Inhibition of Ataxia-Telangiectasia Mutated and RAD3-Related (ATR) Overcomes Oxaliplatin Resistance and Promotes Antitumor Immunity in Colorectal Cancer

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Abstract

Although many patients with colorectal cancer initially respond to the chemotherapeutic agent oxaliplatin, acquired resistance to this treatment remains a major challenge to the long-term management of this disease. To identify molecular targets of oxaliplatin resistance in colorectal cancer, we performed an shRNA-based loss-of-function genetic screen using a kinome library. We found that silencing of ataxia-telangiectasia mutated and RAD3-related (ATR), a serine/threonine protein kinase involved in the response to DNA stress, restored oxaliplatin sensitivity in a cellular model of oxaliplatin resistance. Combined application of the ATR inhibitor VE-822 and oxaliplatin resulted in strong synergistic effects in six different colorectal cancer cell lines and their oxaliplatin-resistant subclones, promoted DNA single- and double-strand break formation, growth arrest, and apoptosis. This treatment also increased replicative stress, cytoplasmic DNA, and signals related to immunogenic cell death such as calreticulin exposure and HMGB1 and ATP release. In a syngeneic colorectal cancer mouse model, combined administration of VE-822 and oxaliplatin significantly increased survival by promoting antitumor T-cell responses. Finally, a DNA repair gene signature discriminated sensitive from drug-resistant patients with colorectal cancer. Overall, our results highlight the potential of ATR inhibition combined with oxaliplatin to sensitize cells to chemotherapy as a therapeutic option for patients with colorectal cancer.

Significance: These findings demonstrate that resistance to oxaliplatin in colorectal cancer cells can be overcome with inhibitors of ATR and that combined treatment with both agents exerts synergistic antitumor effects.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/79/11/2933/F1.large.jpg.
Introduction

Colorectal cancer is the fourth most common cancer worldwide. In 2015, the estimated colorectal cancer incidence was of 1.65 million cases worldwide, and colorectal cancer caused more than 835,000 deaths (1). Importantly, 30% of patients present synchronous metastases and 50%-60% will develop metastases that will require chemotherapy. The current management of colorectal cancer is based on various drugs [5-fluorouracil (5-FU)/leucovorin (LV), capecitabine, irinotecan, oxaliplatin, bevacizumab, cetuximab, and panitumumab], either in combination or as single agents (2). These treatments significantly improved the patients’ overall survival, though tumor resistance is still a frequent cause of therapy failure.

Among the drugs used in colorectal cancer treatment, oxaliplatin is a third-generation platinum compound with a 1,2-diaminocyclohexane carrier ligand, and is used in combination with 5-fluorouracil and leucovorin (FOLFOX regimen). It causes inter- and intrastrand DNA cross-links that stop DNA replication and transcription, leading to apoptotic cell death (3). Oxaliplatin exerts its antitumor effect also by inducing immunogenic cell death (ICD; ref. 4), where the host immune system is primed to recognize and eliminate tumor cells upon drug-mediated stimulation of the host antitumor immunity. Resistance to oxaliplatin is mediated by several factors, such as reduced cellular uptake, impaired DNA adduct formation, alterations in DNA repair genes (e.g., ERCC1 and XRCC1), apoptosis defects, and modifications in the expression levels of copper transporters (ATP7A and ATP7B; refs. 3–7).

Drug combinations are often used to overcome drug resistance and a major effort is made to identify novel drug combinations to improve therapeutic efficiency. High-throughput functional genetic screens (using shRNA or CRISPR/Cas9 approaches) provide a powerful tool to identify not only the mechanisms of drug resistance, but also new targets for efficient drug combinations. In this study, we wanted to identify molecules that could modify molecular alterations that occur in oxaliplatin-resistant colorectal cancer cell lines. To this aim, we performed a kinome-specific shRNA genetic screen showing that ataxia-telangiectasia mutated and RAD3-related (ATR) is implicated in oxaliplatin resistance. Importantly, we could demonstrate that the combination of an ATR inhibitor with oxaliplatin is synergistic in in vitro and in vivo colorectal cancer models by inducing DNA double-strand breaks, leading to apoptosis, and promoting antitumor immunity.

Materials and Methods

Cell culture

The human HCT116, SW48, SW480, SW620, HT29 (from the ATCC and the murine CT26, kindly provided by N. Bonnefoy, colorectal cancer cell lines were grown in RPMI1640 with 10% FCS and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO2. The murine colorectal cancer cell line MC38, provided by N. Bonnefoy, was grown in DMEM with 10% FCS and 1 mM sodium pyruvate at 37°C in a humidified atmosphere with 5% CO2. The oxaliplatin-resistant clones (HCT116-R1, HCT116-R2, SW48-R) were obtained as described previously (5). Briefly, the oxaliplatin-sensitive parental HCT116 and SW48 cell lines were grown in the presence of 5–10 μM/L oxaliplatin and cloned to obtain the resistant HCT116-R1 and HCT116-R2 and SW48-R clones, respectively. The cell lines were tested and authenticated by short-tandem repeat profiling (LGIC Standards and Eurofins Genomics). All experiments were performed at least three times.

Drugs and reagents

VE-822 (ATR inhibitor) was kindly provided by A. Coquelle and dissolved to a final concentration of 50 mM/L in DMSO (Sigma Chemical Co.). Oxaliplatin (5 mg/mL) was from ICM (oxaliplatin ACCORD Cip: 3400957954642) and was diluted in PBS to the final concentration of 12 mM/L. Both stock solutions were aliquoted and stored at −20°C. Poly-β-cornithine was purchased from Sigma and formaldehyde (16%) from Electron Microscopy Sciences. Antibodies against ATR #19334, ATM #2873, phosphorylated ATM (Ser1981) #13050, CDK2 #2546, phosphorylated CDK2 #2561, CHK1 #2360, phosphorylated CHK1 (Ser345) #2341, CHK2 #2662, phosphorylated CHK2 (Thr68) #2661, GAPDH #5174, p21 WAF1/CIP1 #2947, and phosphorylated p53 (Ser15) #9284 were from Cell Signaling Technology. The anti-phosphorylated ATR (Thr1989) GTX128145 antibody was from Genetex, anti-β-tubulin T4026 from Sigma, and anti-p53 (DO-1) sc-126 from Santa Cruz Biotechnology.

