding domains A and B (DBD-A and DBD-B) and contributes most significantly to the RPA–ssDNA interaction (11). The RPA p34 subunit also contains an oligonucleotide binding fold and interacts with additional proteins including XPA, whereas the 14-kDa subunit plays a role in protein stability (12, 13). The RPA–DNA interaction is essential for the formation of the NER preincision complex and proper functioning of the NER pathway (14). Disruption of this essential protein–DNA interaction via small molecule inhibitors (SMI) should reduce the NER efficiency. Previous reports have shown that decreased expression levels of essential NER proteins, such as XPA, result in decreased NER capacity and removal of cisplatin adducts (15–17). Furthermore, increased expression of...
ERCC1-XPF was shown to correlate with cisplatin resistance in ovarian cancer cell lines (18). Taken together, these data suggest that the expression level of essential NER proteins affects the efficiency of the NER machinery. Using SMIs to inhibit RPA–DNA interactions and consequently the function of the NER machinery may increase the efficacy of DNA-damaging chemotherapeutics, particularly in tissues where enhanced repair via NER is a resistance mechanism.

Cisplatin, [cis-diaminedichloroplatinum(III)], is a front-line treatment for a variety of neoplasms, including ovarian, lung, and testicular cancers (19). Innate and acquired resistance to cisplatin therapy is a recurring issue in the clinic and a wide spectrum of responses are observed in cancer patients, warranting the discovery of novel chemotherapeutic treatments (19–22). Cisplatin induces its toxic effects by interacting with DNA, typically by intrastrand linkage of adjacent guanines (GpG). This produces an N-Pt-N cross-link from the imidazole nitrogen (N7), resulting in a 12- to 28-degree kink in the DNA. This kink is then recognized and repaired by the NER machinery (23–25). Disruption of protein–DNA interactions, resulting in a decrease in NER efficiency and DNA repair, may be exploited to increase efficacy of cisplatin and related platinum chemotherapeutics. Previous work has shown that a decrease in NER efficiency elicited by decreasing the expression of essential NER proteins results in increased sensitivity to cisplatin (16, 18). Therefore targeting the RPA–ssDNA interaction via SMIs holds the potential to sensitize cancer cells to platinum-based chemotherapy. Combination treatments involving SMIs may result in increased accumulation of cisplatin adducts and therefore increased efficacy of treatment, potentially decreasing the probability of recurrence/resistance.

Cisplatin damage results in cell-cycle response-arresting cells in the G2 phase, ultimately leading to cellular apoptosis (26, 27). The p53 tumor suppressor is a key element in DNA damage response. It is posttranslationally modified upon the induction of DNA damage resulting in activation (28). Activated p53 transactivates the p21 cyclin-dependent kinase inhibitor, which, in turn, results in cell-cycle arrest (29). In the absence of the ability to repair the lesions, ultimately apoptosis can be induced. Therefore, decreasing NER-catalyzed removal, via inhibition of the RPA–DNA interaction, may result in persistent cisplatin–DNA adducts and increased cellular sensitivity to cisplatin treatment.

We present here characterization of the physiologic effect of SMIs of the RPA–ssDNA interaction in a cell culture model. The core inhibitor isoform structure was identified in a screen of the NCI library (30) and subsequent structure–activity relationship led to the development of halo ester derivatives that act as irreversible inhibitors of RPA in vitro (31, 32). Two of these isobornyl derivatives MCI13E and MCI13F showed cellular activity and were chosen for further characterization. These data presented herein show that the disruption of RPA activity in the lung and ovarian cancer cell models results in increased apoptosis and lengthening of cell-cycle stages. The induction of apoptosis is independent of p53, and these SMIs synergize with cisplatin in combination treatments.

