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p53 dependent and independent sensitivity to oxaliplatin of colon cancer cells

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ABSTRACT

Oxaliplatin is an efficient chemotherapeutic agent used for the treatment of metastatic human colon cancer, but cancer cells are frequently resistant. The aim of this study was to analyse the underlying mechanisms in a panel of 10 human colorectal cancer cell lines submitted to a short (2 h) oxaliplatin treatment period, accordingly to the usual therapeutic procedure in humans. Sensitivity to oxaliplatin was a characteristic of p53 wild-type colon cancer cells. In contrast, all p53-mutated cell lines had a high IC50 to oxaliplatin, with the exception of the V9P cell line. Exposure to oxaliplatin resulted in G0/G1 arrest in p53 wild-type cell lines, and in S phase in p53-mutated cell lines. In our treatment conditions, no DNA accumulation in sub G0/G1 phase, no caspase-3 activation nor PARP cleavage were detected after oxaliplatin treatment, except for the V9P cell line. The major role of the p53–p21 pathway in oxaliplatin sensitivity was confirmed in the p53 wild-type HCT116 cell line, using siRNA duplex, and knockdown of the TAp73 protein also enhanced resistance to oxaliplatin in this cell line. Surprisingly, siRNA duplex invalidation revealed a residual effect of the mutant p53 protein in p53-mutated cell lines. Persistent sensitivity to oxaliplatin of the p53-mutated V9P cell line was associated with oxalipatin-induced apoptosis but TAp73 was not the responsible alternative pathway.

1. Introduction

Oxaliplatin, a third-generation diaminocyclohexane platinum compound, recently emerged as a major drug for the treatment of metastatic colorectal carcinoma in combination with the fluoropyrimidine 5-fluorouracil (5-FU). This combination significantly increased response rates to 40–50% and prolonged overall survival [1]. Oxaliplatin, like cisplatin, acts by generating covalent adducts between platinum and two adjacent guanines or guanine and adenine in cell DNA, leading to disruption of DNA replication and transcription. The advantage of oxaliplatin is a bulkier efficacy as compared to cisplatin, namely in colon cancer [2]. Furthermore, oxaliplatin induces fewer complications, restricted to peripheral sensory neuropathy reversible in a great majority of cases, as compared to other platinum derivates such as cisplatin and carboplatin that induce nephrotoxicity and myelosuppression, respectively [3].

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Resistance to cytotoxic agents remains a major concern in metastatic colon cancer. Indeed, despite clinical benefits of oxaliplatin and other agents, about 50% of patients show no tumour regression under chemotherapy. While the mechanisms of resistance to cisplatin are well studied, only limited data are available to explain resistance to oxaliplatin. Previous reports suggested different mechanisms: an increase in gluthatione-S-transferase, a reduction of cellular drug accumulation and DNA-platinum adducts, different repair of DNA adducts [3], or a decreased susceptibility to apoptosis [4].

The tumour suppressor protein p53 has been shown to exert a pivotal role in determining the cellular sensitivity to a number of therapeutic agents, including 5-FU and cisplatin [5–8]. The expression and stability of the p53 protein, modified after chemotherapy-induced DNA damage, leads to a transcriptional activation of its target genes, including the main mediator of cell cycle arrest p21 and the p53 homologue p73 protein [9]. These proteins regulate cell cycle arrest and apoptosis [9–11], two events intimately related, notably in response to chemotherapeutic agents. The *p53* gene is altered in more than 50% of colorectal cancers, and mutations of p53 are usually associated with drug resistance [10]. As some p53 mutants do not completely loose their function, a mutant p53 ''gain of function'' phenotype has been demonstrated in tumour cells, and consists in increased proliferation, tumourigenicity and chemoresistance [12].

It has been recently shown that the HCT116 cell line harbouring a wild-type p53 protein was sensitive to oxaliplatin treatment, which led to cell cycle arrest and increased apoptosis [4,13–15]. Targeted inactivation of p53 in HCT116 cells resulted in increased resistance to oxaliplatin. However, p53 status alone was not sufficient to predict response to oxaliplatin [13], and several molecular markers such as an increased level of the pro-apoptotic proteins Bax and Bak [4], or the expression of nucleotide excision repair genes [16], could be valuable candidates.