Pooled shRNA “dropout” screen

The TRC kinome library included 1,798 short-hairpin RNA vectors that target 518 genes selected from TRC collection. The library was used to generate lentiviral supernatants as described previously (8). HCT-116-R1 cells were infected with the lentiviral pools at a multiplicity of infection < 0.3 and with cell numbers sufficient to represent the kinome library with a 1,000 times coverage for each shRNA present in the library. After puromycin selection, infected cells were pooled and plated in 15-cm dishes (1 × 10⁶ cells/dish) with sufficient cell numbers to maintain the 1,000-fold coverage. After incubation in the absence or presence of 3 μM/L oxaliplatin for 10 days, cells were harvested, genomic DNA was isolated, and used for recovery of the shRNA inserts by PCR. Indexes and adaptors for deep sequencing (Illumina) were incorporated in the PCR primers. PCR products were purified using the QIAGEN PCR Purification Kit, according to the manufacturer’s protocol. DNA was quantified using a BioAnalyzer, and samples were combined at the same molar ratio. The shRNA sequences were extracted from the sequencing reads and aligned to the kinome library. The matched reads were counted and the read counts were used for analysis. The statistical analysis was done with DESeq, version 1.8.3, using the default settings for “pooled” analysis. The results of the DESeq analysis were used to calculate the ratio between treated and nontreated cells for each individual shRNA.

ATR silencing by shRNAs

Four different shRNA vectors with the puromycin selection marker and specific to ATR were used. Lentiviral production was performed by cotransfecting 293T cells (for lentiviral packaging) with 1 μg of anti-ATR shRNAs, 1 μg of the packaging vector gag-pol, and 1 μg of envelope vector, using FuGene6 (Invitrogen), according to the manufacturer’s instructions. Lentiviral particles were harvested and then used to infect 1 × 10⁶ HCT116 and HCT116-R1 cells for 48 hours. Following shRNA transduction, cells were selected with puromycin.
Inhibition of ATR Overcomes Resistance to Oxaliplatin in CRC

Cell growth inhibition assay (2D assay)

Cell growth was evaluated using the sulforhodamine B (SRB) assay, as described by Skehan and colleagues (9). Briefly, 300–1,000 cells/well were seeded in 96-well plates. After 24 hours, drugs were added in serial dilution. Cells were incubated for 96 hours, after which, they were fixed in trichloroacetic acid solution (final concentration of 10%), and stained with 0.4% SRB solution in 1% acetic acid (Sigma Chemical Co.). Fixed SRB was dissolved in 10 mmol/L Tris-HCl solution and absorbance at 560 nm was read using a Thermo Fisher Scientific Multiskan EX plate reader. The IC_{50} was determined graphically from the cell growth curves.

3D spheroid assay

For spheroid generation, 100 µL/well of cell suspensions at optimized densities (50–300 cells/well) were dispensed in ultralow attachment 96-well round-bottomed plates (Corning B.V. Life Sciences) and cultured at 37°C, 5% CO_{2}, 95% humidity, for 4 days. Depending on the cell sensitivity, spheroids were incubated with VE-822 (0 to 2.5 µmol/L) and/or oxaliplatin (0–30 µmol/L) at day 4 and at day 10 after plating.

At day 14, images were captured with the Celigo Imaging Cytometer (Nexcelom Bioscience) using the "Tumorosphere" application. Cell viability was measured using a CellTiter-Glo Luminescence Cell Viability Assay (Promega), according to the manufacturer's instructions. Luminescence was measured on a 1450 MicroBeta TriLux Luminescence Microplate Reader (Perkin Elmer). The IC_{50} was determined graphically from the cytotoxicity curves.

Quantification of the interaction effect

The interaction between the drugs tested in vitro was investigated with a concentration matrix test, in which increasing concentration of each single drug was assessed with all possible combinations of the other drugs. For each combination, the percentage of expected growing cells in the case of effect independence was calculated according to the Bliss equation (10):

\[ fu_c = fu_Afu_B \]

where \( fu_c \) is the expected fraction of cells unaffected by the drug combination in the case of effect independence, and \( fu_A \) and \( fu_B \) are the fractions of cells unaffected by treatment A and B, respectively. The difference between the \( fu_c \) value and the fraction of living cells in the cytotoxicity test was considered as an estimation of the interaction effect, with positive values indicating synergism and negative values antagonism.

EdU assay

Cell proliferation was measured with the Click-IT EdU Assay (Invitrogen) according to the manufacturer’s protocol. Briefly, 2.5 × 10^{5} HCT116 and 4 × 10^{5} HCT116-R1 cells were plated in 25 cm² flasks, and 24 hours later they were incubated with oxaliplatin (5 µmol/L) and/or VE-822 (1 µmol/L). After 48 hours, cells were incubated in ice-cold PBS, fixed in 75% ethanol, and labeled with 40 µg/ml propidium iodide (Sigma) containing 100 µg/ml RNase A (Sigma). Cell-cycle distribution was then determined with a Gallios Cytometer (Beckman Coulter) and quantified using the Kaluza Software (Beckman Coulter).

Apoptosis assay

A total of 2.5 × 10^{5} HCT116 and 4 × 10^{5} HCT116-R1 cells were plated, and after 24 hours, they were incubated with the indicated drugs (alone or in combination) for 48 hours. Cells were stained with FITC-labeled Annexin V and 7-aminoactinomycin D (7-AAD; Annexin V/7-AAD-Beckman Coulter). For apoptosis determination, Annexin V- and 7-ADD–positive cells were quantified using a Gallios Cytometer and the Kaluza Software (Beckman Coulter).

Western blot analysis

After treatment, cells (1 × 10^{6} cells/µL) were washed (1 × PBS) and directly lysed in Laemmli buffer (4% SDS, 20% glycerol, 1% 2-β mercaptoethanol, 0.004% bromophenol blue, 0.125 mol/L Tris HCl) + benzonase. After denaturation at 95°C for 5 minutes, protein extracts were deposited and separated on SDS-PAGE polyacrylamide (10%, 12%, or 4–20% gradient) gels. They were then transferred (transfer buffer containing 20% ethanol) to nitrocellulose membranes (10% and 12% gels: semi-dry transfer for 1 hour; 4–20% gradient gels: liquid transfer for 2 hours). Membranes were then blocked in PBS/0.1% Tween/5% milk at room temperature for 1 hour under agitation. They were then incubated with the primary antibodies at 4°C by gentle agitation overnight. After three washes in PBS/0.1% Tween, membranes were incubated with the relevant antispecies secondary antibody coupled to horseradish peroxidase diluted to 1/5,000 in PBS/0.1% Tween/5% milk with gentle agitation for 1 hour. After washing, immunoreactions were revealed by chemiluminescence (ECL RevelBlot Plus, and ECL RevelBlot Intense Kits) and visualized using the G-Box Imaging System (Syngene). Western blot analysis was performed using ImageJ software, and quantification was calculated relatively to the loading control associated. The untreated condition was arbitrary fixed at 1 to easily observe the changes induced by transfections or treatments. Each Western blot was performed and quantified between two and four times in each cell line except for ATM total in HCT116 cells, for which quantification could only be done once.

