

Reactivating p53 and Inducing Tumor Apoptosis (RITA) Enhances the Response of RITA-Sensitive Colorectal Cancer Cells to Chemotherapeutic Agents 5-Fluorouracil and Oxaliplatin



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Abstract

Colorectal carcinoma (CRC) is the most common cancer of the gastrointestinal tract with frequently dysregulated intracellular signaling pathways, including p53 signaling. The mainstay of chemotherapy treatment of CRC is 5-fluorouracil (5FU) and oxaliplatin. The two anticancer drugs mediate their therapeutic effect via DNA damage-triggered signaling. The small molecule reactivating p53 and inducing tumor apoptosis (RITA) is described as an activator of wild-type and reactivator of mutant p53 function, resulting in elevated levels of p53 protein, cell growth arrest, and cell death. Additionally, it has been shown that RITA can induce DNA damage signaling. It is expected that the therapeutic benefits of 5FU and oxaliplatin can be increased by enhancing DNA damage signaling pathways. Therefore, we highlighted the antiproliferative response of RITA alone and in combination with 5FU or oxaliplatin in human CRC cells. A panel of long-term established CRC cell lines ($n = 9$) including p53 wild-type, p53 mutant, and p53 null and primary patient-derived, low-passage cell lines ($n = 5$) with different p53 protein status were used for this study. A substantial number of CRC cells with pronounced sensitivity to RITA ($IC_{50} < 3.0 \mu\text{mol/l}$) were identified within established (4/9) and primary patient-derived (2/5) CRC cell lines harboring wild-type or mutant p53 protein. Sensitivity to RITA appeared independent of p53 status and was associated with an increase in antiproliferative response to 5FU and oxaliplatin, a transcriptional increase of p53 targets *p21* and *NOXA*, and a decrease in *MYC* mRNA. The effect of RITA as an inducer of DNA damage was shown by a strong elevation of phosphorylated histone variant H2A.X, which was restricted to RITA-sensitive cells. Our data underline the primary effect of RITA, inducing DNA damage, and demonstrate the differential antiproliferative effect of RITA to CRC cells independent of p53 protein status. We found a substantial number of RITA-sensitive CRC cells within both panels of established CRC cell lines and primary patient-derived CRC cell lines (6/14) that provide a rationale for combining RITA with 5FU or oxaliplatin to enhance the antiproliferative response to both chemotherapeutic agents.

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Introduction

Colorectal carcinoma (CRC) is the most common malignancy of the gastrointestinal tract and the third most common cancer worldwide [1–3]. CRC displays frequently dysregulated intracellular signaling

Table 1. p53 Protein Status and Sensitivity to RITA of Established and Primary Patient-Derived, Low Passage CRC Cells

Cell Line	Microsatellite Status	p53 status	Mutation	Location	p53 Protein	IC ₅₀ RITA (μmol/l)
HROC113	MSI	Wild-type	–	–	Yes	2.72 [1.22-6.07]
HCT116	MSI	Wild-type	–	–	Yes	0.061 [0.037-0.10]
LS174T	MSI	Wild-type	–	–	Yes	0.023 [0.014-0.038]
Colo320	MSS	Mutant	R248W	E7	Yes	20.62 [15.83-26.86]
HROC69	MSS	Mutant	R306X (stop)	E8	Yes	18.24 [12.05-27.59]
HCT15	MSI	Mutant	S241F	E5/E7	Yes	17.88 [11.7-27.4]
HROC32	MSS	Mutant	R282W	E8	Yes	17.54 [7.25-42.45]
HROC107	MSS	Mutant	E285K	E8	Yes	10.06 [4.66-21.75]
DLD1	MSI	Mutant	S241F	E7	Yes	4.95 [3.90-6.28]
HROC183	MSS	Mutant	R282W	E8	Yes	2.12 [0.91-4.93]
SW480	MSS	Mutant	R273H; P309S	E8/E9	Yes	0.23 [0.14-0.37]
HT29	MSS	Mutant	R273H	E8	Yes	0.22 [0.13-0.39]
CaCo2	MSS	Null	E204X (stop)	E6	No	26.51 [16.60-42.33]
HCT116TP53 ^{-/-}	MSI	Null	–	–	No	3.33 [1.52-7.31]

MSI, microsatellite instable; MSS, microsatellite stable.

* Heterozygous mutation.

pathways, including the WNT, MAPK, Pi3K, and p53 signaling pathways [4]. The p53 gene (*TP53*) is one of the most frequently mutated genes in cancer, and about 50% of all human tumors display p53 mutations [5]. *TP53* encodes the tumor suppressor protein p53 that plays an important role as transcription factor in preventing cancer formation. p53 mediates a wide spectrum of distinct features within the cell, e.g., cell growth arrest and cell death [6]. Inhibition of wild-type p53 function in tumors is largely mediated by double minute 2 (MDM2) protein that binds to the N-terminal domain of p53 and targets it for proteasomal degradation by ubiquitination [7,8].

In 2004, Issaeva et al. identified a small molecule inhibitor disrupting the p53-MDM2 interaction, designated RITA (reactivation of p53 and induction of tumor cell apoptosis), that induces both accumulation of wild-type p53 and reactivation of its function [9]. The authors analyzed the antiproliferative effect of RITA in the wild-type p53-expressing CRC cell line HCT116 (*TP53*^{+/+}) and its isogenic p53-lacking (null) variant HCT116 *TP53*^{-/-}. Upon RITA treatment, HCT116 *TP53*^{+/+} cells showed, in contrast to HCT116 *TP53*^{-/-} cells, a downregulation of a significant number of p53-regulated genes, including different oncogenes such as *MYC*, and an upregulation of p53 targets involved in cell cycle arrest, i.e., cyclin-dependent kinase (CDK) inhibitor *p21*, and apoptosis-related genes, i.e. *NOXA* [8,9]. In 2012, with an *in silico* screening methodology, Yu et al. identified anticancer drugs that restore wild-type p53 activity in cell lines expressing mutant p53 [10]. Therefore, developing therapeutics to restore p53 function in malignant cells independent of the p53 status is a promising approach in translational cancer research [11].

