ATR Inhibitors VE-821 and VX-970 Sensitize Cancer Cells to Topoisomerase I Inhibitors by Disabling DNA Replication Initiation and Fork Elongation Responses

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Abstract

Camptothecin and its derivatives, topotecan and irinotecan, are specific topoisomerase I (Top1) inhibitors and potent anticancer drugs killing cancer cells by producing replication-associated DNA double-strand breaks, and the indenoisoquinoline LMP-400 (indotecan) is a novel Top1 inhibitor in clinical trial. To develop novel drug combinations, we conducted a synthetic lethal siRNA screen using a library that targets nearly 7,000 human genes. Depletion of ATR, the main transducer of replication stress, came as a top candidate gene for camptothecin synthetic lethality. Validation studies using ATR siRNA and the ATR inhibitor VE-821 confirmed marked antiproliferative synergy with camptothecin and even greater synergy with LMP-400. Single-cell analyses and DNA fiber combing assays showed that VE-821 abrogates the S-phase replication elongation checkpoint and the replication origin-firing checkpoint induced by camptothecin and LMP-400. As expected, the combination of Top1 inhibitors with VE-821 inhibited the phosphorylation of ATR and Chk1; however, it strongly induced γH2AX. In cells treated with the combination, the γH2AX pattern changed over time from the well-defined Top1-induced damage foci to an intense peripheral and diffuse nuclear staining, which could be used as response biomarker. Finally, the clinical derivative of VE-821, VX-970, enhanced the in vivo tumor response to irinotecan without additional toxicity. A key implication of our work is the mechanistic rationale and proof of principle it provides to evaluate the combination of Top1 inhibitors with ATR inhibitors in clinical trials. Cancer Res; 74(23); 6968–79. ©2014 AACR.

Introduction

Topoisomerases are essential enzymes that catalyze the breaking and rejoining of the phosphodiester backbone to allow the unwinding and disentanglement of DNA during replication and transcription. Among them, Top1 produces transient single-stranded DNA breaks as catalytic intermediates that are referred to as Top1 cleavage complexes (Top1cc; ref. 1). Anticancer Top1 inhibitors act by slowing down the reversal of Top1cc and the religation of DNA (2–4). Persistence of Top1cc leads to collisions with replication forks and transcription complexes, converting them into irreversible Top1 covalent complexes and DNA double-strand breaks, which, if not repaired are lethal. The anticancer activity of Top1 inhibitors is primarily related to replication damage by replication “run-off” and replication-mediated DNA double-strand breaks (3, 5, 6).

Camptothecin and its derivatives are highly selective inhibitors of Top1, and the water-soluble derivatives, topotecan and irinotecan, are effective anticancer drugs against a wide range of tumors. However, they are limited by their chemical instability, ABCG2- and MDR1-mediated efflux, the rapid reversibility of the Top1cc, and dose-limiting bone marrow and gastro-intestinal toxicity (3, 5). To overcome these limitations, we have developed a novel class of Top1 inhibitors, the indenoisoquinolines (2), which are chemically stable, not substrates for the drug efflux membrane pumps, and produce persistent Top1cc (7). LMP-400 (Indotecan; NSC-724998) is one of two indenoisoquinolines currently in phase I clinical trial.

The broad use of camptothecins and the potential of LMP-400 as a clinical agent raise the prospect of rational drug combinations. To address this goal, we conducted a broad range siRNA-based screening to reveal targets that,
when silenced, improve the efficacy of Top1 inhibitors. Using camptothecin and an apoptosis-targeted siRNA library, we recently demonstrated the feasibility of the approach and identified the kinase TAK1 (MAP3K7), which is involved in cancer cell growth and in the NF-kB, p38, JNK pathways, as a novel synthetic lethal pathway for Top1-mediated DNA damage (8). In the present study, we extended this approach to more than 7,000 human genes with four independent siRNAs per gene, and identified siRNAs that were synthetically lethal with Top1 poisoning, prioritizing genes that can be validated with drugs in preclinical development. We focused on ATR (ataxia telangiectasia and Rad3-related protein kinase), a major signal transducer of the replication stress-induced DNA damage response (DDR), which functions in parallel with ATM (ataxia telangiectasia mutation stress protein kinase), a major transducer of the DNA double-strand break responses (9–12).