Materials and Methods

Chemicals
Dulbecco’s Modified Eagle’s Media (DMEM), RPMI, FBS, penicillin/streptomycin, and trypsin were purchased from Mediatech, Inc. (Cellgro). Annexin V–FITC/propidium iodine (PI) Vybrant Apoptosis Assay Kit and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody were purchased from Invitrogen. Dimethyl sulfoxide (DMSO) and SDS was purchased from Fisher Scientific. Cell Counting Kit-8 (CCK-8) solution was purchased from Dojindo Laboratories, and primary antibodies against p53 and ataxia-telangiectasia mutated (ATM) were supplied by Abcam. Secondary antibodies were purchased from Bio-Rad and Santa Cruz. All other reagents and chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

Cell lines
Tumor cell lines A549 (lung; CCL-185), H1299 (p53-deficient lung; CRL-5803), and H460 (HTB-177) were obtained from the American Type Culture Collection, verified via short tandem repeat profiling or testing, and were not passaged over 6 months following resurrection. The A2780 line was obtained from Tom Hamilton at Fox Chase Cancer Center. The A349, H1299, and A2780 cells were maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. H460 cells were maintained in RPMI media containing 1% penicillin/streptomycin and 10% FBS. Cells were maintained in a humidified incubator at 37°C with 5% CO2 supplementation.

Cell viability assays
A crystal violet cell viability assay was conducted to determine cytotoxicity of SMI. Lung cancer cell lines, A549 and H460, were plated in 24-well dishes at 5 x 10⁴ total cells. Forty-eight hours after plating, the cells were treated for 24 hours with variable concentrations of SMIs. The media was aspirated, cells washed with 500 µL of PBS/EDTA, and stained for 10 minutes with crystal violet solution (50% ethanol and 0.75% crystal violet). Stained cells were resuspended in 500 µL DMSO containing 1% SDS, and absorbance was measured at 590 nm in a SpectraMax M5 plate reader (Molecular Devices) and analyzed using SoftMax Pro 5.2. Percentage viability was taken as an absorbance percentage against control untreated cells. Values plotted represent 4 separate plate replicates, and each plate replicate contained 3 assay replicates per plate.

Cells of the ovarian cancer cell line A2780 are difficult to use for crystal violet analysis due to their enhanced sensitivity to treatment. Therefore, the CCK-8 was used to
determine the viability of A2780 cells in addition to the p53-null lung cancer cell line H1299, following a 24-hour treatment with log dosing of SMI. Cells were plated (1 × 10^5) in 24-well dishes and treated with inhibitor after 24 hours. After treatment media was aspirated and 1 mL of DMEM was added in addition to 100 μL of CCK-8 solution. Absorbance at 450 nm was measured over 4 hours in a SpectraMax M5 plate reader and values are presented as the percentage of untreated control at the 2-hour time point for 3 separate plate replicates each containing 3 assays per plate.

**Flow cytometry**

After determining the SMI cytotoxicity, H460, A549, and H1299 lung cancer cell lines were analyzed for apoptosis with the Annexin V–FITC/PI Vybrant Apoptosis Assay Kit (Invitrogen). Cells were plated in 6-well dishes (2 × 10^5 cells total) and treated for 24 (H1299) or 48 hours (H460 and A549) with increasing concentrations of SMI. Following treatment, both adherent and nonadherent cells were collected, processed via the manufacturer’s protocol (Invitrogen), and analyzed on a FACScan flow cytometer (Becton Dickinson). Data were analyzed using WinMDI software (The Scripps Research Institute, San Diego, CA), and results are presented as a percentage of control (untreated cells).

Cell-cycle analysis was also carried out on the 3 lung cancer cell lines by PI staining to identify the potential arrest resulting from treatment with SMIs. Briefly, cells were plated in 6-well dishes (2 × 10^5 cells total) and treated for 6 or 12 hours with increasing concentration of inhibitor. Adherent and nonadherent cells were collected, washed twice with PBS containing 2% bovine serum albumin, resuspended in 70% cold ethanol, and incubated overnight at −20°C. Cells were again collected and stained with PI (1 μg/mL) and RNase A (25 μg/mL) for 30 minutes at 37°C followed by 1.5 hours at 4°C in the dark. Flow cytometry was conducted as earlier, and data were analyzed on a histogram with events plotted against the FL2-A parameter. Cell-cycle distribution was analyzed using the ModFit software, and data presented represent 3 independent trials.