The chemotherapy treatment of CRC is mainly limited to the currently available drugs 5-fluorouracil (5FU) and oxaliplatin (OXA). Both antineoplastic drugs demonstrate significant CRC cell death induction caused by DNA damage [12,13]. In addition to its ability to activate wild-type p53 and reactivate mutated p53 function, it has been shown that RITA can induce DNA damage signaling [14]. It is expected that the therapeutic benefits of 5FU and OXA can be increased by enhancing DNA damage signaling pathways. Therefore, we tested the antiproliferative effect of RITA alone and in combination with 5FU and OXA on established CRC cell lines and primary patient-derived CRC cell lines [15–17] to increase the DNA damage-triggered signaling and, therefore, the therapeutic effect of both anticancer drugs. We found a substantial number of RITA-sensitive CRC cells (IC₅₀ < 3 μmol/l RITA) with different p53 status within both panels of CRC cell lines (6 of 14 cell lines). In RITA-sensitive cells, RITA was involved in

increasing the antiproliferative response to 5FU and OXA with induction of DNA damage, increased transcriptional levels of p53 targets *p21* and *NOXA*, and decreased levels in *MYC* mRNA. In contrast, RITA-resistant CRC cells (IC₅₀ > 3 μmol/l) demonstrated uninfluenced transcription levels of *p21* and *NOXA*. In most of these cells, RITA failed to induce DNA damage.

Material and Methods

Cell Lines and Culture Conditions

Long-term established human CRC cell lines expressing wild-type or mutant p53 ($n = 7$) plus two p53-negative (null) cell lines (Table 1) were routinely cultured in recommended medium: CaCo2, Colo320, DLD1, HCT15, and LS174T were cultured in RPMI 1640; HCT116, HCT116 *TP53*^{-/-}, and HT29 were cultured in McCoy's; SW480 was cultured in DMEM/Ham'sF12. The cell lines were directly obtained from American Type Culture Collection, USA (www.atcc.org); Cell Lines Services GmbH, Germany (www.cell-lines-service.de); and German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Germany (www.dsmz.de). All media were used with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mmol/l of glutamine, 50 mmol/l of mercaptoethanol, and 1% nonessential amino acids (Invitrogen Life Technologies GmbH, Germany). Cell line authentication was validated by short-tandem repeat genotyping by Cell Lines Service (www.clsgmbh.de) before starting the experiments. Five primary patient-derived, low-passage CRC cell lines (HROC cell lines; Table 1) isolated from resection specimens [15–17] were cultured in DMEM/Ham'sF12 with supplements as described above. Molecular analysis of tumor-associated genes, e.g., *TP53*, was done as described [15–17]. The cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C (standard incubator conditions). Cell lines were regularly tested by reverse transcription (RT) quantitative polymerase chain reaction (RT-qPCR) for mycoplasma contamination (Minerva Biolabs GmbH, Germany) and used for less than 15 passages after revitalization. All procedures involving patient material were approved by the ethics committee of the Medical Faculty, University of Rostock, Germany (reference number II HV 43/2004), in accordance with generally accepted guidelines for the use of human material. Informed patient consent was obtained in writing.

Long-term established and primary patient-derived, low-passage CRC cells (HROC) were treated with serial dilutions of RITA (10⁻⁵

to 10^{-8} mol/l) in culture medium (with supplements as described) for 72 hours under standard incubator conditions. The IC_{50} values and their 95% confidence intervals (CIs; in brackets) were calculated in $n = 3$ independent experiments performed for each cell line tested by using GraphPad Prism 5.0 software. Data on *TP53* mutation status for established CRC cell lines were taken from the IARC *TP53* mutation database (p53.iarc.fr/). Molecular analysis for *TP53* mutation for HROC cell lines was done as described [15–17]. The microsatellite status of the permanent CRC cell lines was taken from reference [18], and the microsatellite status of patient-derived, low-passage CRC cells was determined by one of the authors (M.L.). HCT15 and DLD1 were generated from the same cancer specimen and demonstrated different chromosome changes [19]. CRC cells are arranged according to p53 protein status and decreasing IC_{50} values for RITA (indicating increased sensitivity to RITA).

Reagents

RITA (NSC 652287), obtained from Calbiochem (Merck Millipore, Germany), was set up in a stock solution of 10^{-3} mol/l with 100% dimethyl sulfoxide (DMSO; Sigma Aldrich, USA), and aliquots were stored at -20°C . The chemotherapy agents 5FU (stock solution of 0.38 mol/l) and OXA (stock solution of 2.5 mmol/l) were purchased from the local hospital pharmacy and used at final concentrations of 10^{-3} to 10^{-8} mol/l. RITA was used at final concentrations of 10^{-5} to 10^{-8} mol/l, and the final concentration of DMSO ranged between 1% for 10^{-5} mol/l RITA and 0.001% for 10^{-8} mol/l RITA.

Cell Viability Assay and Determination of IC_{50} Values

Exponentially growing cells (5×10^3 cells/well in 200 μl of culture medium) were cultured in 96-well flat-bottom tissue plates (Greiner Bio-One, Germany). The next day, culture medium was replaced, and the cells were treated with RITA, 5FU, or OXA at concentrations as indicated for 72 hours under standard incubator conditions. Cell viability was determined by crystal violet (CV) staining (0.5% CV in 25% methanol) as described previously [20]. Briefly, after CV staining, the absorbance was measured with a microplate reader at a wavelength of 570 nm. The IC_{50} values (half-maximal inhibitory concentration) and their 95% CIs were calculated with nonlinear regression fit to a sigmoidal dose-response curve (log of compound concentration versus normalized response) using Prism 5 statistical software (GraphPad Software, Inc., USA).

RT-qPCR

Total RNA was extracted from 10^6 cells with Trizol, as recommended by the manufacturer (Invitrogen Life Technologies, Germany). Samples were quantified by Nanodrop ND-1000 Spectrophotometry (Thermo Fisher Scientific, USA), and RNA integrity was assessed using the Experion automated electrophoresis station (Bio-Rad Laboratories Inc., USA). RNA (0.7 μg) was reverse transcribed using the iScriptcDNA synthesis kit from Bio-Rad. The cDNA synthesis of 1:2 diluted cDNA was performed by heating at 25°C for 5 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes. qPCR was performed with MESA Green qPCR Master Mix Kit for SYBR Green containing Meteor Taq hotstart polymerase (Eurogentec GmbH, Germany). qPCRs were performed on a CFX96 real-time PCR system (Bio-Rad) operated by CFX Manager Software (version 3.0). The cycling protocol was 5 minutes at 95°C , 40 cycles of 15 seconds at 95°C , 60 seconds at 60°C , and 5 minutes at 72°C . Postamplification melting curves were controlled to exclude