RPA32 and γH2AX analysis

HCT116 (4,000 to 9,000 cells/well) or HCT116-R1 (8,000 to 16,000 cells/well) cells were plated in black-sided 96-well, flat-bottomed plates (Greiner) precoated with poly-L-ornithine. After 24 hours, cells were incubated with VE-822 (1 µmol/L) and/or oxaliplatin (12.5 or 50 µmol/L) for 3, 6, 16, and 24 hours. Then, RPA not bound to chromatin was extracted with PBS/0.2% Triton X-100 on ice for 90 seconds. All steps were carried out carefully to keep cells attached to the plate. Between each step, cells were washed with PBS containing 1 mg/ml BSA. First, cells were fixed in PBS/4% paraformaldehyde (PFA) for 30 minutes and permeabilized with PBS/0.2% Triton X-100 at room temperature for 10 minutes. After blocking with PBS/1% BSA for 30 minutes, primary antibodies against RPA32 (Abcam, ab2175) and γH2AX (Cell Signaling Technology, #9718) were diluted in PBS with
1 mg/mL BSA and added to each well at room temperature for 1 hour. Then, the secondary antibodies (anti-mouse A448, Invitrogen, A1029; and anti-rabbit A568, Thermo Fisher Scientific, A10111), diluted in PBS/1 mg/mL BSA, were added at room temperature for 45 minutes. After this step, cells were kept in the dark and incubated with 1 μg/mL DAPI at 37°C for 30 minutes. Cells were then washed and left in PBS for analysis with the Celigo cytometer imaging system.

DNA fiber spreading

HCT116-R1 cells were seeded in 6-well plates (0.5 × 10^6/well). After 1 day, cells were incubated with VE-822 (1 μmol/L) and/or oxaliplatin (12.5 μmol/L) for 24 hours. Then, cells were labeled by two consecutive pulses of 15 minutes of each of the thymidine analogues iodo-deoxyuridine (IdU; final concentration: 20 μmol/L) and chloro-deoxyuridine (CldU; final concentration: 200 μmol/L). Then, the medium was removed and replaced by fresh medium containing 400 μmol/L of thymidine for 2 hours (chase period). After this step, cells were trypsinized, washed, and diluted in cold PBS to 1 × 10^6 cells.

In a humid chamber, the DNA of approximately 2,000 cells from the cell suspension was spread along a microcopy slide using lysing buffer (200 μmol/L Tris-HCl, 50 μmol/L EDTA, 0.5% SDS in milliQ H2O), and fixed in acetic acid:methanol (1:3) for 10 minutes, after which, slides were dried.

For immunostaining, slides were washed with H2O (2 × 5 minutes) and denatured in 2.5 mol/L HCl for 1 hour. After washing in PBS, slides were saturated in PBS/5% BSA for 1 hour, and then incubated with the anti-Cludu antibody (Bio-Rad OBT0030) in PBS/0.1% Triton X-100 at 37°C for 1 hour. After washes in PBST (2 × 5 minutes) and in PBS (1 × 5 minutes), slides were incubated with the secondary antibody (Thermo Fisher Scientific A1006) in PBST at 37°C for 45 minutes (in the dark). After washing, slides were incubated with anti-idU (BD Biosciences 347580) and anti-ssDNA (DHSB supernatant) antibodies in PBST at 37°C for 1 hour, washed, and incubated with the relevant secondary antibody (Thermo Fisher Scientific A21123 and A21241) in PBST at 37°C for 45 minutes. Then, slides were washed, air-dried, and mounted with Prolong Gold (Thermo Fisher Scientific P10144). Micrographs of labeled fibers were taken with a Fluorescence Microscope (Zeiss or Leica DMRM) and for each condition, at least 100 track lengths were measured. Each experiment was repeated at least two or three times.

Calreticulin exposure

A total of 1.5–2 × 10^6 cells were plated in 6-well plates, and after 24 hours, the medium was changed and cells were incubated with oxaliplatin (25 μmol/L) and/or VE-822 (1 μmol/L) for 48 hours. Cells were collected, washed in cold PBS/10% FBS, and stained with 7-AAD (Beckman Coulter) for 30 minutes. After washing in PBS/10% FBS, cells were fixed with 0.25% PFA for 5 minutes. After washing, cells were incubated with the anti–CRT-Alexa647 antibody (ab196159, Abcam) for 1 hour, followed by washing and fixation in 1% PFA for 5 minutes. Calreticulin (CRT) exposure was assessed using a Gallios Cytometer (Beckman Coulter). The fluorescent intensity of CRT-positive cells was gated relative to 7-AAD–negative cells.

ATP and HMGB1 release

ATP and HMGB1 release in the cell supernatant were analyzed after incubation with oxaliplatin (12.5–100 μmol/L) and/or VE-822 (1 μmol/L) for 24 hours and 48 hours. Supernatants were collected and centrifuged to remove dying cells. ATP release was measured with the ATPlite Luminescence Assay System (PerkinElmer), according to the manufacturer’s instructions. HMGB1 release was quantified with the HMGB1 ELISA Kit (IBL International), according to the manufacturer’s instructions.

Cytosolic DNA

Cells grown on glass coverslips were fixed in 80% ice-cold methanol for 20 minutes. Control samples were preincubated at 37°C with 200 mg/mL RNase A (Sigma) and/or DNase for 20 minutes. Samples were blocked with 1% BSA/PBS/Triton X-100 for 1 hour, and then incubated with the primary antibodies overnight [anti-ssDNA (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA) or anti-dsDNA (MAB1293, Millipore) in PBS/0.1% Tween]. After removal of unbound primary antibodies by washes with PBS/0.1% Tween, secondary antibodies were added at room temperature for 1 hour. Coverslips were then washed in PBS/0.1% Tween before mounting with DAPI-containing Prolong Gold. Images were taken using a 63× objective and a Zeiss microscope.