A549 cells were also treated sequentially or concurrently with combination treatment using cisplatin and SMIs. Sequentially treated cells were plated (3 × 10^5 total cells) in 6-well dishes and treated 12 hours later with cisplatin (5 μmol/L) for 12 hours, at which time SMIs (7 μmol/L) were added as indicated. Cells were then processed (as described earlier for cell-cycle analysis) at 6, 12, and 24 hours after the addition of SMIs. Concurrently, treated cells were plated in 6-well dishes, media was changed 12 hours after initial plating, and cells were treated with cisplatin (5 μmol/L) and SMIs (7 μmol/L) after an additional 12 hours. Cells were then processed at 6, 12, and 24 hours post–combination treatment for PI staining as described earlier.

To keep the cisplatin treatment constant and monitor cell-cycle progression, a similar methodology was applied. Twelve hours after initial plating, A549 cells were treated with cisplatin (2.5 μmol/L). Following either 12 or 24 hours of cisplatin treatment, MCI13E was added to the media (5 μmol/L) and cells were processed 12 or 24 hours later and analyzed for PI incorporation as discussed earlier (see Supplementary Fig. S1).

**Combination index studies**

Combination index studies conducted with combination SMI and cisplatin treatment were used to determine the level of synergy or antagonism between the 2 compounds. Briefly, A549 cells were plated as described earlier in 24-well dishes (5 × 10^4 cells per well) and treated either concurrently or sequentially with single-agent cisplatin or MCI treatment or with combination treatments. For sequential treatment, A549 cells were treated with cisplatin (0, 0.9, 1.8, 3.7, or 15 μmol/L) for 24 hours and then the inhibitor was added to the media for an additional 24 hours (0, 0.75, 1.5, 3, 6, or 12 μmol/L for MCI12E and 0, 0.25, 0.5, 1, 2, or 4 μmol/L for MCI13F). Concurrently, treated cells were again treated with both cisplatin and inhibitor for a full 48 hours. Following 48 hours of total treatment, cells were analyzed for viability by the crystal violet assay described earlier, and combination index values were calculated according to the Chou and Talalay method (33), as we have previously described (34, 35).

**Real-time PCR**

A549 cells were plated in 10-cm dishes at a density of 1.5 × 10^6, allowed to adhere, and treated for 24 to 48 hours with cisplatin or SMIs (5 μmol/L). For sequential treatment, A549 cells were exposed to cisplatin for 24 hours followed by the addition of SMIs for another 24 hours as described earlier. Concurrently, treated cells were incubated simultaneously with both cisplatin and MCl for 24 hours. RNA was extracted with a RNeasy Mini Kit (QIAGEN) per the manufacturer’s protocol. cDNA was generated with Applied Biosystem’s (ABI) High Capacity RNA-to-cDNA Kit. Validated gene primer/probe sets, also from ABI, were used for quantitative real-time PCR (qRT-PCR). ABI’s ΔΔC, relative quantification methodology was used for the analyses. Data were normalized to the housekeeping gene GAPDH and calibrated to the mock-treated samples. Results presented are the average of 2 separate cell treatments/RNA isolations with duplicate TaqMan Assays per RNA sample set.

**Western blot analysis**

Cells lines (A549, H460, or H1299) were plated in 10-cm dishes (3 × 10^6) and treated with the approximately IC_{50} concentration of SMIs in the absence or presence of cisplatin (5 μmol/L for A549 and H460 and 10 μmol/L for H1299 cells). Twenty-four hours following treatment, the media was aspirated and adherent cells were washed with 5 mL of PBS/EDTA. Adherent cells were scraped from the plates into 100 μL of RIPA buffer (10 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 0.1% SDS,
1% Triton X-100, 1% deoxycholate, and 5 mmol/L EDTA) and cellular debris was pelleted by centrifugation (10,000 × g, 10 minutes, 4°C). The supernatant was collected into a 1.5-mL microfuge tube, and total protein concentration was determined (Bio-Rad) standardized against bovine serum albumin. Equal amounts of protein (40 μg) were loaded onto SDS-PAGE and following electrophoresis proteins were transferred to polyvinylidene difluoride membranes. Proteins were detected with various antibodies (1:2,000 dilutions) and goat antimouse or goat antirabbit horseradish peroxidase secondary antibodies (1:2,500 or 1:3,000), respectively. Chemiluminescence and an image reader (LAS-3000; Fujifilm) were used to visualize bands, whereas Multi Gauge V3.0 software was used for data analysis.