ATR is activated in response to replication stress (single-stranded DNA associated with replication complexes and stalled replication forks) induced by UV, hydroxyurea, or chemotherapeutic drugs such as camptothecins. In turn, ATR activates the cell-cycle kinase Chk1 by phosphorylation (at serine 345), leading to a suppression of origin firing and DNA elongation, and cell-cycle arrest in S/G1, which promote repair and prevent premature mitosis, thereby maintaining genomic stability (9, 10).

Potent and selective ATR inhibitors are in clinical development (13–17) with the rationale to target cancer cells under replication stress resulting from oncogene addiction (14, 18, 19) or chemotherapeutic replication inhibitors such as Top1 inhibitors or gemcitabine (20–22). In the present study, we explore the molecular and preclinical rationale for combining Top1 inhibitor with ATR inhibitors.

Materials and Methods

Cell lines, chemicals, and drugs

HT29 and COLO 205 colon and MDA-MB-231 breast carcinoma cells were grown in RPMI medium with 10% FBS (Gibco-BRL) at 37°C in 5% CO2. HT29 and MDA-MB-231 cells, camptothecin, LMP-400, and VE-821 were obtained from the Developmental Therapeutics Program (DCTD, NCI), where cells were authenticated by short tandem repeat DNA fingerprinting. COLO 205 colorectal adenocarcinoma cells were purchased from the ATCC. HCT116 wild-type and p53 knockout cells were given by Bert Vogelstein (Johns Hopkins University). All the cell lines were grown in 5% CO2. HT29 and MDA-MB-231 cells were purchased from the ATCC. HCT116 wild-type and p53 knockout cells were given by Bert Vogelstein (Johns Hopkins University). All the cell lines were confirmed with the absence of Mycoplasma contamination (MycoAlert; Lonza). VX-970 (VE-822) was produced with the absence of Mycoplasma contamination (MycoAlert; Lonza). VX-970 (VE-822) was produced by Vertex Pharmaceuticals.

siRNA screening

For transfections, 20 μL of serum-free media containing Lipofectamine RNAiMax (0.05 μL) was added to wells of 384-well plates (Corning 3570) containing siRNA (0.8 pmol). Lipid and siRNA were allowed to complex for 45 minutes at ambient temperature before addition of 600 MDA-MB-231 cells to yield final transfection mixtures containing 20 nmol/L siRNA in RPMI, 10% FBS. Camptothecin (20 nmol/L, ~EC30) or vehicle (0.1% DMSO) was added to the entire plate 48 hours after transfection, and viability (CellTiter Glo; Promega) was assayed 72 hours later on a PerkinElmer Envision 2104 Multi-label Reader.

The druggable genome screening was conducted using the Qigian human druggable genome library version 4.1, which targets nearly 7,000 human genes with approximately four siRNAs per gene. Each siRNA is arrayed in an individual well. Qiagen’s AllStars Negative control and Qiagen’s AllStars Cell Death control were incorporated on all screening plates for normalization and as positive transfection control, respectively. Plates were rejected and rescreened if they exhibited an assay z’-factor of less than 0.4 or other apparent defects. Follow-up dose–response tests were carried under analogous assay conditions.

To select candidate genes modulating camptothecin activity, the log2 ratio of vehicle-treated cell viability (%siNeg) to camptothecin-treated cell viability (%siNeg) was calculated for each siRNA. Redundant siRNA Analysis (RSA; ref. 23) was performed on the ratios to rank gene candidates in terms of their ability to sensitize MDA-MB-231 to camptothecin. RSA rankings can be found in Supplementary Table S1. All screen data have been deposited on PubChem (AID 743121).