In vivo studies

Xenografts. HCT116/R1 and MC38 tumor cells (2 × 10^5 and 5 × 10^5, respectively) were injected subcutaneously in the left flank of 8-week-old female athymic nu/nu and C57BL/6 mice (Charles River Laboratories; n = 6–8). Tumors were detected by palpation and measured with a calliper weekly. Mice were euthanized when the tumor volume reached 1,500 mm^3. Ethical approvals were obtained by the local ethics committee (Ethics Committee approved by the French Ministry, animal facility approval C34-172-27, personal approval (Céline Gongora) 34.142 and protocol approval CEEA-LR-1193 and CEEA-LR-1194).

Tumor treatment. When tumors reached the volume of 100 mm^3, mice were treated for 4 weeks with: (i) 0.2 mL of 0.9% sodium chloride solution by intraperitoneal injection + D-α-tocopherol polyethylene glycol succinate (TPGS; gavage; control group), twice per week; (ii) 5 mg/kg oxaliplatin by intraperitoneal injection once per week; (iii) 30 mg/kg VE-822 (dissolved in TPGS) by gavage twice per week; or (iv) 5 mg/kg oxaliplatin once per week + 30 mg/kg VE-822 twice per week.

Statistical analysis. A linear mixed-regression model, containing both fixed and random effects, was used to determine the relationship between tumor growth and days after grafting. Data were first transformed using the natural log scale to better fit the assumptions of the linear mixed model. The fixed part of the model included variables corresponding to the number of post-graft days and the different treatments. Interaction terms were built into the model; random intercepts and random slopes were included to take time into account. The model coefficients were estimated by maximum likelihood and considered significant at the 0.05 level. Statistical analyses were performed using the STATA 10.0 Software (StataCorp).

Detection of CD8-positive T cells

Treated and control mice harboring mouse MC38 cell xenografts were sacrificed 3 weeks after graft/treatment. Spleens were recovered in ice-cold PBS containing 0.5% BSA and 2 mmol/L...
characteristics in terms of MSI/MSS status, origin, and KRAS, BRAF, PIK3CA, and TP53 gene mutation status (Supplementary Table S1). Using 2D (SB) and 3D (spheroid growth) cell culture assays, we confirmed that the three oxaliplatin-resistant cell lines displayed the highest oxaliplatin IC₅₀ values (Fig. 1A).

To identify genes the inhibition of which confers sensitivity to oxaliplatin in HCT116-R1 cells (synthetic lethal interactions with oxaliplatin; ref. 8), we performed a loss-of-function genetic screen using the TRC lentiviral kinase shRNA library that targets 518 human kinases (Fig. 1B). After infection of HCT116-R1 cells with the library and culture in the absence/presence of 3 μmol/L oxaliplatin (the oxaliplatin concentration required to kill all sensitive HCT116 cells, but inefficient in HCT116-R1 cells, see Fig. 1B) for 10 days, shRNAs were amplified by PCR and their relative abundance was determined by next-generation sequencing using the barcode identifiers present in each shRNA vector. We considered only shRNA vectors that were significantly depleted (by at least 50%) upon incubation with oxaliplatin, and selected genes represented by multiple shRNAs matching this criterion. Only few of the 1,798 shRNAs in the library met these criteria (Fig. 1C), among which, three (out of four) were independent shRNAs targeting ATR. To validate this finding, we infected HCT116-R1 cells with shRNAs against ATR or luciferase (control; Fig. 1D) and confirmed that ATR silencing sensitized HCT116-R1 cells to oxaliplatin (Fig. 1E; Supplementary Fig. S1A and S1B). Moreover, comparison of the oxaliplatin IC₅₀ values of HCT116-R1-shATR and HCT116 cells indicated that upon ATR knockdown, resistant HCT116-R1 cells became as sensitive to oxaliplatin as HCT116 cells (Fig. 1F). These findings demonstrate that inhibition can be sufficient to overcome oxaliplatin resistance, at least in HCT116-R1 cells.

We then tested whether ATR or its target protein checkpoint kinase 1 (CHK1) were constitutively activated (i.e., phosphorylated) in HCT116-R1 cells compared with sensitive HCT116 cells. Western blot analysis showed that neither ATR nor CHK1 were activated in HCT116-R1 cells (Fig. 1G). In addition, analysis of a transcriptome dataset from patients with mCRC (18) showed that ATR expression was significantly higher in primary tumors (n = 20) and hepatic metastasis (n = 19) than in normal tissue (n = 17) samples (Fig. 1H). This indicates that ATR is aberrantly expressed in colorectal cancer tumor samples and might be a potential therapeutic target.

The ATR inhibitor VE-822 and oxaliplatin combination is synergistic in colorectal cancer cells

Currently, two ATR inhibitors (ATRIs) are clinically available (VX-970 and AZD-6738). As VX-970 (called VE-822 thereafter) is more advanced in terms of clinical development, we used this ATRI. We evaluated the interaction between oxaliplatin and VE-822 in HCT116-R1 cells and in seven additional colorectal cancer cell lines, using a full-range concentration matrix approach and the SRB cytotoxicity assay (Fig. 2A). Specifically, we quantified the percentage of cell growth (blue matrix) and the additive, synergistic and antagonistic effects (black, red, and green matrices, respectively). We observed a synergistic interaction between oxaliplatin and VE-822 in all tested colorectal cancer cell lines, with the highest values in HCT116-R1, HCT116-R2, and SW48-R cells and the smallest for SW480. This indicates that VE-822 can efficiently overcome oxaliplatin resistance. The distribution of the interaction effects was not always homogeneous over the concentration matrices. For instance, focal areas with higher

Results

A functional genetic screen reveals that ATR is implicated in oxaliplatin resistance in colorectal cancer cells

We assessed oxaliplatin sensitivity in five colorectal cancer cell lines (HCT116, SW48, SW480, SW620, and HT29) and three oxaliplatin-resistant derivatives (HCT116-R1, HCT116-R2, and SW48-R) that were established by long-term growth in the presence of oxaliplatin (5). These cell lines display different molecular