However, well validated indicators of sensitivity to oxaliplatin, and of the cellular pathways responsible for this sensitivity, have been poorly used in previous studies: this included IC50, cell cycle distribution and usual molecular markers of apoptosis. Moreover, most studies focused on one defined colon cancer cell line. For this reason, we tested oxaliplatin sensitivity in 10 well-characterized cell lines, with an oxaliplatin exposure time close to that used in human tumour therapy [17]. Our results confirm the p53 dependent oxaliplatin sensitivity of most colon cancer cell lines, demonstrate that blockage of the cell cycle plays a major role, as compared to apoptosis, in oxaliplatin induced cell death, and suggest that alternative pathways to p53 are responsible for oxaliplatin sensitivity in a sub-group of colon cancer cell lines.

2. Materials and methods

2.1. Cell culture and drug treatment

The human colorectal cancer cell lines were a gift from Dr. Hamelin (Inserm U762, Paris, France), and the HCT116 isogenic derivatives with a targeted inactivation of p53 or p21 were obtained from Dr. Dumontet (Inserm U590, Lyon, France). Cell lines were maintained in minimum essential medium (DMEM) (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (streptomycin 10 μg/ml and penicillin 10,000 IU/ml) in a humidified 5% CO_2–95% air incubator at 37 °C. Fresh solutions of oxaliplatin (Eloxatin (Sanofi Aventis, Paris, France); 5 mg/ml), cisplatin (Dako Pharma; 1 mg/ml) or transplatin (Sigma, Saint-Quentin Fallavier, France; 1 mg/ml) were mixed in complete cell culture medium at the desired concentrations, added to adherent cells for 2 h, then removed and replaced with fresh medium.

2.2. Drug sensitivity assay

Growth inhibition assays were performed by seeding 10,000 cells per well in 96-well plates for 24 h. The cells were then treated with a range of concentrations of oxaliplatin (0–1350 μM), cisplatin (0–450 μM) or transplatin (0–450 μM) for 2 h. After drug treatment, the cells were washed with DMEM, and incubated in complete DMEM (containing 10% FBS) for 72 h. Subsequently, 20 μl of (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) dye (5 mg/ml) were added to each well, and the plates were incubated at 37 °C for 2 h. The surviving cells converted MTT to formazan crystals which are dissolved in 100 μl of isopropanol–HCl (9/1), generating a blue-purple color. The absorbance was monitored at 540 nm using a plate-reader (Model 680, Bio-Rad, Marnes-La-Coquette, France). The survival is expressed as a percentage of corresponding untreated controls. To calculate the IC50, the concentration required to inhibit survival by 50%, regression analysis was carried out by plotting the percentage survival against the log of the drug concentration, as shown on Fig. 1A.

2.3. Flow cytometry

Cells were seeded at 4×10^5 cells/well in six-well plates. After 24 h, cells were treated with their respective IC50s of oxaliplatin for 2 h. Twenty-four hours post-treatment, cells were harvested in 5 ml PBS/0.5 mM EDTA and pelleted by centrifugation (400 × *g*, 4 °C, 5 min). Cell pellets were washed twice with citrate/sucrose/DMSO and stained with propidium iodide, according to the kit's instructions (Cycle Test Plus staining kit, BD Pharmingen, Le Pont-de-Claix, France). For each sample, data were obtained through two gates, FSC versus SSC and FL2W versus FL2A, to exclude emission of debris and doublet nuclei. Red fluorescence (PI) emission was measured on a linear scale of 1024 channels. 20,000 nuclei were counted for each sample.

For BrdU incorporation, oxaliplatin-treated cells (10^6) were exposed to BrdU (10 μM, BD Pharmingen) during 15 min. Cells were trypsinized, and stained using FITC-conjugated anti-BrdU mouse monoclonal antibody (BD Pharmingen) according to the manufacturer's instructions. Briefly, cells were fixed with 1 ml frozen 70% ethanol, incubated for 30 min at 4 °C, and permeabilized with 1 ml of 2N HCl–0.5% Triton X-100 during 30 min. After washing steps, acid residues were neutralized with 1 ml of 100 mM Borax during 5 min. Cells were incubated with FITC-conjugated anti-BrdU mouse monoclonal antibody