Results

SMIs of RPA-DNA–binding activity decrease cell viability, induce apoptosis, and lengthen cell-cycle stages

Previous work from our laboratory identified and characterized the in vitro activity of MCI13E (Fig. 1A), a SMI of RPA that blocks the RPA–DNA interaction (ref. 31, 34; Fig. 1B). Considering that RPA is an essential protein that plays a central role in DNA replication, we assessed the activity of the SMI MCI13E in assays measuring cellular viability. Experiments were carried out in non–small cell lung cancer (NSCLC) cell lines A549, an adenocarcinoma, and the large cell carcinoma H460 cell line. Both lung cancer cell lines displayed a decrease in viability with increasing concentrations of SMI (Fig. 1C), with similar sensitivity and IC50 values of approximately 5 μmol/L. We also did not detect any change in the levels of endogenous RPA as a function of MCI13E treatment (Supplementary Fig. S2).

Decreased viability in the staining assay can be a function of numerous factors. These include induction of cell death, decreased rates of proliferation, or induction of cell-cycle arrest. To assess induction of cell death, we assayed phosphatidylserine flipping, a measure of early apoptosis, in conjunction with PI staining, a measure of membrane integrity. Treatment of H460 and A549 cells produced a significant increase in Annexin V reactivity and PI staining (Fig. 1D), showing the induction of a classical apoptotic pathway following treatment with MCI13E.

Figure 1. A, chemical structure of MCI13E. B, MCI13E inhibits RPA–DNA binding in vitro. RPA was mock treated (lane 1) or treated with 50 μmol/L MCI13E (lane 2), and DNA binding to a single-stranded 30-bp DNA (12.5 nmol/L) was assessed by electrophoretic mobility shift assay as previously described by Anciano and colleagues (31). C, MCI13E displays single-agent cytotoxic activity against the NSCLC cells A549 and H460. Cytotoxicity was analyzed via crystal violet analysis as described in Materials and Methods 48 hours following MCI13E treatment. Data represent the average ± SD of 4 replicate experiments, with each replicate containing triplicate determinations. D, MCI13E induced death via apoptosis in NSCLC cell lines. Cell were treated with the indicated concentrations of MCI13E for 48 hours and analyzed for the induction of apoptosis via Annexin V/PI staining. E, percentage survival was calculated for each cell line using untreated cells as a control. Data presented are the average ± SD from 4 replicate experiments.
MCI13E. Quantification of the extent of cell death resulted in IC$_{50}$ values of approximately 5 µmol/L (Fig. 1E).

To determine the cellular mechanism by which apoptosis is induced by SMI treatment, we sought to assess effects on cell-cycle progression. Interestingly, treatment of A549 cells with increasing doses of MCI13E produced a greater proportion of cells in the G1 phase of the cell cycle whereas H460 cells show an increase in the proportion of cells in S-phase (Fig. 2). Cell-cycle progression was analyzed at 6, 12, and 24 hours of treatment with increasing concentrations of SMI, and the 6- and 12-hour time points are presented in Fig. 2. Although cell-cycle stages lengthened by 6 hours, there is no significant further increase after an additional 6 or 12 hours of treatment (Fig. 2 and data not shown). This suggests that the effect of MCI13E occurs early and does not persist. This may also be due to chemical instability of the α-halo ester moiety of MCI13E, resulting in reduced activity.

Considering this difference in cell type, we sought to determine the effects of MCI13E on other cell types. We selected the p53-null NSCLC cell line H1299 and the epithelial ovarian cancer cell line A2780. MCI13E was highly potent against A2780 cells with an IC$_{50}$ of approximately 1 µmol/L (Fig. 3), whereas the H1299 cell line displayed a similar IC$_{50}$ (~4 µmol/L) to the other NSCLC cell lines despite the p53 status (Fig. 3). Also note that this IC$_{50}$ value was determined following 24 hours of treatment, similar to that of the A2780 cell line, again showing sensitivity to the SMI. Cell-cycle progression was also measured in the H1299 cell line following 6, 12, and 24 hours of treatment with MCI13E, and an increased percentage of cells accumulated in S-phase (Fig. 3). As with the H460 cell line, an additional 6 or 12 hours of treatment did not result in a significant increase in the percentage of cells accumulating in S-phase.