primer-dimer artifacts and contaminations. mRNA expression levels of *MYC*, *p21*, and *NOXA* were normalized to those of the reference genes *PPIA* (peptidylprolyl isomerase A) and *ACTB* (β -actin), calculated with the $\Delta\Delta\text{Cq}$ method [21] and displayed as fold change. The following primer pairs were used: *PPIA* (PubMed ID: NM_021130.3), forward: GCTGGACCCAACACAAATGG, reverse: CAAACACCACATGCTTGCCA (82 bp); *ACTB* (NM_001101), forward: CCTTGCCATCCTAAAAGCC, reverse: CACGAAAGCAATGCTATCAC (96 bp); *MYC* (NM_002467.4), forward: CACCAGCAGCGACTCTGA, reverse: GATCCAGACTCTGACC TTTTG (102 bp); *p21* (NM_000389.4), forward: GGATTCGCCGAGGCACCCGAG, reverse: GCCGCATGGGTTCTGACGGA (80 bp); *NOXA* (NM_021127.2); forward, CGAAGATTACCGC TGGCCTA, reverse: TGAAGTGTCTCCCCAGCC (74 bp).

Western Blotting

Western blotting was performed as we described previously [2,22]. In brief, 1×10^6 cells each were lysed in precooled RIPA buffer (Pierce, Thermo Fisher Scientific) containing phosphatase and proteinase inhibitors and 2.5 mmol/l dithiothreitol (Sigma-Aldrich). Afterward, samples were mixed in 5 \times loading buffer (Fermentas, Thermo Fisher Scientific), denatured at 95°C for 5 minutes, and chilled on ice. Equal amounts of proteins (15 μg) were loaded on a 10% polyacrylamide gel (sodium dodecyl sulfate polyacrylamide gel electrophoresis), electrophoresed, and then blotted by semidry transfer onto a nitrocellulose membrane (Schleicher & Schuell, Germany). After a blocking step with 5% nonfat milk (Merck KGaA, Germany) for 1 hour, the membranes were incubated with primary antibodies diluted in TBST/5% nonfat milk for 1 hour at room temperature or overnight at 4°C (TBST; 5 mmol/l TRIS, 15 mmol/l NaCl, 0.1% Tween 20, pH 7.5). Antibodies were as follows: anti-MYC (Abcam, Y69, 1:1000), anti-p53 (Abcam, DO1, 1:1000), anti-phospho-p53 (Cell Signaling, Ser15, 1:1000), and anti-phospho-histone H2A.X (Cell Signaling, Ser139, 1:1000). After washing with PBS, membranes were incubated with 1:10,000 diluted horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse (both antibodies from Dako, Denmark) for 60 minutes at room temperature. A monoclonal mouse anti-GAPDH (clone GAPDH-71.1, diluted 1:10,000, Sigma-Aldrich) was used as loading control. Immunoblots were visualized by highly sensitive chemiluminescent detection reagent (Amersham, GE Healthcare, UK) with subsequent CCD-based imaging (FluorChem System; Biozym Scientific GmbH, Germany).

Calculation of Fold Sensitization Factor

To analyze whether RITA sensitizes CRC cells to 5FU or OXA, the sensitization factor (SF) was calculated as follows: $[IC_{50} \text{ 5FU}] / [IC_{50} (5FU + 1 \mu\text{mol/l RITA})]$ or $[IC_{50} \text{ OXA}] / [IC_{50} (OXA + 1 \mu\text{mol/l RITA})]$ [23].

Statistical Analysis

Experiments were performed at least three times with replicate samples. Data are plotted as means \pm SD or means \pm SEM. The means were compared using analysis of variance plus Bonferroni's *t*-test. A *P*-value of $<.05$ was considered to indicate a statistically significant result.

Results

Expression and Mutation Status of p53 in CRC Cells

Western blot was used to evaluate p53 protein levels in the panel of nine established CRC cell lines (Figure 1): two cell lines expressing wild-type p53 (22%), five cell lines expressing mutant p53 (56%),