Gene expression profiling and DNA repair score

ATR expression was assessed using the dataset GSE62322 ([n = 17 normal mucosa, n = 20 primary tumor, and n = 19 hepatic metastasis tissue samples from patients with metastatic colorectal cancer (mCRC)] from the prospective single-center study REC2 (9, 10). To build the DNA repair score, the gene expression datasets from two cohorts were used. The Tsuji dataset (GSE28702) included 80 patients with stage IV colorectal cancer treated with FOLFOX. Shingo Tsuji (Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan) kindly provided the OS data. The Del Rio dataset (GSE72970) included 36 patients with stage IV colorectal cancer treated with FOLFOX and also the clinical data (11). Gene expression data were normalized with the MAS5 algorithm and gene expression was assessed using the dataset GSE62322 –17). The DNA repair score was the sum of the Cox beta coefficients for 1 hour. After extracellular staining, cells were fixed and permeabilized, according to the manufacturer’s protocol; BD Biosciences) for 6 hours. To identify IFN-γ-secreting CD8-positive T cells, cells were washed once with PBS and incubated with antibodies against surface markers CD45-BV786 (clone 30F11), CD8-BV605 (clone 53-6.7), and the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) at 4°C for 1 hour. After extracellular staining, cells were fixed and permeabilized, according to the Bioscience fixation and permeabilization procedures, and intracellular staining ([anti-IFN antibody V421 (clone XMG1.2)] was performed at 4°C overnight. Then, samples were washed, fixed in 1% PFA, and processed for data acquisition and analysis using a Cytoflex Flow Cytometer (Beckman Coulter) and the Flowjo 10 software.

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synergy were often located in concentration regions that corresponded to high VE-822 concentration.

Next, we assessed whether ATR inhibition influenced oxaliplatin cytotoxic activity in mice xenografted subcutaneously with HCT116-R1 cells. To this aim, we treated mice with 0.9% sodium chloride solution (control group), oxaliplatin, VE-822, or the oxaliplatin + VE-822 combination. Oxaliplatin and VE-822 alone had no effect on tumor growth compared with control. Conversely, the VE-822 + oxaliplatin combination was effective in limiting tumor growth, even not really marked but significant ($P = 0.018$; Fig. 2B) and extending survival (Kaplan–Meier survival analysis, $P = 0.0216$; Fig. 2C). This result confirms our previous in vitro findings, and provides evidence that ATR inhibition also enhances oxaliplatin therapeutic effect in resistant colorectal cancer cells in vivo.

The VE-822 + oxaliplatin combination induces apoptosis, reduces cell proliferation, and activates the ATM–CHK2 pathway

We then investigated whether the ATRi VE-822 + oxaliplatin combination had a synergistic effect also on cell death and/or cell
proliferation. First, cell-cycle analysis showed that each compound had only a minor effect on the cell-cycle distribution of HCT116-R1 cells. Conversely, the VE-822 + oxaliplatin combination reduced significantly the number of S-phase cells and increased that of cells in the sub-G1 phase (Fig. 3A). We confirmed this finding using the EdU assay. Oxaliplatin and VE-822 alone had no effect on cell proliferation, whereas in combination they inhibited cell proliferation, as indicated by the reduction of EdU-positive HCT116-R1 cells (from 75% in untreated cells to 18% in oxaliplatin + VE-822–treated cells; \( P = 0.0094 \); Fig. 3B).

Next, we assessed apoptosis by quantifying Annexin-V+ HCT116-R1 cells (Fig. 3C). Only the VE-822 + oxaliplatin combination significantly and strongly induced apoptosis compared with untreated cells and the drugs alone. Moreover, p53 and p21 expression levels were increased by the drug combination, whereas phosphorylation of the ATR target CDK2 was decreased compared with cells without treatment or incubated with each single drug (Fig. 3D; Supplementary Fig. S2B and S2C). Altogether, these results demonstrate that the synergy between the ATRi and oxaliplatin impairs cell-cycle progression and proliferation, and promotes cell death.

To assess the effect of these drugs on the ATR signaling pathway, we performed immunoblotting analysis using HCT116-R1 (Fig. 3E) and HCT116 cells (Supplementary Fig. S2A–S2C) after incubation with oxaliplatin and/or VE-822. In both cell lines, oxaliplatin treatment promoted ATR and CHK1 phosphorylation. As expected, this effect was inhibited when cells were incubated with oxaliplatin and VE-822. As the ATR and ATM

Figure 2.
ATR inhibition combined with oxaliplatin alters colorectal cancer cell viability in vitro and in vivo. A, The indicated colorectal cancer cell lines were incubated with increasing concentrations of oxaliplatin and the ATR inhibitor VE-822, and cell viability was assessed with the SRB assay (2D) and by measuring spheroid growth (3D) to obtain the viability matrix. Drug concentrations were as follows: VE-822 (from 0.0075 to 2.56 \( \mu \)mol/L for 2D and from 0.01 to 2.52 \( \mu \)mol/L for 3D), oxaliplatin (from 0.84 and 30 \( \mu \)mol/L for 2D and from 0.09 and 30 \( \mu \)mol/L for 3D). The synergy matrix was calculated as described in Materials and Methods. B, Effect of the VE-822 + oxaliplatin combination on the tumor volume in nude mice xenografted with HCT116-R1 cells. Mice received oxaliplatin, VE-822, or both or vehicle (NT, normal tissue). C, Kaplan–Meier survival analysis of the mice described in B.
pathways cross-talk with each other, we also evaluated ATM and CHK2 expression (Fig. 3E, right). VE-822 induced CHK2 phosphorylation, whereas oxaliplatin did not have detectable effect. Conversely, the oxaliplatin and VE-822 combination significantly increased ATM and CHK2 phosphorylation, indicating that in the presence of oxaliplatin, the VE-822–induced inhibition of the ATR signaling pathway leads to activation of the ATM pathway. Altogether, these results demonstrate that the combination of oxaliplatin + VE-822 has a cytotoxic and cytostatic effect in colorectal cancer cell lines, and that it can effectively inhibit the ATR pathway and activate ATM signaling.