**Synergy with cisplatin**

Analyses thus far indicate that inhibition of RPA is effective in eliciting effects consistent with inhibition of the role of RPA in cell-cycle progression and DNA replication. To assess whether SMI treatment impacts the role of RPA in DNA repair, we assessed how MCI13E or MCI13F treatment influences cellular sensitivity to cisplatin. MCI13F is an isobornyl derivative identified by Anciano and colleagues that shows an IC$_{50}$ value similar to that of MCI13E in vitro (31) and in vivo (data not shown) but contains an iodide in place of the bromide. Cisplatin-induced DNA damage is primarily repaired via NER and homologous recombination. Considering that both cisplatin and MCI treatment possess single-agent activity in the NSCLC cell lines, we used combination index studies.
to analyze the effectiveness of combination treatments. We initially undertook a concurrent approach, similar to that we described for another SMI of RPA, TDRL-505 (34). Briefly, A549 cells were treated for 48 hours with MCI13E, cisplatin, or combination treatment, with viability determined via crystal violet staining. The results from these experiments revealed an antagonistic effect (Table 1), suggesting that either cisplatin was rendering the SMI ineffective or vice versa. The analysis of cell-cycle effects induced by MCI suggested that this compound might have a relatively short half-life eliciting its effects relatively quickly whereas cisplatin typically requires at least 48 hours to produce effects. We therefore pursued a sequential treatment protocol, first treating cells with

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<th>Treatment</th>
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<th>Combination index</th>
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<td>Sequential</td>
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NOTE: A549 cells were treated sequentially or concurrently with cisplatin and MCI13E or MCI13F for a total of 48 hours and cell viability was determined. Combination index values were calculated on the basis of the Chou–Talalay method.
cisplatin for 24 hours and then adding the SMI and incubating for an additional 24 hours. The results from these experiments displayed synergy according to the Chou–Talalay method (33). Interestingly, we observed slight differences with MCI13E, synergizing with cisplatin at higher dosing concentrations whereas MCI13F showed synergy with cisplatin at all tested concentrations (Table 1).

**Cell-cycle analysis of combination treatments shows a difference in cycle lengthening**

As discussed earlier, MCI13E synergizes with cisplatin following sequential treatment whereas concurrent cisplatin/SMI treatment results in an antagonistic effect. Knowing that MCI13E induced a lengthening of the G1 stage of the cell cycle for A549 cells, we wanted to determine whether alteration in cycle progression with either sequential or concurrent cisplatin/SMI treatment could account for the differences in drug interactions. A549 cells were plated and treated sequentially or concurrently with cisplatin/SMI and processed for cell-cycle analysis. The experiment was carried out by 2 separate dosing schedules, one keeping the time of SMI treatment consistent between the sequential and concurrent schedules and the other keeping cisplatin treatment time consistent. These data from the latter experiment are presented (Fig. 4) as a reduced dose of SMI corresponding to the combination index studies and show, under both schedules, that the cisplatin-induced S and G2 accumulation at 24 and 36 hours posttreatment is modestly impacted by SMI treatment. These results suggest that differences in cellular response to the combined treatment schedule are not a function of differential cell-cycle arrest between the concurrent and sequential dosing schedules.

**Transcriptional and posttranslational modifications following combination SMI and cisplatin treatments**

Although no significant alteration in cell-cycle progression was observed, we sought to determine how activation of the DNA damage signaling pathways was impacted by the 2 treatment protocols. Using quantitative PCR, we analyzed the transcriptional activation of a series of genes encoding proteins involved in the DNA damage response. Three of these, \( \text{XPC} \), \( \text{DDB2} \), and \( \text{p21} \), showed consistent increase in the expression of cisplatin-treated cells as expected for \( \text{p53} \)-responsive genes. Interestingly, in the concurrently treated cells, the expression of each gene was reduced compared with the cisplatin control whereas in the sequentially treated cells, only \( \text{p21} \)

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**Figure 4.** Cell-cycle analysis with combination treatment of cisplatin and MCI. Sequential (A) and concurrent (B) treatment regimens for maintaining constant cisplatin treatment time. C, A549 cells were treated sequentially or concurrently with single-agent or combination treatment of cisplatin (Pt) and/or MCI13E (E) and analyzed for cell-cycle distribution via PI and flow cytometry. Data represent the average percentage of cells in G1, S, or G2 phase of the cell cycle. This experiment was repeated twice with similar results.
showed a decrease in expression compared with the cisplatin control (Fig. 5). These quantitative PCR data were further confirmed by Western blot analysis of p21 expression following the various treatment methods (Supplementary Fig. S3). This suggests that p53 is differentially activated without a corresponding change in cell-cycle distribution (Fig. 5).