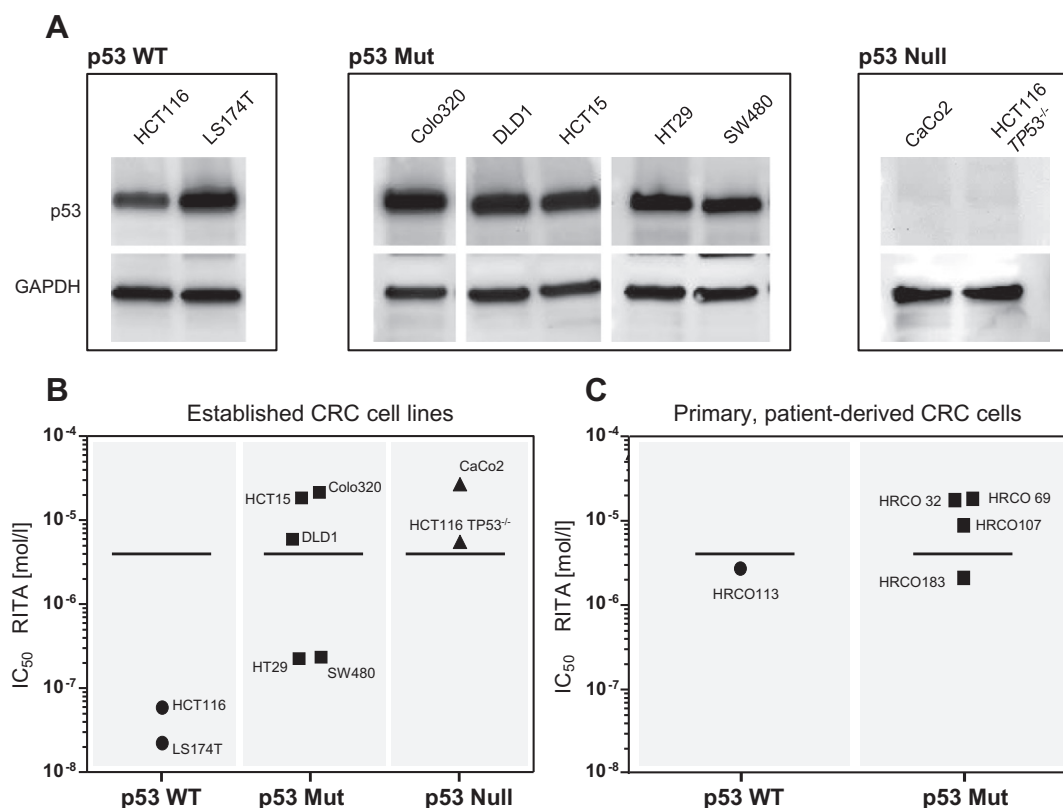


Figure 1. p53 protein status and RITA sensitivity of CRC cells. Expression levels of p53 were assessed by Western blot in CRC cell lines expressing wild-type (WT) or mutant (Mut) p53 protein or lacking p53 (p53 null) used as controls (A). Protein p53 was detected with anti-p53 antibody DO1 (53 kDa). GAPDH was used as a loading control and detected with anti-GAPDH antibody (37 kDa). Representative blot is shown out of $n = 3$ independent experiments. Sensitivity of long-term established CRC cell lines (B) and primary patient-derived, low-passage CRC cell lines (C) to RITA was expressed as IC₅₀. Cells were treated with serial dilutions of RITA (10^{-8} to 10^{-5} mol/l) in culture medium for 72 hours at standard incubator conditions. Cell proliferation was determined by CV staining in hexaplicates of $n = 3$ independent experiments performed for each cell line tested. The IC₅₀ values were calculated using GraphPad Prism 5.0 software. Horizontal lines indicate the cutoff level of 3 μ mol/l RITA to differentiate between RITA-sensitive and RITA-resistant cells.

and two p53-null cell lines CaCo2 and HCT116 TP53^{-/-} (22%), which were used as controls (Table 1). The panel of five primary patient-derived CRC cell lines includes one cell line with wild-type p53 protein (20%) and four cell lines with mutant p53 protein (80%) (Table 1).

Identification of RITA-Sensitive CRC Cell Lines

The sensitivity of established and primary patient-derived CRC cell lines to RITA (IC₅₀) was heterogeneous and ranged from 0.023 μ mol/l (LS174T) to 20.62 μ mol/l (Colo320) after 72 hours of culture (Table 1). The six RITA-sensitive CRC cell lines (IC₅₀ RITA < 3.0 μ mol/l) identified in this study were the three wild-type p53-expressing cell lines HCT116 (0.061 μ mol/l), HROC113 (2.72 μ mol/l), and LS174T (0.023 μ mol/l) and the three mutant p53-expressing cell lines HROC183 (2.12 μ mol/l), HT29 (0.22 μ mol/l), and SW480 (0.23 μ mol/l). Nonmalignant control cells used in this study were less sensitive to RITA. For human dermal (NHDF) and lung (MRC5) fibroblasts, we determined IC₅₀ values for RITA of 20.0 μ mol/l and 46.9 μ mol/l, respectively (not shown). This result underlines the obvious antiproliferative effect of RITA in both wild-type and mutant p53-expressing CRC cells, and it does not seem to be strictly dependent on wild-type p53. In addition to determining the IC₅₀ values of RITA for the CRC cell lines, we validated the antiproliferative effect of 1 μ mol/l RITA, which is described as a cytotoxic concentration to induce cell death

in different p53 WT-expressing cancer cell lines [8]. We found that the antiproliferative effect of 1.0 μ mol/l RITA was higher in CRC cells with IC₅₀ < 3.0 μ mol/l RITA and lower in CRC cells with IC₅₀ values greater than 3.0 μ mol/l. For the p53-null HCT116 TP53^{-/-} (and all other cell lines with higher IC₅₀ for RITA), we found >86% viable cells after incubation with RITA for 72 hours in contrast to HCT116, HROC113, HROC183, HT29, LS174T, and SW480, which demonstrated viable cells below 30% (not shown).

RITA-Induced Changes in RITA-Sensitive CRC Cells

RITA affects p53 protein accumulation in RITA-sensitive CRC cell lines HCT116, HROC113, and LS174T expressing wild-type p53 but not in RITA-sensitive CRC cell lines HROC183, HT29, and SW480 expressing mutant p53 (Figure S1). Next, we examined whether RITA influenced p53 activity by assessing the expression of p53 targets *p21*, involved in cell cycle arrest, and *NOXA*, involved in apoptosis. We found that RITA increased the transcriptional levels of both *p21* and *NOXA* in RITA-sensitive CRC cells HCT116, HROC113, and LS174T (wild-type p53), and HROC183, HT29, and SW480 (mutant p53) (Figure 2). Because p53 can suppress the transcription of different oncogenes that promote cell growth and proliferation [9], we tested whether RITA can inhibit expression of *MYC* mRNA, a global transcriptional regulator with an important role in colorectal cancer development [24]. Analysis of the