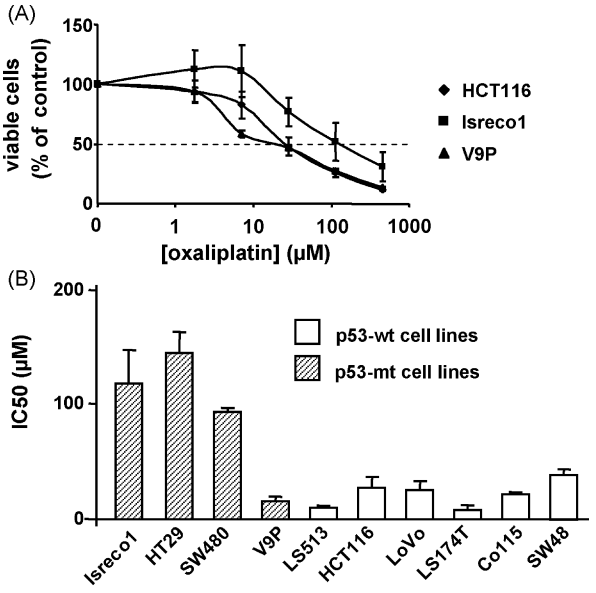


Fig. 1 – Effect of oxaliplatin on cytotoxicity of colorectal cancer cell lines. (A) Survival curves for HCT116, Isreco1 and V9P colon cancer cell lines treated with oxaliplatin (derived from MTT assays). Curves represent the average of values from at least three independent experiments in triplicates. (B) Cells were treated for a 2-h period with increasing concentrations of oxaliplatin, and number of viable cells was determined 72 h later using MTT assay as described in Section 2. IC50 values represent the oxaliplatin concentration required to inhibit cell growth by 50% relative to untreated cells 72 h after exposure. Results are the mean ± S.E.M. of at least three independent experiments, each performed in triplicate. wt: wild-type, mt: mutated-type.

during 30 min at room temperature. After centrifugation (800 × *g*, 10 min), cells were stained with 50 μg/ml propidium iodide (PI) during 10 min in dark. Cells were analysed by flow cytometry, exciting at 488 nm and measuring the BrdU-linked green fluorescence (FITC) through a 514 nm bandpass filter and the PI-linked red fluorescence through a 600 nm wavelength filter.

All analyses were performed on a Dako flow cytometer (Carpinteria, USA), using the FloMax program.

2.4. *Immunoblotting*

Subconfluent cell monolayers were trypsinized, washed twice in cold PBS and lysed in cold solubilisation buffer (50 mM HEPES, 150 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 1% Triton X-100, 10 μg/ml aprotinin, 2 μM pepstatin, 20 μM leupeptin, 5 μM benzamidine and 0.2 mg/ml phenylmethylsulfonylfluoride (PMSF), pH 7.5) for 15 min at 4 °C. Lysates were clarified by centrifugation at 4 °C for 15 min at 10,000 × *g*, and protein concentrations were determined using a modified Bradford method (Bio-Rad). Cell lysates were diluted in 4× SDS-PAGE buffer (62 mM Tris–HCl, 20% 2-mercaptoethanol, 8% SDS, 40% glycerol and 0.16% bromophenol blue), boiled for 5 min and resolved on 8% or 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes, which were blocked using 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20. Membranes were then exposed overnight at 4 °C in the same buffer with the appropriate monoclonal antibodies: anti-p53 (clone DO7, Dako); anti-p21 (clone sc-397, Santa Cruz, Tebu, Le Perray en Yvelines, France); anti-PARP (clone C2-10, BD Biosciences, Erembodegem, Belgium); anti-p73α/β (clone ER15, Neomarkers, Interchim, Montluçon, France); anti-ΔNp73 (clone 38C674, Imgenex, Clinisciences, Montrouge, France); anti-tubulin-α (Sigma). After incubation with the secondary antibody conjugated to horseradish peroxydase, blots were revealed using the ECL method (Covalab, Lyon, France).

2.5. *Immunocytochemistry*

Cells were seeded in four-well Lab-Tek chamber slides (Nunc, Dutscher, Brumath, France) at 10^4 cells/well. Twenty-four hours after seeding, cells were treated with their respective IC50s of oxaliplatin during 2 h. Oxaliplatin was then replaced by complete DMEM, and cells were incubated for 72 h. Cells were washed, fixed for 5 min in 200 μl/well of cold methanol, and washed five times with PBS-10% FBS. Primary anti-p53 antibody (1/200) was deposed in two-wells for 1 h. Cells were then washed five times with PBS-10% FBS, incubated for 30 min at room temperature and sheltered from light with secondary anti-mouse antibody conjugated with FITC (1/25, Beckman Coulter, Roissy, France), and washed with PBS. The coverslips were briefly dried off and mounted on a microscope slide with one drop of Fluoprep (Invitrogen). A Zeiss microscope with digital camera was used for viewing and for digital photography (ACT1 program).