The VE-822 + oxaliplatin combination induces replication stress, leading to DNA break formation

ATR plays an essential role in the cell response to replication stress (RS; ref. 19). As accumulation of ssDNA and DNA damage is an RS feature, we quantified levels of RPA, a protein covering persisting ssDNA and γH2AX, marker of DNA damage, in HCT116-R1 cells incubated with VE-822 and/or oxaliplatin. For this, we used a specific fluorimetric assay that allows to correlate the respective protein levels in cell lysates (Fig. 4A). Compared with untreated cells, RPA levels were significantly increased in cells incubated with oxaliplatin, but not with VE-822 (Fig. 4B). This effect was even more pronounced when oxaliplatin administration was combined with VE-822. Similar results were obtained for γH2AX, indicating that the VE-822 + oxaliplatin combination acts synergistically to induce DNA damage (Fig. 4B).

Temporal analysis of RPA (ssDNA) and γH2AX (DNA damage) labeling showed that oxaliplatin alone induced only a slight increase in RPA staining, but not in γH2AX or RPA+γH2AX labeling (Fig. 4C, left). Notably, combined VE-822 + oxaliplatin treatment increased the number of double γH2AX- and RPA-positive cells over time (Fig. 4C, right), indicating replication catastrophe driven by excessive ssDNA accumulation (20).

**Figure 3.**

ATR inhibition combined with oxaliplatin alters the cell-cycle distribution profile and induces cell death. HCT116-R1 cells were incubated or not with oxaliplatin (5 μmol/L) and/or VE-822 (1 μmol/L) as indicated for 48 hours, and then the cell-cycle distribution was analyzed using the propidium iodide staining method (A), and the number of Edu-positive cells (B) and Annexin-V-positive cells (C) was quantified by flow cytometry. Data are the mean ± SD of at least three independent experiments. *P < 0.05, compared with control; #, compared with the drug combination. D and E, Western blot analysis of p53, p21, CDK2 (D), the ATR-CHK1 and ATM-CHK2 signaling pathways (E) in HCT116-R1 cells incubated or not with oxaliplatin (2.5 μmol/L) and/or VE-822 (1 μmol/L) as indicated for 24 hours. Tubulin and GAPDH were used as loading controls.
To explore the impact of both drugs on RS, we analyzed replication fork speed in HCT116-R1 cells (Fig. 4D). VE-822 (1 mM for 24 hours) strongly affected replication in HCT116-R1 cells, decreasing fork speed by about 40%, as previously shown with the ATRi VE-821 (21). Oxaliplatin adducts were also reported to affect polymerase progression (22), and we describe here for the first time that oxaliplatin (12.5 mM for 24 hours) decreased significantly replication fork speed by 10%. The VE-822 + oxaliplatin combination inhibited replication fork speed stronger than each drug on its own, and in some cases even led to replication fork arrest. These results show that the VE-822 + oxaliplatin combination induces a dramatic decrease in replication fork speed, leading to RS and replication catastrophe.

As exposure to genotoxic agents and replication fork stalling increase the levels of cytosolic DNA (23), we assessed whether the VE-822 + oxaliplatin combination affected the cytosolic DNA levels. In fact, we observed that VE-822 or oxaliplatin alone slightly increased the cytosolic DNA level, whereas the drug combination had a much stronger effect (Fig. 4E). This concurs
with the observation that the VE-822 + oxaliplatin combination increases ssDNA and dsDNA levels which could then lead to activation of the immune system.

**The VE-822 + oxaliplatin combination promotes the antitumor T-cell response**

Oxaliplatin has been described to induce ICD in vitro and in vivo in different preclinical cancer models (4, 24). ICD is a specific cell death characterized by the release or expression of damage-associated molecular patterns (DAMP), such as adenosine-5'-monophosphate (ATP) and HMGB-1 release and CRT translocation. We therefore decided to test the effect of the combined VE-822 + oxaliplatin administration in vivo.

For this, we first asked whether the ATRi VE-822 synergized with oxaliplatin also in mouse CT26 and MC38 colorectal cancer cells. To this aim, we used the concentration matrix approach and SRB cytotoxicity tests with four oxaliplatin and five VE-822 concentrations in 2D (SRB) and 3D (spheroid growth) cell assays (Supplementary Fig. S3A). Like in human colorectal cancer cell lines, we observed a strong synergistic interaction between oxaliplatin and VE-822 in both mouse cell lines, with higher values in the 3D than in the 2D assay.

We then monitored ICD in vitro by assessing CRT exposure, ATP secretion, and HMGB1 release (25) in mouse MC38 cells and in oxaliplatin-resistant human HCT116-R1 cells (Fig. 5A–C; Supplementary Fig. S3B and S3C for HCT116 cells). Quantification of CRT translocation to the outer leaflet of the plasma membrane by flow cytometry of live cells (Fig. 5A) showed that oxaliplatin and the drug combination, but not VE-822 alone, strongly increased CRT translocation, in both cell lines. Also, ATP secretion was promoted by the drug combination in both cell lines (Fig. 5B). HMGB1 release was significantly increased only in MC38 cells incubated with the VE-822 + oxaliplatin combination (Fig. 5C). These results reveal that the ICD induction by oxaliplatin is significantly increased in combination with the ATRi VE-822.

To test whether the VE-822 + oxaliplatin combination increased ICD also in vivo, we grafted the syngeneic MC38 cells in immunocompetent C57/B16 mice (Fig. 5D). When tumors reached a volume of 100 mm³, we treated them or not with oxaliplatin, VE-822, or the oxaliplatin + VE-822 combination. Like in nude mice (Fig. 2B), VE-822 had no effect on tumor growth. Conversely, oxaliplatin and particularly the oxaliplatin + VE-822 combination decreased tumor growth (Fig. 5D).

Importantly, Kaplan–Meier survival analysis showed that oxaliplatin + VE-822 was more efficient than control (P = 0.00471), and also oxaliplatin (P = 0.0237) and VE-822 alone (P = 0.0218; Fig. 5E). The higher efficiency of the drug combination in immunocompetent mice suggests the establishment of a specific antitumor immune response. To test whether VE-822 amplifies oxaliplatin immunogenic effect, we sacrificed mice at week 3 after treatment and quantified IFNγ-producing CD8-positive T cells in the spleen in untreated animals and mice treated with oxaliplatin alone or combined with VE-822 (Fig. 5F). After stimulation with irradiated MC38 cells (target cells), IFNγ-producing CD8-positive T cells (effector cells) were significantly increased (P = 0.0121) in mice that received oxaliplatin + VE-822 compared with those treated only with oxaliplatin. Altogether, these results provide evidence that ATR inhibition can reverse resistance to oxaliplatin and amplify oxaliplatin-induced ICD in vitro and in vivo.