Ingenuity Pathway and STRING (24) analyses were performed to identify enriched pathways and protein–protein interactions among these 42 candidates. For Ingenuity Pathway Analysis (IPA), a core analysis was performed using only direct relationships and used the approximately 7,000 genes represented in the screen as background. For STRING, relationships were mined using only experiment- and database-determined relationships of at least "medium confidence."

Cytotoxicity

Cells were seeded in 96-well plates 24 hours before drug addition. After a 72-hour treatment, cell survival was determined using the ATPlite 1step Kit (PerkinElmer Life Sciences). Luminescence was measured by an Envision 2104 Multilabel Reader. Synergism was assessed using the CompuSyn Software.

Analyses of EdU incorporation, cell cycle, and apoptosis

HT29 cells were incubated with 50 μmol/L 5-ethyl-2′-deoxyuridine (EdU) for 30 minutes before adding Top1 inhibitors for 1 hour. Cells were harvested 18 hours after Top1 inhibitors. EdU was detected by flow cytometry (Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay; Invitrogen), and DNA using propidium iodide (PI). Apoptotic cells were detected 48 hours after Top1 inhibitors using Annexin V/PI containing (FITC Annexin V Apoptosis kit; BD Biosciences). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

Immunofluorescence microscopy staining of replication foci using CldU and IdU

HT29 cells were grown in 4-well chamber slides (Nalge-Nunc) and labeled with 100 μmol/L chlorodeoxyuridine (CldU) or iododeoxyuridine (IdU) for 45 minutes. Slides were prepared (6) and images visualized with a Zeiss LSM 780 confocal microscope.
DNA fiber assays
Molecular combing was performed as described (6). Briefly, at the end of the ClDu pulse, trypanized cells were embedded in low-melting agarose. After digestion with β-agarase (New England Biolabs), DNA was combed on silanized surfaces (Microsurfaces, Inc.), and replicas were detected with anti-IdU and anti-CldU antibodies. Images were captured with the software Attovision using the epifluorescence microscope Pathway (Becton Dickinson). Signals were measured using ImageJ (open source from National Cancer Institute, NIH) with custom-made modifications.

Immunoblotting
Total cell lysates were electrophoresed on 4% to 20% Tris-glycine or 3% to 8% Tris-acetate polyacrylamide gels, and transferred onto nitrocellulose membranes. Antibodies against GAPDH, ATR, and pS345-Chk1 were obtained from Cell Signaling Technology, γH2AX and pT68-Chk2 from Abcam, and Top1 from BD Biosciences. Antibody against pT1989-ATR was generated by the Pharmacodynamics Assay Development and Implementation Section (PADIS) of the Laboratory of Human Toxicology and Pharmacology at the National Laboratory for Cancer Research (Frederick, MD). After incubation with secondary antibody, signals were detected by enhanced chemiluminescence (Pierce).

IdU and γH2AX staining
After incubation with 100 μmol/L IdU for 75 minutes with or without drugs for 1 hour, HT29 cells were fixed at different time intervals with 4% paraformaldehyde for 10 minutes. Slides were prepared (6) and images visualized by Zeiss LSM 780 confocal microscope.

Xenografts
MFl athymic, nude mice were treated in accordance with the Animal (Scientific Procedures) Act and the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia. Mice weighing 20 to 30 g (Harlan UK Ltd) were inoculated subcutaneously with 1 × 10^7 COLO205 cells in 100 μL of a serum-free media and Matrigel mixture (1:1). When the tumors reached about 250 mm^3, the mice were randomized in groups of 8 animals. VX-970 [VE-822, 60 mg/kg in a solution in 10% Vitamin E Tocopheryl Polyethylene Glycol Succinate (Vite TPGS)] was administered by oral gavage on days 0, 1, and 2 of each 4-day cycle. Irinotecan (20 mg/kg or 40 mg/kg in PBS) was administered by intraperitoneal bolus injection on day 0 of each 4-day cycle. The control group was treated with vehicle (10% Vite TPGS in water) on days 0, 1, and 2 of each 4-day cycle. A total of 4 treatment cycles were given. Tumors were measured twice a week by caliper, and volumes were calculated using the formula (length × width^2)/2. Body weight was assessed twice a week.