2.6. *RNA extraction and semi-quantitative RT-PCR*

Total RNA was extracted from 10^7 cells with Trizol (Invitrogen) and 2 μg of RNA were used for RT. The cDNA corresponding to the gene of interest was synthesized using the SuperScript First-Strand synthesis System (Invitrogen) as recommended by manufacturer. p53 was then amplified using the following primers: 5′-CCTATGGAAACTACTTCCTG-3′ (forward) and 5′-ACTTGCACGTACTCCCCTG-3′ (reverse). Internal control was human cyclophilin A amplification (forward primer 5′-CTTGTCCATGGCAAATGCTG-3′; reverse primer 5′-GTGATCTTCTTCTTGCTGGTCTTG-3′). A PCR amplification cycle included denaturation at 94 °C for 45 s, followed by 25 (cyclophilin A) or 30 cycles (p53) at 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 5 min.

2.7. *Small interfering RNA (siRNA) transfections*

The double-stranded 21 bp oligonucleotide RNAs, forming a 19 bp duplex core with 2 nucleotides 3′ overhang, were synthesized by Eurogentec (Seraing, Belgium). The following oligonucleotides were used: p53 sense 5′-CAAUGGUUCACUGAAGACC-3′; p53 antisense 5′-GGUCUUCAGUGAACCAUUG-3′; p73 sense 5′-CGGAUUCCAGCAUGGACGU-3′, p73 antisense 5′-ACGUCCAUGCUGGAAUCCG-3′; control non-silencing sense 5′-UUCUCCGAACGUGUCACGU-3′, control non-silencing

antisense 5′-ACGUGACACGUUCGGAGAA-3′. The 5′-fluorescein-labeled p53 duplex (same sequence as p53) was from Xeragon (Qiagen, Courtaboeuf, France). All siRNA sequences were subjected to BLAST search to confirm the absence of homology to any additional known coding sequences in the human genome. Cells were transfected with siRNA at approximately 20–30% confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The final p53 and p73 siRNA concentrations were 200 and 50 nM, respectively. Five hours after transfection, medium was replaced by complete DMEM. For MTT assays, cells were allowed to recover for 24 h prior to transfer into 96-well plates and oxaliplatin treatment 48 h after transfection.

2.8. p53 transcriptional assays in yeast

The *Saccharomyces cerevisiae* haploid strains yIG397, YPH-p21, YPH-bax and YPH-PIG3, containing RGC (Ribosomal Gene Cluster), p21, BAX and PIG3 promoter sequences, respectively, were previously described [18]. These strains contain the ADE2 reporter gene under the p53 responsive element. They were transformed with an appropriate yeast expression vector harbouring human wild-type p53 cDNA, or mutant p53 cDNA from Isreco1 or V9P cell lines under an ADH1 promoter. The yeast functional assay was performed using yeast plated on synthetic dropout minimal medium minus leucine plus adenine (5 μg/ml) and incubated for 3 days at 30 °C in a humidified atmosphere. The expression of wild-type p53 conferred adenine prototrophy resulting in white, normal size colonies, while the expression of a mutant p53 unable to transactivate the ADE2 gene conferred adenine auxotrophy and the growth of small red colonies.

2.9. Determination of caspase-3 activity

Caspase-3 activity was measured using the Caspase-3 Colorimetric Assay (R&D Systems, Lille, France). Cells were scrapped and homogenized in 50 μl of lysis buffer. Proteins were dosed, and diluted in dilution buffer to obtain 80 μg in 50 μl final in a 96-well plate. 50 μl of reaction buffer containing 0.5 μl of fresh DTT and 5 μl of caspase-3 colorimetric substrate (DEVD-pNA) were added to each sample, and incubated for 2 h at 37 °C. The absorbance was read on a microplate reader using 405 nm wavelength light. Cells treated with the apoptosis effector tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) were used as positive controls of caspase-3 activity.

2.10. Statistical analysis

The data are the mean values ± S.E.M. of at least three separate experiments. Results were analysed using a Student's *t*-test, to assess the statistical significance of difference between control and treatment groups. A statistically significant difference was considered to be present at $P < 0.05$.