To assess p53 activation directly, we determined the phosphorylation level of serine-15 by differing combination treatment protocols. Interestingly, bromide MCI13E and the iodide MCI13F had minimal single-agent effects on p53 phosphorylation whereas concurrent treatment with cisplatin produced a dramatic increase (Fig. 6). This increase was not seen in the sequentially treated cells and is consistent with the transcriptional profiling analysis in which differences were observed depending on treatment schedule. The analysis of total p53 was also analyzed, via Western blot analysis, and a similar increase in total protein following concurrent treatment was observed. Following sequential treatment, however, total p53 was below the detection limit, which we attribute to the difference in treatment times and the half-life of p53 (Supplementary Fig. S3). We also assessed a series of factors including DNA-dependent protein kinase, ATM and Rad3-related (ATR) kinase, ATM kinase, checkpoint kinase (CHK)-1, and CHK2. Robust levels of phosphorylation and activation were observed only for ATM and in this case there was no significant difference between the concurrent sequentially treatments, suggesting that the differential activation of p53 was not applicable to ATM.

**Discussion**

Targeting protein–DNA interactions with small molecules holds the potential to disrupt numerous essential cellular processes that could be used therapeutically. This approach holds much promise but presents significant challenges. Advances in screening technologies including
high-throughput screening and in silico screening of chemical libraries have aided in the identification of SMIs of a small number of protein–DNA interactions, and previously our laboratory identified 2 different classes of SMIs capable of inhibiting the interaction of RPA with DNA (31, 34). Here, we describe the cellular effects of a novel isoborneol haloacetate SMI (MCI13E), which irreversibly inhibits RPA binding to ssDNA in vitro. We recently reported another RPA inhibitor, TDRL-505, a reversible inhibitor that disrupts RPA binding through the p70 central oligonucleotide binding fold–DNA interaction (31, 34). However, MCI13E does not act via the p70 central DNA-binding domain A/B. Considering that MCI13E and TDRL-505 target 2 distinct regions of RPA (31), it is not surprising that we observe some different cellular effects of treatment with these compounds. Both MCI13E and TDRL-505 possess single-agent cytotoxic activity likely owing to the role of RPA in S-phase replication. However, MCI13E treatment induces death via classical apoptosis (Fig. 2) whereas TDRL-505, a reversible inhibitor that disrupts RPA binding through the p70 central oligonucleotide binding fold–DNA interaction (31, 34). The schedule-dependent synergy of MCI13E could be magnified because of this initial difference. It is easy to attribute the differential effects of these 2 classes of compounds to the different mechanisms by which they inhibit RPA. However, we cannot rule out potential of off-target effects or differential activity as a function of compound stability and/or cellular uptake.

In addition to single-agent cytotoxic activity, inhibition of RPA results in increased sensitivity to cisplatin (Fig. 4; ref. 34). The role of RPA in NER is 2-fold. First, RPA interacts with damaged duplex DNA and forms the preincision complex with XPA and transcription factor II H (36). Second, RPA aids in the resynthesis and relegation in the final step of the NER pathway (37). More recently, mutational analysis of RPA has revealed differential effects on DNA repair activity, sometimes producing a dramatic reduction in ssDNA binding and abrogating repair but with a less effect on replication (14). Consistent with the importance of ssDNA binding in repair, we observe a greater than additive effect when combining MCI13E treatment with cisplatin. Interestingly, this is a schedule-dependent phenomenon and synergy was observed only in cells treated sequentially with cisplatin than with MCI13E. Schedule-dependent synergy was not observed for TDRL-505–cisplatin interactions (34). The schedule-dependent synergy of MCI13E could not be attributed to alterations in cell cycle but could point to compound stability and reactivity, as the halo ester may be relatively unstable in medium. The potential also exists for a direct MCI13E–cisplatin interaction that inactivates either or both compounds.