Results
RNAi screening identifies ATR as a top synthetic lethal gene for Top1 inhibitors
To determine pathways and associated drug targets to improve the use of Top1 inhibitors, we conducted a druggable genome screen targeting nearly 7,000 human genes in MDA-MB-231 cells (8). To select candidate genes that modulate camptothecin activity, the log2 ratio of vehicle-treated to camptothecin-treated cell viability was calculated for each siRNA, and RSA (23) was used to rank gene candidates for their ability to sensitize to camptothecin. RSA identified 42 candidates with P ≤ 0.001 (Supplementary Table S1). IPA on these top candidates revealed an expected enrichment in canonical cell cycle and DNA repair–related pathways (Supplementary Table S2), and both IPA and STRING (24) identified enrichments for known protein–protein interactions (Fig. 1A and Supplementary Fig. S1). Evasion of apoptosis represents one of the fundamental hallmarks of cancer and can be driven by upregulation of prosurvival factors. Accordingly, we found that several antiapoptotic factors ranked high in the screen. They included BCL2L1 (BCLXL) and the complex comprising MAP3K7 (TAK1), MAP3K7IP2 (TAB2), and TRAF6, which is consistent with our prior screen (8). These results indicated that the screen identified relevant genes.

To further investigate top candidate genes, the effects of siRNAs were examined with camptothecin concentration responses. Follow-up was performed in two rounds. The first involved testing many of the same sequences from the primary screen, plus additional siRNAs from the same vendor (Qiagen). In all, at least four sequences were tested for 79 of 133 genes scoring P < 0.005 by RSA (Supplementary Table S3). In the second round, three additional siRNAs were obtained from a different vendor (Ambion) for 47 of the candidate genes to further distinguish the most robust hits and reduce the likelihood of false positives from off-target effects. Figure 1B lists those with more than half of ≥7 siRNAs yielding >4-fold sensitization. The corresponding curves can be found in Supplementary Fig. S2. ATR was among the most potent and robust targets, and additional testing showed that depletion of ATR similarly affected LMP-400 (Fig. 1C). Because of the ongoing development of clinical ATR and Top1 inhibitors, and our interest in Top1-mediated DNA replication damage and S-phase checkpoint regulation (6, 25, 26), the remaining part of our study focuses on the combination of ATR inhibitors with camptothecin and LMP-400.

VE-821 potentiates the cytotoxicity of both camptothecin and LMP-400
VE-821 was chosen for our molecular analyses because of its well-documented effects on ATR (13, 15, 21). Cells were exposed to camptothecin or LMP-400 for 1 hour because Top1 inhibitors are usually administered as short-time infusions. Yet, they produce extended cell-cycle arrest and checkpoint responses (6, 26, 27). VE-821 treatments were started together with the Top1 inhibitors but continued after Top1 inhibitor removal to maximize the impact of checkpoint inhibition (6, 25). Three colon carcinoma cell lines were examined in addition to the MDA-231 breast cancer cells: HT29 and a pair of isogenic HCT116 wild-type and p53 knockout cells to study the contribution of p53 to the potentiation of drug lethality.
VE-821 alone had minimal effect on cell viability up to 10 μmol/L (Fig 2). Consistent with the results obtained in cells depleted for ATR by RNAi (Fig. 1C), VE-821 sensitized MDA-MB-231 cells to LMP-400. Analysis was conducted with two active siRNAs (Qiagen) tested in B. ATR siRNAs, n = 3. Negative control siRNA (siNeg), n = 16. Both siRNAs reduced ATR transcript levels by more than 80% as determined by real-time PCR. Catalog numbers are listed in the chart legends along with calculated EC50s and confidence intervals (CI).