3. Results

3.1. Sensitivity to oxaliplatin of 10 human colon cancer cell lines is correlated with their p53 status except for V9P cells

We systematically determined the sensitivity to oxaliplatin of a panel of 10 human colon cancer cell lines, 6 with a wild-type p53 protein (LS513, HCT116, LS174T, LoVo, CO115, SW48) and 4 with a mutated p53 protein (Isreco1, HT29, SW480, V9P). All cell lines had been previously characterized according to microsatellite instability status, LOH at multiple loci and hMLH1 methylation [19] (Table 1). Using a MTT assay, we determined the IC50 of each cell line, which represented oxaliplatin concentration necessary to inhibit cell growth by 50% relative to untreated cells, 72 h after a 2 h treatment (Fig. 1A and B). Interestingly, all p53 wild-type cell lines showed a high sensitivity to oxaliplatin, whereas all but one (the V9P cell line) p53-mutated cell lines were resistant to this treatment. The p53 status thus appears as a major but not exclusive determinant of sensitivity to oxaliplatin in colon cancer cell lines.

3.2. Cell cycle phase distribution following oxaliplatin treatment is dependent on the p53 status of colon cancer cell lines

As the p53 protein is a major regulator of cell cycle and apoptosis following a genotoxic damage [9–11], we first

Table 1 – Main characteristics of the colon cancer cell lines

Cell line	p53 LOH[a] 17	p53 mutation	Microsatellite instability status[b]	Gene of MMR system altered[c]
Isreco1	+	Y163H	MSS	–
HT29	+	R273H	MSS	–
SW480	+	R273H	MSS	–
V9P	+	G245D	MSS	–
LS513	–	–	MSS	–
HCT116	–	–	MSI-H	hMLH1
LoVo	–	–	MSI-H	hMSH2
LS174T	–	–	MSI-H	hMLH1
Co115	–	–	MSI-H	hMLH1
SW48	–	–	MSI-H	hMLH1

[a] + and – indicate presence or absence of LOH, respectively.
[b] MSS: microsatellite-stable, MSI-H: microsatellite-instability high.
[c] – indicates absence of altered mismatch repair system gene. From Gayet et al. [19].