**Identification of DNA repair genes associated with overall survival and response to oxaliplatin-based treatment in patients with colorectal cancer**

Our results indicated that the ATRi VE-822 strongly synergizes with oxaliplatin in colorectal cancer cells. Therefore, we asked whether some DNA repair genes that are deregulated in colorectal cancer are associated with overall survival (OS) and/or response to oxaliplatin-based treatment. To this aim, we used microarray gene expression data from primary tumors before treatment (FOLFOX regimen) from two independent cohorts of patients with colorectal cancer: (i) Tsuji cohort (n = 80 patients (26)), and (ii) Del Rio cohort (n = 36 patients; ref. 27). We defined a list of 176 genes involved in the six major DNA repair pathways [base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination repair (HR), non-homologous end joining (NHEJ), and FANC] using the REPAIRtoire database (http://repairtoire.genesilico.pl) and literature data (14).

Using the Maxstat R function and Benjamini–Hochberg multiple testing correction, we found that 12 of these 176 genes had a prognostic value (5 genes with poor and 7 with good prognostic values; Fig. 6A, left). We used this DNA repair gene signature to create the DNA repair score. In the Tsuji cohort, patients with a high DNA repair score were characterized by stronger expression of the five bad prognostic genes and weaker expression of the seven good prognostic genes (Fig. 6A, right). Using the Maxstat R function, we found the maximum difference in OS with a DNA repair score = −4.2464 that split patients in the Tsuji cohort into a low survival group (67.5% of patients, median OS = 12 months), and a high survival group (32.5% of patients, median survival not reached; P = 6.58 × 10⁻⁵; Fig. 6B). The DNA repair score, computed using the Tsuji cohort parameters, was also prognostic in the Del Rio cohort (Fig. 6C, left). The median OS of patients with high DNA repair score (i.e., higher than −4.2464) was 17 months, whereas it was not reached for patients with low DNA repair score (P = 0.00181). The Del Rio cohort also included data on the response after the first-line FOLFOX chemotherapy, according to the WHO criteria. Using the same DNA repair score threshold (−4.2464), we found a significant difference in the median progression-free survival (PFS) between groups (6 months for patients with high DNA repair score and 21 months for patients with low DNA repair score; P = 0.0189; Fig. 6C, right). These findings confirm that the ATR and DNA damage response pathways have an important prognostic and therapeutic value in patients with colorectal cancer.

**Discussion**

Despite the advances in mCRC management, the 5-year survival rate is still 12% (28). One of the causes of treatment failure is the resistance to therapy that occurs in 90% of patients with mCRC (29). Hence, the aim of this study was to identify new therapeutic targets to overcome oxaliplatin resistance in colorectal cancer. Using a "chemical synthetic lethality screening," also called "dropout screen," we found that ATR inhibition synergizes with oxaliplatin to induce cancer cell death. The combination of oxaliplatin with the ATRi VE-822 was cytotoxic and cytostatic, both in vitro and in vivo.

The ATR protein kinase is a member of the PI3K-related kinase (PIKK) family and activates CHK1 (30). ATR is activated by RPA-dependent phosphorylation of the ATR protein kinase is a member of the PI3K-related kinase (PIKK) family and activates CHK1 (30). ATR is activated by RPA-dependent phosphorylation of ATR, resulting in the inhibition of DNA replication and cell cycle progression. The inhibition of ATR by VE-822 results in the activation of CHK1, which inhibits the cell cycle checkpoint, leading to cell cycle arrest. The combination of oxaliplatin and VE-822 results in the inhibition of DNA replication, cell cycle progression, and cell death.

In conclusion, the combination of oxaliplatin and VE-822 results in the inhibition of DNA replication, cell cycle progression, and cell death. This combination has the potential to improve the efficacy of oxaliplatin-based treatments in patients with colorectal cancer. Further studies are needed to investigate the mechanisms of action of the VE-822 combination and to evaluate its clinical efficacy in patients with colorectal cancer.
and repair of replication forks (31–33). Here, we found that the ATRi + oxaliplatin combination promoted cell death and inhibited cell proliferation, as observed in the presence of high repli-
cation stress, DNA damage, and activation of the ATM pathway.
These data are in agreement with the model presented by Toledo
and colleagues (20), suggesting that VE-822 might increase rep-
licative stress generated by oxaliplatin and causing RPA shortage.
If ssDNA is not stabilized by RPA, its phosphorylation by ATR
becomes unstable. Thus, in unstable ssDNA regions, DNA dam-
age increases, promoting the activation of the ATM–CHK2 path-
way and the presence of cytosolic DNA. Finally, these molecular
events lead to the inhibition of replication and induction of cell
death. Moreover, as cancer cells may be defective in G1–S check-
points, they might require the ATR–CHK1 pathway to repair DNA
damage (34), thus making them more sensitive/suitable to ATR
inhibition than normal cells.

To the best of our knowledge, this is the first report that clari-
ifies the molecular mechanisms underlying the synergism between

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**Figure 5.**
Oxaliplatin combined with the ATR inhibitor VE-822 induces immune death signaling. **A,** CRT exposure at the cell surface of mouse MC38 and human HCT116-R1 colorectal cancer cells was assessed after 48-hour incubation with oxaliplatin (25 μmol/L) and/or VE-822 (1 μmol/L), or not (control) by flow cytometry analysis. **B,** ATP and HMGB1 release in the supernatant was assessed using luminescence and ELISA assays, respectively, in MC38 and HCT116-R1 cells incubated with oxaliplatin (2.5 or 100 μmol/L) and/or VE-822 (1 μmol/L) for 24 hours or 48 hours. Data are the mean ± SD of at least three independent experiments. *, P < 0.05, compared with control; #, compared with the drug combination. **C,** ATP and HMGB1 release in the supernatant was assessed using luminescence and ELISA assays, respectively, in MC38 and HCT116-R1 cells incubated with oxaliplatin (2.5 or 100 μmol/L) and/or VE-822 (1 μmol/L) for 24 hours or 48 hours. Data are the mean ± SD of at least three independent experiments. *, P < 0.05, compared with control; #, compared with the drug combination.