To further examine the effects of VE-821 on cell-cycle progression and because Top1 inhibitors selectively target replicating cells, we followed the cells that were in S-phase during the Top1 inhibitor treatments using EdU pulse labeling (6). FACS profiles of EdU incorporation versus DNA content revealed the progression of control cells and VE-821–treated cells through the cell cycle, indicating their normal progression through G2–M toward the next S-phase (Fig. 3B, left). As expected, camptothecin and LMP-400 produced a marked delay in S-phase progression with cells blocked in G2 without advancing to the next cell cycle (Fig. 3B, left). The non–EdU–labeled cells (i.e., those that were not in S-phase at the time of Top1 inhibitor treatment) progressed also very slowly with more cells in G2 after camptothecin treatment than in the control cells. In the case of LMP-400, most of the non–EdU–labeled cells were in G2, and only few in G1, indicating that only few cells were able to initiate a new cell cycle. VE-821 allowed cell-cycle progression of the cells that had been targeted in S-phase by the Top1 inhibitors (Fig. 3B, right); the cells were more broadly distributed throughout the cell cycle, with a fraction of labeled cells having progressed through G2–M and being in S-phase of the next cell cycle. Together, these experiments demonstrate that VE-821 efficiently abrogates the intra–S-phase and G2 checkpoints induced by camptothecin and LMP-400.

VE-821 abrogates the intra–S-phase checkpoint induced by camptothecin and LMP-400, and induces apoptosis

Top1 inhibitors alone produced a marked delay in S-phase progression (6, 7, 26): 18 hours after treatment with camptothecin and LMP-400, cells accumulated in late and mid S-phase, respectively (Fig. 3A, left). VE-821 abrogated this S-phase delay, enabling cells to move through the G2–M checkpoint after camptothecin while accumulating in G2–M after LMP-400 (Fig. 3A, right). The more pronounced cell-cycle effect of LMP-400 is consistent with its more potent and persistent Top1 inhibition and greater cytotoxicity (Fig. 2; ref. 7).
Vehicle

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No.

Table 1. Cell viability of indicated cell lines treated with varying concentrations of VE-821 and Top1 inhibitors

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Concentration (µmol/L)</th>
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<tr>
<td></td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>10</td>
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<tr>
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<tr>
<td></td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>0.3</td>
</tr>
<tr>
<td>HCT-116 p53+/+</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
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<tr>
<td></td>
<td>10</td>
<td>0.8</td>
</tr>
<tr>
<td>HCT-116 p53−/−</td>
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<td>1.5</td>
</tr>
<tr>
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VE-821 rapidly inhibits the DNA elongation checkpoint induced by Top1 inhibitors

Together with inhibition of replication origin firing, camptothecin produces a rapid fork elongation response (6). To elucidate the effect of Top1 inhibitors and VE-821 on checkpoint control of replication fork progression, DNA fibers were analyzed to visualize single replicons (6, 29). Cells were sequentially pulse labeled with IdU and CldU (Fig. 4A).

Figure 4B and E shows representative images for untreated and VE-821-treated cells, respectively. After 8 hours (the time interval between the two pulses), new replication foci formed, whereas those that were active 8 hours earlier did not incorporate IdU any longer. As a result, the green (CldU) and red (IdU) foci generally do not colocalize, demonstrating cell-cycle progression through S-phase.

In the absence of VE-821, camptothecin- and LMP-400–treated cells (Fig. 4C and D) showed a pronounced attenuation of IdU foci, demonstrating S-phase checkpoint with inactivation of late replication origins (6). By contrast, in the presence of VE-821, IdU foci were restored in spite of camptothecin or LMP-400 (Fig. 4F and G). Most CldU and IdU foci remained distinct, indicating initiation of new origins. Yet, in some cells, some CldU foci colocalized with IdU foci, suggesting persistence of replication foci beyond 8 hours. These results demonstrate that VE-821 blocks origin firing inhibition in cells treated with Top1 inhibitors.
replication fork slowdown was abrogated by VE-821, as the ratio CldU:IdU shifted back to 0.98 and 1.02 for the combinations of VE-821 with camptothecin or LMP-400, respectively. These results demonstrate that VE-821 abrogates the DNA elongation checkpoint.