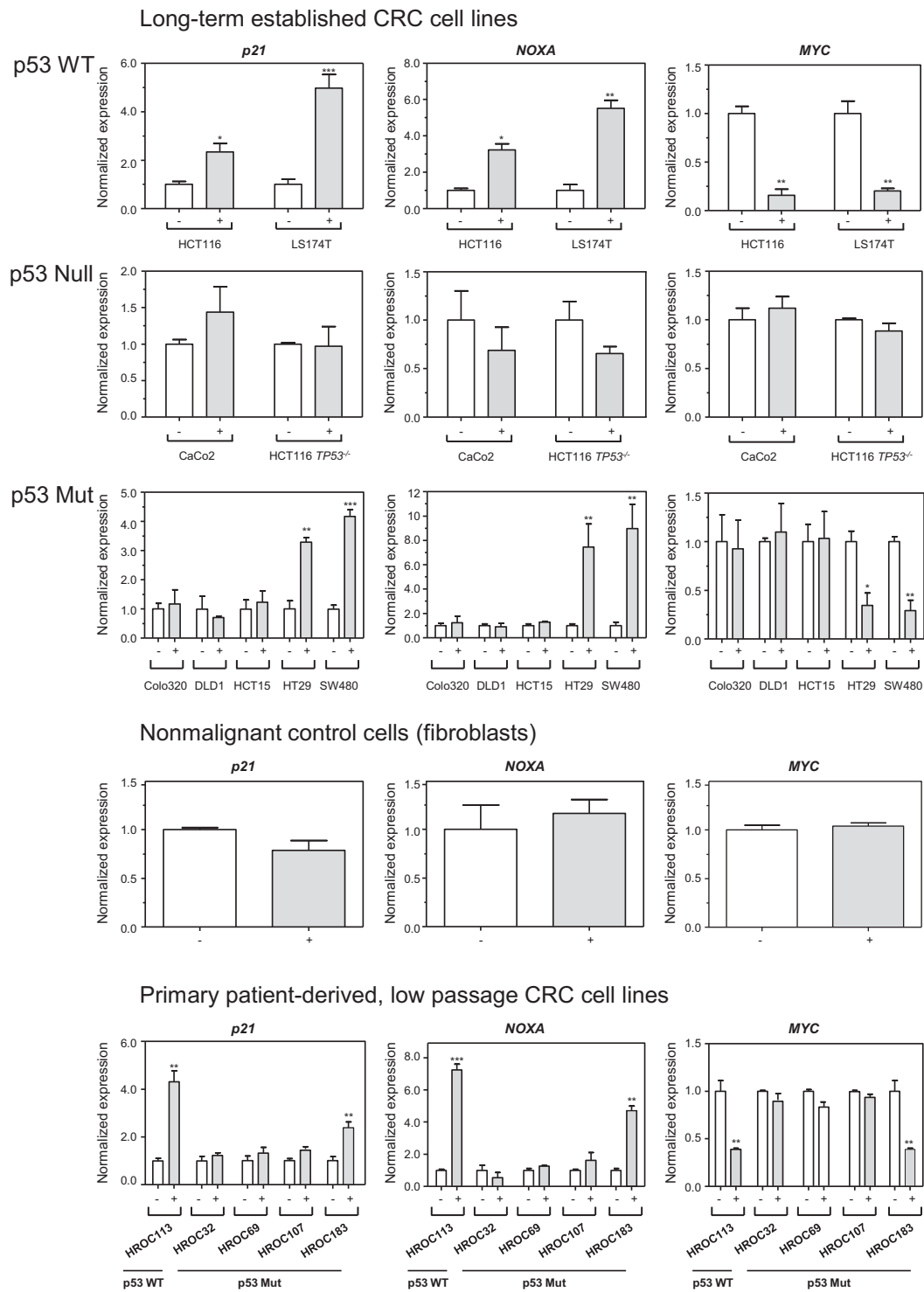


Figure 2. Transcriptional upregulation of p53 targets *p21* and *NOXA* and downregulation of *MYC* independent of expression p53 wild-type or mutant protein in RITA-sensitive CRC cell lines. Cells (long-term established and primary patient-derived, low-passage CRC cell lines) were incubated with $1.0 \mu\text{M}$ /l RITA (+) or with 0.1% DMSO (-) in culture medium for 6 h. Wild-type p53-expressing cell lines (HCT116, HROC113, LS174T), mutated p53-expressing cell lines (Colo320, DLD1, HCT15, HROC32, HROC69, HROC107, HROC183, SW480), p53-null cells lines (CaCo2, HCT116 *TP53*^{-/-}), and fibroblasts were analyzed for transcriptional levels of *MYC* oncogene and p53-target genes *p21*, and *NOXA* by RT-qPCR. mRNA levels were normalized and displayed as fold change relative to untreated (0.1% DMSO) samples. Expression from DMSO (0.1%)-treated samples was set to 1.0. Results are shown as mean \pm SEM for $n = 3$ independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$ (versus untreated samples).

transcriptional activity of the *MYC* gene revealed a decrease in mRNA levels in the six RITA-sensitive cell lines without effects in the RITA-resistant cells (Figure 2).

RITA Can Induce DNA Damage in CRC Cells

Previous reports on the mechanism of RITA suggested DNA with induction of DNA damage as a primary way of action for RITA