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oxaliplatin and the ATRi VE-822 in human cells, a long time after the studies on fission yeast by Perego and colleagues showing the relevance of ATR homologue (rad3) in the sensitivity to oxaliplatin (35). It also shows that oxaliplatin can induce replicative stress, the activation of ATR–CHK1 pathway and ssDNA accumulation (and RPA levels) on its own, and, as well as increased DNA damages and ICD when combined with VE-822.

Acquired resistance to oxaliplatin has limited the management of colorectal cancer and other tumor types. Our loss-of-function genetic screen allowed showing that the kinase ATR is, at least in part, responsible of this resistance, and suggests that the ATRi and oxaliplatin combination therapy could be useful in the clinic. Similar screenings have already demonstrated that ATR, CHK1, and WEE1 are involved in the sensitivity to cisplatin in triple-negative breast cancer (36). Moreover, ATR inhibition sensitized cancer cells to chemotherapy, for instance in ovarian cancer (in combination with cisplatin and gemcitabine), in pancreatic cancer (with gemcitabine), in breast cancer (with camptothecin) and in lung cancer (with cisplatin, oxaliplatin, gemcitabine, etoposide, and SN38; ref. 37). However, none of these studies reported that the combination of ATRi with chemotherapeutic drugs or radiotherapy can overcome drug resistance, or its molecular effects. Moreover, the role of ATR in oxaliplatin resistance is somewhat controversial because at least two studies have shown opposite effects. First, Lewis and colleagues (2009) found that ATR contributes to the survival of fibroblast and osteosarcoma cell lines after cisplatin, but not oxaliplatin treatment (38). Conversely, Hall and colleagues, demonstrated in 35 lung cancer cell lines that ATR inhibition sensitizes cells to DNA-damaging drugs (cisplatin, oxaliplatin, gemcitabine, etoposide, and SN38; ref. 37). Moreover, they found that while the combination of the ATRi VX-970 with cisplatin or etoposide is always synergistic, the combination with oxaliplatin is synergistic in only 60% of the tested cell lines and antagonistic in the others (37). These findings suggest that the response to the oxaliplatin and ATRi combination is cell type-specific. Our results showed synergistic effects in all tested colorectal cancer cell lines, even in oxaliplatin-resistant colorectal cancer cell lines.

Figure 6.
Identification of prognostic genes that are regulated by oxaliplatin treatment in two independent cohorts of patients. A, Left, The DNA repair gene signature obtained after Maxstat R function and Benjamini–Hochberg multiple testing correction. Five genes have poor and seven genes good prognostic value. Right, clustergram of the DNA repair score genes ordered from best to worst prognosis. The level of the probe set signal is displayed from low (deep blue) to high (deep red) expression. B, Patients with colorectal cancer (N = 80; Tsuji cohort) were ordered according to the increasing DNA repair score. C and D, High DNA repair score in the primary colorectal cancer could predict shorter OS and PFS. Patients from the Tsuji cohort (N = 80) and the Del Rio cohort (N = 36) were divided into two groups based on the DNA repair score threshold of −4.2464 (group D, low DNA repair score, score lower than −4.2464; group 1, high DNA repair score, score higher than −4.2464).

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lines, suggesting that colorectal cancer is a potential target for the synergistic interaction between oxaliplatin and ATR.

The synergistic effects of oxaliplatin and VE-822 also promoted ICD signals. It has been recently shown that oxaliplatin antitumor activity is due, at least in part, to ICD activation (1). Tumor cells incubated with the accurate concentration of oxaliplatin are able to produce ATP (laborious to be measured), release the DNA-binding protein HMGB1 in the tumor microenvironment, and expose the chaperone protein CRT to the outer part of the plasma membrane. These three signals favor the priming of the host antitumor immune response, and thus an efficient elimination of tumor cells, which could result in a long-term antitumor immunity. Here, we found that in the tested colorectal cancer cell lines, oxaliplatin could induce these three signals, as expected, and for the first time that ATR + VE-822 potentiates this effect.

Cytosolic DNA can be recognized by DNA sensors, leading to the expression of type I IFNs (39) that has tumor-suppressive effects (40). Moreover, replication fork stalling may induce the formation of extended ssDNA regions, dsDNA breaks, and the recruitment of DNA damage response protein complexes. These in vitro results were confirmed by the results in immunocompetent mice, where treatment with the VE-822 + oxaliplatin combination promoted tumor growth inhibition and tumor-specific CD8-positive T-cell activation. Although the combination of oxaliplatin + ATR inhibition results in immunogenic cell death, it is not clear how much of the observed tumor size reduction is actually due to a cancer cell autonomous effect of the combined therapy versus immunogenic cancer cell death.

The clinical efficacy of oxaliplatin has been largely demonstrated, and its mechanism of action is well known. As a platinum derivative, its primary target is DNA, where it can form intra- and interstrand cross-links. The repair of these lesions mostly occurs through the NER pathway that involves ERCC1, 2, 3, 4, 5. Indeed, the association between ERCC1 expression and response to platinum derivatives has been widely reported particularly in advanced non-small-cell lung, bladder, head-and-neck and esophagus cancer (for cisplatin) and in colorectal cancer (for oxaliplatin).

In agreement, the gene expression–based predictive DNA repair score we developed for OS and PFS of patients with colorectal cancer undergoing oxaliplatin treatment validates the importance of ERCC1 in oxaliplatin resistance, and identifies four other NER genes (GTF2H3, HMGN1, RPA2, and GTF2H2) as involved in resistance to this drug. This confirms the impact of the NER pathway in the repair of oxaliplatin-induced lesions. Moreover, 50% of the genes in the DNA repair signature belong to the HRR pathway (SCARB, XRCC2, DMCI, BARD1, C12orf50, and RPA2). This could be surprising, but is consistent with our results showing that ATR, the major protein of the HRR pathway, is implicated in resistance to oxaliplatin. Thus, the DNA repair score could become a biomarker of oxaliplatin response in patients with colorectal cancer and might allow identifying the patients who will most benefit from the oxaliplatin + ATR combination.

Altogether, our data indicate that the ATR + oxaliplatin combination is efficient in colorectal cancer cells, and also in oxaliplatin-resistant colorectal cancer cells. Our results provide a rational mechanistic basis for clinical trials with the oxaliplatin + ATR combination.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Inhibition of Ataxia-Telangiectasia Mutated and RAD3-Related (ATR) Overcomes Oxaliplatin Resistance and Promotes Antitumor Immunity in Colorectal Cancer

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