DDR molecular biomarkers induced by VE-281

Because VE-281 directly targets ATR (13, 15) upstream from Chk1, we determined autophosphorylation of ATR on threonine 1989, a DNA damage activation site (30, 31), and Chk1 phosphorylation on serine 345, which is mediated by ATR. We also determined histone H2AX phosphorylation on serine 139 (γH2AX), a validated pharmacodynamic biomarker for DNA damage and apoptosis (32–34).

Camptothecin and LMP-400 quickly induced the rapid phosphorylation of Chk1, Chk2, and H2AX, whereas phosphorylation of ATR was delayed and best detectable after drug removal. Chk1 and ATR remained phosphorylated for 18 hours, consistent with the persistent checkpoint activation for hours after Top1 inhibitor removal (6, 27). VE-821 inhibited the phosphorylation of both ATR and Chk1 without reducing Chk2 phosphorylation, confirming the specificity of VE-821 for ATR. Notably, VE-821 strongly induced γH2AX over time (Fig. 6B and C). LMP-400 showed more pronounced effects than camptothecin, consistent with the enhanced Top1 inhibitory potency of LMP-400 (ref. 7; Figs. 2 and 3).

VE-821 markedly enhances γH2AX

Next, we focused on γH2AX using single-cell analyses (6) to determine the relationship between the γH2AX and DNA replication foci (labeled by short IdU pulses), and the possible induction of γH2AX pan-staining, as a hallmark of ATR-dependent replication stress (14, 18) or apoptosis (34).

Consistent with previous results (6, 7), in the absence of VE-821, camptothecin- and LMP-400–induced γH2AX foci mostly colocalized with DNA replication foci (Fig. 6D, left plots, second and fifth rows; E, right, top row). At the 1-hour treatment time, VE-821 had no significant impact on camptothecin- or LMP-400–induced γH2AX foci intensity and distribution (Fig. 6D and E).

Next, we examined the γH2AX response at later times in the presence and absence of VE-821. Consistent with the Western blotting results (Fig. 6B and C), in the absence of VE-821, camptothecin-induced γH2AX foci decreased over time, whereas LMP-400–induced γH2AX foci were more persistent. Notably, in the presence of VE-821, the γH2AX pattern changed. Instead of well-defined foci, γH2AX staining became diffuse and appeared like a ring. This ‘γH2AX ring’ increased over time (Fig. 6D, right). Figure 6E shows detailed images of a typical cell following LMP-400 + VE-821 cotreatment, demonstrating that γH2AX accumulates at the periphery of enlarged nuclei without colocalization with the replication foci that were damaged by Top1 inhibitors. Similar results were obtained with camptothecin, although with a lesser intensity. Together, these results demonstrate that ATR inhibition by VE-821 produces intense γH2AX pan-staining.

In vivo potentiation of irinotecan by VX-970

To assess the effect of ATR inhibition on tumor responses to Top1 inhibitors, we used VX-970 (VE-822), which is a close
analogue of VE-821 with improved potency and absorption, distribution, metabolism, and excretion (ADME) properties that make it usable in vivo as an ATR inhibitor (16, 22). First, we assessed the combined effect of VX-970 and the active metabolite of irinotecan, 7-ethyl-10-hydroxycamptothecin (SN-38) in vitro on COLO205 colorectal cancer cell viability. Strong synergy was observed between the two agents at concentrations of VX-970 as low as 80 nmol/L; VX-970 decreased the IC50 of SN38 by \( \frac{1}{8} \text{-fold} \) (Supplementary Fig. S3).