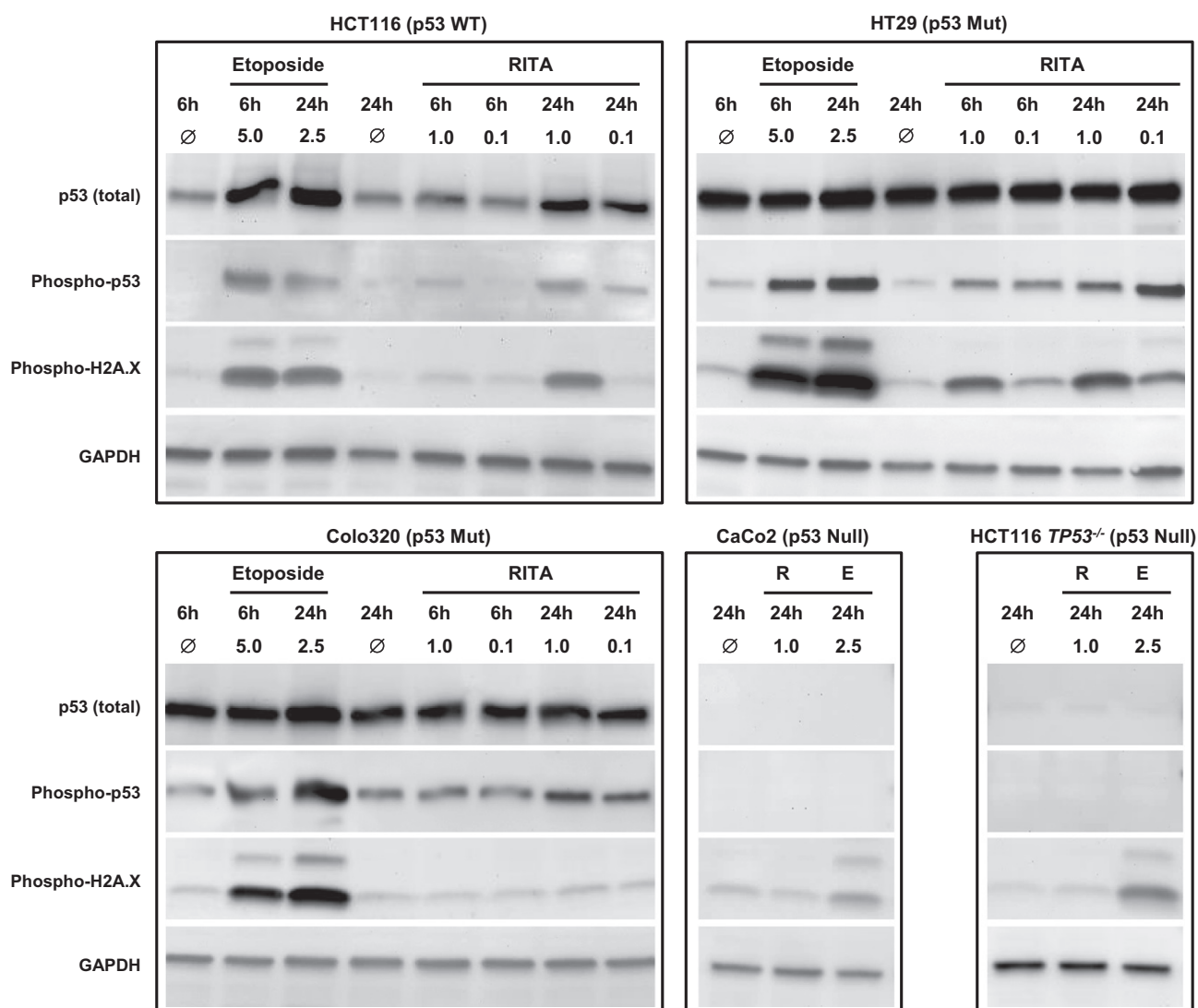


Figure 3. RITA-triggered induction of DNA damage in long-term established, RITA-sensitive CRC cells. Shown are the results for the RITA-sensitive cell lines HCT116 and HT29 and RITA-resistant cell lines Colo320, CaCo2, and HCT116 *TP53*^{-/-}. Results for LS174T, DLD1, HCT115, and SW480 are shown in Supplementary Figure 2. Cells were treated with 1.0 and 0.1 $\mu\text{mol/l}$ RITA (R), 2.5 and 5.0 $\mu\text{mol/l}$ etoposide (E), or 0.1% DMSO (\emptyset) in culture medium for 6 and 24 hours. Protein extracts from 10^6 CRC cells were analyzed by Western blot with antibodies against total p53 (57 kDa), phosphorylated p53 (phospho-p53; 53 kDa), and phosphorylated H2A.X (phospho-H2A.X; 15 kDa). GAPDH (37 kDa) was used as a loading control. Results are representative of at least three independent experiments.

[14]. Therefore, we analyzed our CRC cells for signs of DNA damage. An early common event in the DNA damage response is the accumulation of phosphorylated histone H2A.X at Ser139 ($\gamma\text{H2A.X}$). The topoisomerase inhibitor etoposide was used as control for induction of DNA damage. In all cell lines, etoposide led to a strong induction of histone H2A.X phosphorylation. In contrast, RITA was shown to induce massive accumulation of $\gamma\text{H2A.X}$ in RITA-sensitive cells, whereas there was nearly no effect in RITA-resistant cells (Figures 3 and S2).

Because p53 plays a major role in cellular response to DNA damage [25], we also analyzed the phosphorylation of p53 on key serine residue 15 (Ser15). Following RITA treatment, activated p53 was detectable in wild-type and the majority of mutant p53-expressing CRC cells (Figures 4 and S2). This is in line with evidence from the literature that mutated p53 can still exhibit normal p53 activity [26].

RITA-Enhanced Antiproliferative Response of RITA-Sensitive CRC Cells to 5FU and OXA

We showed that RITA is involved in the induction of DNA damage. The effect of 5FU and OXA as inducer of DNA damage is well described [12,13], and a possible combined effect of RITA and 5FU or RITA and OXA is assumed. Therefore, we investigated in established and primary patient-derived CRC cell lines whether RITA influenced the antiproliferative response of 5FU and OXA, respectively. We found that the six cell lines HCT116, HROC113, and LS174T (wild-type p53) and HROC183, HT29, and SW480 (mutant p53) identified as RITA-sensitive demonstrated an obvious increase in the antiproliferative response to 5FU or OXA in combination with RITA (Table 2). RITA clearly sensitized RITA-sensitive cells to 5FU and OXA with sensitization factors between 27-fold and >100-fold (Table 2). In RITA-resistant cells, RITA did not increase sensitivity to either anticancer drug with sensitization factors between 2.5-fold and <1.0-fold.