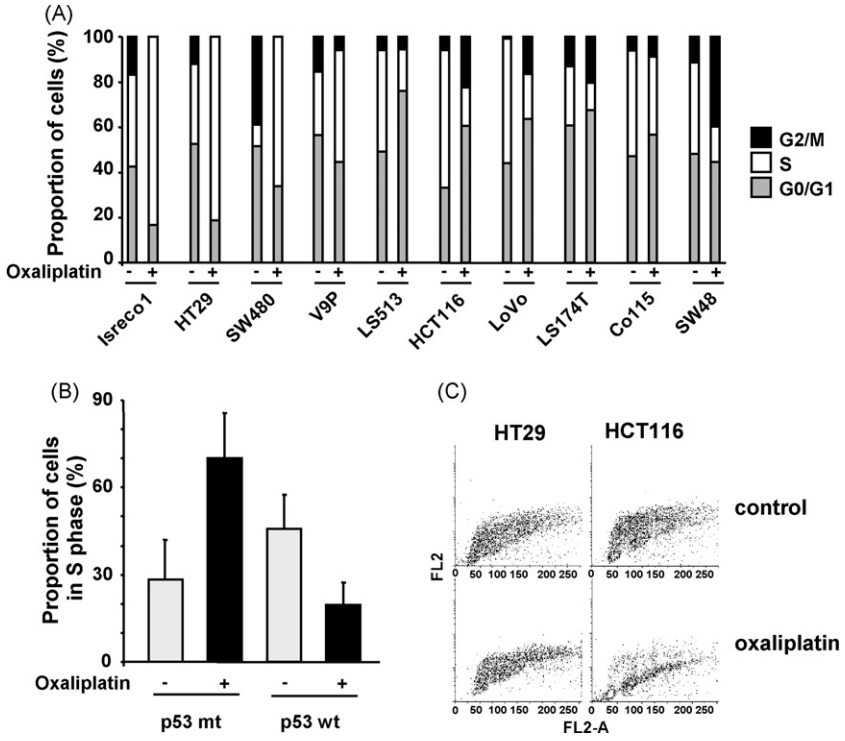


Fig. 2 – Effect of oxaliplatin on cell cycle distribution in colorectal cancer cell lines. (A) A panel of cell lines were treated during 2 h with oxaliplatin used at their respective IC50s. After propidium iodide staining, cell cycle distribution was determined by flow cytometry 24 h after the beginning of oxaliplatin exposure. Results are representative of at least three independent experiment; usual error magnitude for each point never exceeded 6%, but error bars are omitted to enhance viewing of the histogram. (B) Mean modifications of S phase in the four p53-mutated and the six p53-wild type colon cancer cell lines 24 h after oxaliplatin treatment. (C) BrdU incorporation analysed by flow cytometry in representative p53-mutated HT29 and p53 wild-type HCT116 cell lines 24 h after a 2 h-exposure to oxaliplatin.

investigated cell cycle modifications after exposure to oxaliplatin. Colon cancer cell lines were incubated for 2 h with oxaliplatin at concentrations corresponding to the previously determined IC50s. Oxaliplatin treatment decreased the proportion of S phase cells in all six p53 wild-type cell lines from a mean 45.7% (±12.0%) to a mean 19.6% (±7.8%) (Fig. 2A and B), and this effect was maintained during 96 h at least (not shown). In contrast, the proportion of S phase cells increased from a mean 28.3% (±13.7%) to a mean 69.9% (±15.6%) in the four p53-mutated cell lines (Fig. 2A and B), including the oxaliplatin sensitive V9P cell line. However this arrest was transient, as cell cycle distribution returned to the normal situation after 72 h (not shown). BrdU incorporation experiments confirmed the accumulation of cells in the S phase 24 h after oxaliplatin treatment of HT29 cells, and in G0/G1 phase in case of HCT116 cells (Fig. 2C). Our results thus demonstrate that cell cycle blockage by oxaliplatin treatment is dependent on the p53 status of colon cancer cell lines.

3.3. *The p53–p21 pathway is a major determinant of sensitivity to oxaliplatin of the p53 wild-type HCT116 cell line*

We next analysed the effect of oxaliplatin on p53 expression after a 2-h exposure of colon cancer cell lines. For this purpose, we selected 3 representative cell lines: HCT116 (p53 wild-type, low IC50), Isreco1 (p53-mutated, high IC50) and V9P (p53-mutated, low IC50). Immunoblotting of cell lysates revealed a sustained increased level of p53 protein after a 2-h exposure to oxaliplatin in HCT116 cells, which was maintained at least 120 h after treatment (Fig. 3A). In contrast, p53 protein was constitutively expressed in both Isreco1 and V9P cells, and oxaliplatin treatment did not increase the protein expression level. These results were confirmed by immunocytochemistry, as expression of the p53 protein was increased after oxaliplatin only in HCT116 cells (Fig. 3B). As expected, p53 immunoreactivity was detectable in both untreated and oxaliplatin-treated Isreco1 and V9P cells, a pattern known to reflect the accumulation of a mutated p53 protein escaping degradation in the proteasome. Moreover, using RT-PCR, we showed no increased p53 mRNA levels following oxaliplatin exposure (Fig. 3C). We then concluded that oxaliplatin-induced p53 protein surexpression in HCT116 cells was actually due to the blockage of its degradation by the proteasome. We then tested the role of p53 in the sensitivity to oxaliplatin of colon cancer cells by two different strategies: (i) by knocking down the protein, using p53 siRNA at 200 nM (concentration inducing a maximal depletion of the protein in HCT116 cells), and (ii) using an isogenic derivative of HCT116 cells with targeted inactivation of p53 (HCT116 $p53^{-/-}$ cells). Transfection of a fluorescent p53 siRNA, followed by flow cytometry analysis, resulted in a mean transfection rate of 69% in HCT116 cells (Fig. 4A). Knock-down of p53 in HCT116