Next, we tested the combination in mice bearing subcutaneous COLO205 tumors. Mice were treated with either irinotecan (dosed i.p. on day 0 of a 4-day cycle), VX-970 (dosed by oral gavage on days 0, 1, and 2 of each 4-day cycle), or the combination of the two together. After short periods of tumor growth, treatment with 20 mg/kg irinotecan led to 88% tumor growth inhibition and at the maximum tolerated dose (MTD) of 40 mg/kg, complete tumor growth inhibition was observed (compared with starting tumor volumes, Fig. 7A and C). Although VX-970 had no impact on tumor growth when dosed as a single agent at 60 mg/kg, it was highly effective when dosed in combination with 20 mg/kg irinotecan, where substantial tumor regression was observed.
Notably, the antitumor activity for the combination was greater than that observed with irinotecan alone when dosed at its MTD. The combination was well tolerated with no increased body weight loss when compared with single-agent irinotecan treatment (Fig. 7B).

Discussion

Although camptothecins are therapeutically effective, they are not curative as single agents, and novel combinations are needed to improve their efficacy. In this study, we used siRNA screening to identify combinations of drug-targeted proteins and pathways.
We identified significant candidate genes involved in apoptosis. BCL2L1, an antiapoptotic member of the Bcl2 family, also known as BCLXL, whose expression is increased in various cancers (35, 36) and which inhibits proapoptotic factors such as BAX and BAK (36), scored as a top sensitizer. Small-molecule inhibitors of BCL2/BCL-XL such as Obatoclax or ABT-737 have been used in monotherapy or in combination with various agents notably Top inhibitors (37, 38). Depletion of TRAF6, MAP3K7, and MAP3-K7IP2, three genes involved in NF-κB activation and in a kinase complex comprising TAK1 (MAP3K7), TAB1, TAB2 (MAP3-K7IP2), and TRAF6 (39), also sensitized to camptothecin. We also found RNF31 (HOIP), which activates the NF-κB pathway through the polyubiquitylation of NEMO (NF-κB essential modulator, IKKγ) in the canonical IKK complex (40). These results are consistent with a previous screen (8).

The DNA damage sensing kinase, ATR, which was also among the top candidates, was chosen for further analyses as ATR inhibitors are entering clinical trials. After recognition of stalled replication forks, ATR regulates the intra–S-phase checkpoint by stabilizing replication forks, regulating cell cycle and DNA damage repair (9, 10). siRNA of three ATR targets Chk1, BRCA1, and UPF1 (41) also scored as top candidates, as did the PPP2R1A subunit of the protein phosphatase PP2A, which is involved in the regulation of the cell-cycle checkpoints (42).

As the primary cytotoxic mechanism of Top1 inhibitors in dividing cells is by generation of replication-fork collisions that convert Top1cc into irreversible DNA lesions (5), ATR and its downstream targets, Chk1, are crucial factors for the DNA damage response to Top1 inhibitors (5, 6, 25). Accordingly, inhibition of ATR by siRNA or VE-821 and its clinical derivative, VX-970, sensitized tumor cells to Top1 inhibitors. Loss of p53 influenced cell sensitivity to the combination of ATR and Top1 inhibitors as demonstrated in an isogenic system (14–16, 43).
Combinations of Topoisomerase I and ATR Inhibitors

Figure 7. The clinical ATR inhibitor VX-970 potentiates the efficacy of irinotecan in the colorectal cancer COLO205 mouse xenograft model. Mice bearing COLO205 tumors (volume ~250 mm³) were treated with vehicle, irinotecan (20 mg/kg or 40 mg/kg administered by intraperitoneal bolus injection on day 0 of each 4-day cycle), VX-970 (60 mg/kg administered by oral gavage on days 0, 1, and 2 of each 4-day cycle), or the combination of both drugs (using the schedule for administration of each compound alone). Treatment was continued for 4 cycles. Each group consisted of 8 mice. Animals receiving irinotecan or the combination of irinotecan and VX-970 were allowed to recover after treatment to assess tumor regrowth. A, TGI is tumor growth inhibition calculated as 100 - %T/C, where %T/C is the ratio of tumor growth on drug treatment (T) to vehicle-treated controls (C). b, regression is calculated as a percentage.