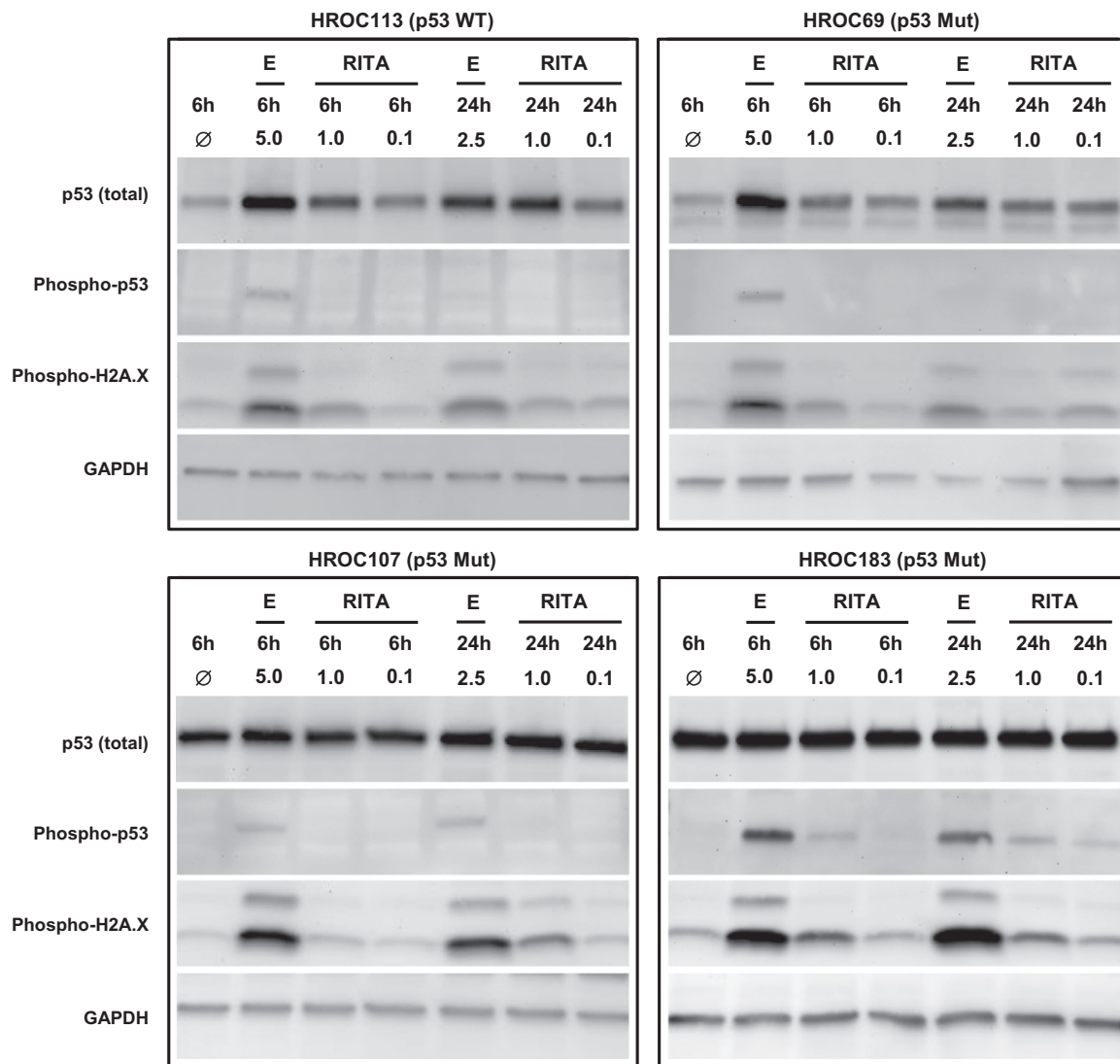


Figure 4. RITA-triggered induction of DNA damage in primary patient-derived, RITA sensitive CRC cells. Shown are the results for the RITA-sensitive cell lines HROC113 and HROC183 and RITA-resistant cell lines HROC69 and HROC107. Results for HROC32 are shown in Supplementary Figure 2. Cells were treated with 1.0 and 0.1 $\mu\text{mol/l}$ RITA, 2.5 and 5.0 $\mu\text{mol/l}$ etoposide (E) or with 0.1% DMSO (\emptyset) in culture medium for 6 hours and 24 hours. Protein extracts from 10^6 CRC cells were analyzed by western blot with antibodies against total p53 (57 kDa), phosphorylated p53 (phospho-p53; 53 kDa), and phosphorylated H2A.X (phospho-H2A.x; 15 kDa). GAPDH (37 kDa) was used as a loading control. Results are representative of at least three independent experiments.

CRC cells (established cell lines and patient-derived, low-passage CRC cells) were treated with serial dilutions of 5FU (10^{-3} to 10^{-8} mol/l) or OXA (10^{-3} to 10^{-8} mol/l) in culture medium for 72 hours under standard incubator conditions. Each dilution step of 5FU and OXA was supplemented with 1.0 $\mu\text{mol/l}$ RITA (a concentration that is described to affect cell functions [9]). Cell proliferation was determined by CV staining in hexaplicates of three independent experiments performed for each cell line tested. The IC_{50} values were calculated using GraphPad Prism 5.0 software. The results are expressed as mean with the corresponding 95% CIs (in brackets). The SF expresses the ratio between IC_{50} values of drugs alone and drugs plus 1 $\mu\text{mol/l}$ RITA: $[\text{IC}_{50} \text{ 5FU}] / [\text{IC}_{50} \text{ (5FU+ 1 } \mu\text{mol/l RITA)}]$ or $[\text{IC}_{50} \text{ OXA}] / [\text{IC}_{50} \text{ (OXA+ 1 } \mu\text{mol/l RITA)}]$ [23]. RITA-sensitive cells are HCT116, HROC113, and LS174T (wild-type p53) and HROC183, HT29, and SW480 (mutant p53). CRC cells are arranged according to Table 1.

Discussion

RITA was identified from the compound library of the National Cancer Institute for its antiproliferative effect in cell-based screens with wild-type p53-expressing and p53-deficient cells [9]. The p53 activator activity of RITA was found in HCT116 cells in a dose-dependent manner in contrast to its p53-lacking HCT116 $TP53^{-/-}$ counterpart. RITA was successfully tested *in vivo* and demonstrated potent antitumor activity against HCT116-derived tumors [9] and neuroblastoma-derived tumors [27] in a mouse xenograft model without mediating systemic toxicity.

RITA is known to disrupt the p53-MDM2 complex and hereby reduces degradation of wild-type p53 and reactivates its transcriptional function [9,11]. We found in wild-type p53-expressing CRC cells HCT116, HROC113, and LS174T both accumulation of p53 and elevated transcriptional levels of p53 targets *p21* and *NOXA*. Di Marzo et al. presented data that RITA also reactivates function of

Table 2. Enhanced Antiproliferative Response of RITA-Sensitive CRC Cells to Combined Treatment of 5FU and OXA with RITA

Cell Line	IC ₅₀ 5FU (μmol/l)	IC ₅₀ 5FU + RITA (μmol/l)	SF	IC ₅₀ OXA (μmol/l)	IC ₅₀ OXA + RITA (μmol/l)	SF
HROC113	166.7 [85.09-326.5]	0.044 [0.01-0.2]	>100	1.36 [0.60-3.10]	0.00061 [0.00022-0.0017]	>100
HCT116	0.12 [0.07-0.19]	0.0045 [0.002-0.012]	27	0.92 [0.45-1.92]	0.0039 [0.0014-0.0109]	>100
LS174T	0.67 [0.38-1.18]	0.0020 [0.0007-0.0060]	>100	1.66 [0.08-3.13]	0.0023 [0.00079-0.0064]	>100
Colo320	166.7 [43.2-343.5]	776.0 [392-1038]	<1.0	39.82 [19.68-80.57]	915.8 [353.3-2374]	<1.0
HROC69	614.8 [316.3-1195]	703.3 [330.6-1496]	<1.0	474.1 [174.8-1286]	614.0 [253.5-1487]	<1.0
HCT15	14.77 [4.37-49.92]	9.52 [3.01-30.14]	1.6	12.12 [7.09-20.73]	22.9 [8.36-62.73]	<1
HROC32	858.1 [495-1488]	885.3 [161.2-4862]	1.0	1715 [699.7-4203]	775.5 [340.2-1768]	2.2
HROC107	1717 [737.4-3997]	939.2 [182.0-4846]	1.8	602.5 [248.4-1461]	709.6 [81.3-2777]	<1.0
DLD1	4.00 [1.17-13.70]	1.60 [0.47-5.4]	2.5	20.75 [11.99-35.93]	11.6 [4.11-32.79]	1.8
HROC183	496.0 [259.5-947.9]	0.10 [0.003-0.5]	>100	83.29 [28.51-243.3]	0.056 [0.012-0.27]	>100
SW480	7.30 [3.10-17.17]	0.068 [3.10-17.17]	>100	7.43 [3.29-16.76]	0.0065 [0.0018-0.023]	>100
HT29	9.89 [3.18-30.75]	0.082 [0.02-0.34]	>100	10.78 [5.22-22.26]	0.0042 [0.0014-0.013]	>100
CaCo2	163 [95.6-514]	95.1 [30.05-300.9]	1.7	11.96 [4.65-30.8]	39.3 [14.11-109.5]	<1.0
HCT116TP53 ^{-/-}	0.72 [0.36-1.43]	1.1 [0.52-2.2]	<1.0	8.33 [4.27-16.23]	8.0 [4.73-13.74]	1.0