Recent studies have also shown the role of RPA in DNA damage–induced signal transduction through the ATR-interacting protein, which, in turn, initiates the recruitment of ATR (38, 39). The formation of this complex and the activation of ATR by TOPBP1 results in the phosphorylation of CHK1, which can phosphorylate p53 to block cell-cycle progression to allow time for the repair of damaged DNA (38). Although the low levels of endogenous CHK1 precluded definitive analysis, p53 activation was assessed and differential phosphorylation was observed in cells that were subject to concurrent versus sequential cisplatin–MCI13E treatment. These data suggest that the synergy observed in sequential treatment could be a function of reduced p53 activation compared with that observed with the concurrent treatment (Fig. 6). Similarly, the dramatic increase in p53 activation in the concurrent treatment is likely, in part, responsible for the significant antagonism observed between cisplatin and MCI in the concurrent treatment protocol (Table 1).

Interestingly, single-agent treatment with SMI MCI13E or MCI13F only slightly increases the expression level of p21 as compared with the untreated control whereas single-agent cisplatin treatment produced a dramatic increase in p21 mRNA expression. Combination studies using concurrent treatment resulted in an increase in p21 mRNA level. However, this increase was maintained at a reduced level compared with the appropriate cisplatin control. Sequential treatment resulted in a similar pattern; however, the differences between single-agent cisplatin treatment and sequential combination treatment were not as pronounced. Taken together, these data suggest that apoptosis and the lengthening of the cell cycle may be primarily due to the increase in p21 mRNA expression level. Furthermore, the moderate differences in mRNA expression apparent with sequential treatment versus the concurrent control likely contribute to the synergy observed with sequential treatment. However, the slight decrease in p21 mRNA expression comparing single-agent cisplatin and combination concurrent treatment does not correlate with the increase in the phosphorylation of p53 detected via Western blot analysis. It is possible, however, that the increase in phosphorylated p53 may not translate directly into an increase in p21 expression or that this is required for p21 as p53 independent mechanisms may be involved, a possibility supported by results with H1299 p53-null cells. Further characterization of DNA repair proteins DDB2 and XPC shows similar trends in mRNA expression levels as that with p21. Taken together, the mRNA expression data are supportive of the synergistic and antagonistic results noted earlier for each treatment method. It also suggests that the inhibitors are altering not only the binding activity of RPA–DNA but also the expression...
of other DNA repair proteins (XPC and DDB2) and tumor suppressor protein p21.

ATM, a protein kinase similar to ATR, initiates cell-cycle arrest in response to DNA double-strand breaks (DSB). ATM, which is recruited to the site of a DSB via the MRN complex (MRE11-Rad50-NSB1), phosphorylates H2AX (γH2AX) and CHK2 resulting in cell-cycle arrest and a lengthening of the S-phase of the cell cycle (38). Again, ATM phosphorylation increases moderately with cisplatin and combination MCI13E treatment in all cell lines analyzed and no detectable CHK2 or γH2AX signal was detected. Taken together, these data suggest that the cell-cycle lengthening following single-agent MCI or combination treatment is not due to an increase in ATM activation. Furthermore, combination treatment of A549 cell lines with MCI13E or MCI13F and ionizing radiation results in, at best, an additive increase in cell death (data not shown). These data, in addition to the ATM data, suggest that the SMIs do not elicit their cytotoxic effects by damaging both DNA strands resulting in a DSB.

Although our data show an increase in cellular apoptosis and synergy between the SMIs and cisplatin, the direct interaction between the RPA protein and SMIs remains unclear. MCI13E is thought to react with cysteine residues, of which RPA contains 13. Four cysteine residues reside within the zinc finger domain, whereas the remaining residues are dispersed throughout the protein.

Mutation analysis of each individual cysteine holds potential to reveal the direct interaction between the SMI and RPA. Moreover, in vitro, a direct SMI–RPA interaction would further confirm the specificity of the interaction already confirmed in vitro (31). Overall, these identified SMIs of RPA binding hold great potential for further characterizing the RPA–DNA interaction and delineating the specific role of individual RPA domains in DNA repair and replication. The synergy with chemotherapeutics-like cisplatin suggests potential clinical benefits as well.

Disclosure of Potential Conflicts of Interest

J.J. Turchi has ownership interest (including patents) in NERx Biosciences.

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