However, cellular sensitivity did not strongly correlate with *TP53* mutational status in heterogeneous cell panels (16). Our results are in agreement with recent reports describing the sensitization of cancer cells to combinations of VE-821 with multiple agents including camptothecin, topotecan, etoposide, gemcitabine, cisplatin, and ionizing radiation (15, 20, 21). Moreover, similar to the disruption of Chk1 by UCN-01, CHIR-24, and AZD7762 (6, 25, 44, 45), disruption of ATR by VE-821 abrogated the cell-cycle arrest induced by camptothecin and LMP-400. The addition of VE-821 forced progression through S-phase and G2-M for a proportion of the cells exposed to camptothecin, whereas the LMP-400–treated cells progressed through S-phase but were still not able to efficiently complete mitosis.

Single-cell analyses of DNA replication foci using Cl DuIdU sequential pulses revealed that the combination of VE-821 with Top1 inhibitors induced unscheduled DNA synthesis and allowed late-replicating chromosomal domains to initiate replication. Because each replication focus in immunohistochemistry experiments contains multiple origins, we used molecular combing (DNA fiber assays) to visualize single replicons (6, 29) and measure the impact of ATR on the elongation of damaged replicons. We previously demonstrated that camptothecin and LMP-400 inhibit DNA elongation (6, 25), but here, using comparable conditions, we show that LMP-400 is a more potent inhibitor of DNA elongation than camptothecin. Yet, VE-821 acted very rapidly to inhibit this checkpoint response elicited by camptothecin or LMP-400.

DNA double-strand breaks induced by Top1 inhibitors are mediated both by replication or transcription collisions (5, 46). A recent study describing a method to quantify the spatial distance between γH2AX foci and replication factories using confocal microscopy estimated a close relationship between γH2AX foci and replication factories (47). γH2AX foci induced by LMP-400 were more persistent than those induced by camptothecin, which is consistent with the chemical stability and persistence of LMP-400–induced Top1cc after drug removal (7). As shown by immunoblotting, inhibition of ATR by VE-821 led to an accumulation of γH2AX induced by Top1 inhibitors, whereas VE-821 by itself did not induce γH2AX. Previous reports (15, 20, 21, 48) are in agreement with our findings showing phosphorylation of H2AX on Ser139 (γH2AX) by ATM and/or DNA-PK after stalled replication, especially in the absence of ATR (14, 49). Furthermore, VE-821 has been shown to inhibit homologous recombination repair, as evidenced by a decrease in RAD51 foci (21).

A notable finding of the present study is the induction of intense γH2AX rings in cells cotreated with Top1 inhibitors and VE-821, starting a few hours after Top1 inhibitors and followed by a pan-staining that persisted until the formation of apoptotic bodies. Yet, this diffuse staining starting at the nuclear periphery is not initially associated with classical apoptotic markers. Rather, it may be related to the diffuse nuclear γH2AX staining induced by ATR inhibition, which was used as an endpoint in a high-throughput cell-based screen to identify ATR inhibitors (14). From a pharmacodynamic perspective, the intense γH2AX diffuse nuclear staining might be useful to evaluate combination therapy with Top1 and ATR inhibitors, and could be tested as a response biomarker in tumor samples, circulating tumor cells and surrogate tissues, such as plucked hairs (50). On the basis of our study, additional pharmacodynamic biomarkers for ATR inhibitors could be inhibition of Chk1 phosphorylation on Ser345 (21, 48) and inhibition of ATR autophosphorylation on threonine 1989.

Beyond its molecular and mechanistic insights, our study provides the proof of concept that ATR inhibitors are worthy of consideration for clinical trials in combination with Top1
inhibitors. Indeed, the clinical derivative of VE-821, VX-970 (16), showed remarkable synergism in combination with irinotecan, a widely used Top1 inhibitor. Further studies are warranted to examine VX-970 and other ATR inhibitors in combination with irinotecan, topotecan, or the non-camptothecin indenoisoquinolines, such as LMP-400 and LMP-776 (2).

Disclosure of Potential Conflicts of Interest
P.M. Reaper, C.S. Barnes, J. Jones, P. Charlton, and J.R. Pollard have ownership interest (including patents) in Vertex Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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