mutated p53 in malignant mesothelioma [28]. This is in line with results of our study as we identified three CRC cell lines (HROC183, HT29, and SW480) harboring mutant p53 with pronounced sensitivity to RITA. These cells also demonstrated an increase in transcriptional levels of *p21* and *NOXA*. The transcriptional activation of p53 targets indicates that RITA can influence mutant p53 function. The effect of RITA on mutated p53 protein is described as the induction of a conformational change that promotes a refolding of mutated p53 protein into a wild-type p53 conformation [29]. Before RITA was found to reactivate p53 function, its genotoxic activity was described [14]. Interesting in this context are NMR results that indicate RITA's failure to block the formation of the p53-MDM2 complex [30]. From our results, it is obvious that the antiproliferative effect of RITA is associated with its ability to induce DNA damage.

We identified two phenotypes of CRC cells regarding the antiproliferative response to RITA: cell lines (6/14) with pronounced sensitivity (IC₅₀ < 3.0 μmol/l) to RITA and cell lines (8/14) with little sensitivity (IC₅₀ > 3.0 μmol/l) to RITA. RITA-sensitive CRC cells expressed wild-type p53 (HCT116, HROC113, and LS174T) and mutant p53 (HROC183, HT29, and SW480). This observation indicates that the antiproliferative effect of RITA in human CRC cells is not strictly related to their p53 protein status. It is important to note that we also showed an antiproliferative effect of RITA in primary patient-derived, low-passage CRC cells that is also independent to their p53 status. These cell lines may be more representative of CRC tumors in patients than long-term established CRC cell lines.

We confirmed the antiproliferative effect of RITA in p53 wild-type-expressing HCT116 cells in contrast to its isogenic p53-null variant HCT116 TP53^{-/-}. In this p53-deficient cell line, RITA failed to induce phosphorylation of histone H2A.X as an early event of DNA damage. Wanzel and coworkers interpreted this observation as proof that RITA resistance is not mediated by p53 but rather by defects in DNA damage signaling [29]. We found that RITA had the same effect in hepatocellular cancer cells HepG2 (p53 wild-type) and Hep3B (p53 null). An induction of elevated levels of phosphorylated H2A.X was observed in HepG2 but not in Hep3B (not shown). In the present study, we defined a cutoff level of 3 μmol/l RITA to differentiate between RITA-sensitive and RITA-resistant CRC cells. This cutoff level allows us to group HCT116 as RITA-sensitive cells with IC₅₀ value of 0.061 μmol/l RITA and HCT116 TP53^{-/-} as RITA-resistant cells with IC₅₀ value of 3.33 μmol/l RITA (Table 1). The same classification is possible for HepG2 cells as RITA-sensitive cells (IC₅₀ RITA: 0.09 μmol/l [95% CI: 0.05-0.17]) and Hep3B as RITA-resistant cells (IC₅₀ RITA:

6.1 μmol/l [95% CI: 2.9-12.9]). The p53-deficient cell lines we tested were RITA resistant and did not show a RITA-induced accumulation of phosphorylated H2A.X. They share this characteristic with the most mutant p53-expressing CRC cell lines that were RITA resistant. This result indicates that the absence of a genotoxic effect of RITA is not dependent on the presence of p53.

Accumulation of phosphorylated histone variant H2A.X induces a complex molecular machinery involved in the DNA damage response. This includes the detection of damaged DNA, its repair, and the induction of cell death or senescence in case DNA repair was unsuccessful [31]. Many anticancer drugs, for example, etoposide, which was used as positive control in this study, are inhibitors of DNA topoisomerases that participate in the overwinding or underwinding of DNA and catalyze DNA breaks and ligation of DNA ends. RITA damages DNA by acting as a DNA cross-linker and not as a DNA strand breaker by inhibition of topoisomerases [14]. The differences in the induction of DNA damage response by etoposide and RITA may be delivered by different signaling pathways. Wanzel et al. found that the depletion of FancD2 that is involved in the repair of DNACross-links restores the genotoxic effect of RITA in RITA-resistant cells [29]. Therefore, cell resistance to RITA seems indeed influenced by different DNA-damage signaling pathways.

One important result of this study is that a substantial number of CRC cell lines were RITA sensitive (6 of 14 cell lines with IC₅₀ < 3.0 μmol/l RITA) independent of p53 protein status. In these cells, RITA increased the antiproliferative response to 5FU and OXA, respectively. The mechanism of how RITA enhances DNA damage signaling pathways in combination with 5FU and OXA needs further investigation. In summary, the induction of DNADamage and DNA-damage signaling seems to be the primary ability of RITA and not its potential to reactivate p53.

In this study, we did not find a specific antiproliferative response of CRC cells to RITA according to the molecular subtype of the cell lines (e.g., *KRAS* or *MSI*). But, we found that two of the three RITA-sensitive cell lines with mutated p53 demonstrated an R273H mutation (HT29 and SW480). It would be interesting to further investigate if RITA sensitivity of CRC cells correlates with the R273H mutation or other known tumor mutations. This information would be important for planning further strategies in cancer treatment with P53 reactivators.

Conclusions

The results underline a primary effect of RITA, inducing DNA damage by phosphorylation of H2A.X independent of p53 protein

status. Further studies are needed to address the differences of RITA-induced DNA damage responses in RITA-resistant and -sensitive CRC cells. Data from a screen of RITA responses to a panel of human established and primary patient-derived CRC cells have revealed an increase in the antiproliferative response to 5FU and OXA in RITA-sensitive cells. Our results *in vitro* provide a rationale for combining RITA with the most frequently used clinical chemotherapeutic compounds 5FU and OXA.

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Competing Interests

The authors declare no conflict of interest.

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