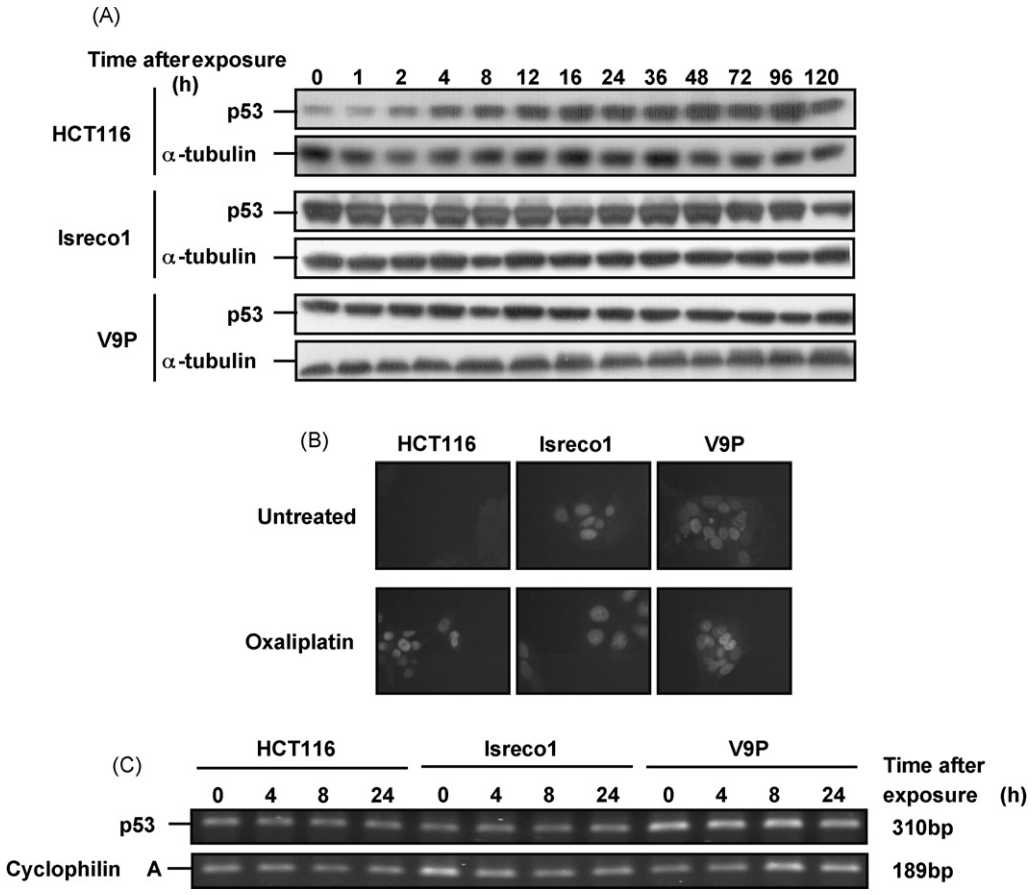


Fig. 3 – Expression of p53 in oxaliplatin-treated HCT116, Isreco1 and V9P cell lines. (A) Cells were treated with corresponding IC50 of oxaliplatin during 2 h, and p53 expression was determined by immunoblotting at different exposure times. Levels of α-tubulin on the same blot were shown as a loading control. (B) Immunocytochemical analysis showing induction of p53 expression in p53 wild-type HCT116 cells but not in p53-mutated Isreco and V9P cells. Results are representative of at least three independent experiments. (C) RT-PCR analysis of p53 in HCT116, Isreco1 and V9P cells, before and 4, 8 or 24 h after a 2 h-oxaliplatin exposure. Total RNA from each cultured cell line was subjected to RT-PCR. Cyclophilin A was used as an internal control.

cells was effective at 24 h, maximal at 72 h, before a slight re-expression of the protein at 96–120 h (not shown). Considering these results, siRNA transfection was performed 48 h before oxaliplatin treatment, and IC50 was determined using MTT 120 h after transfection. Knock-down of p53 protein in the p53 wild-type cell line HCT116 led to a significant increase of oxaliplatin IC50, which rose to 2.6-fold (70 ± 10 μM versus 27 ± 2.8 μM), as compared to the values in cells transfected with a non-silencing siRNA (Fig. 4B). Similarly, IC50 of HCT116 $p53^{-/-}$ cells was significantly higher as compared to the value in parental HCT116 cells (90 ± 20 μM versus 21 ± 7 μM), and comparable to previously determined values in resistant cell lines Isreco1 and SW480 (Fig. 1). To ensure that this increased resistance was not explained by a lower proliferation rate of transfected cells, we checked by MTT assay the number of viable cells from 0 to 120 h after transfection, and observed no change (data not shown). Moreover, oxaliplatin induced HCT116 $p53^{-/-}$ cell blockage in the S phase (Fig. 4C), reproducing the effect of oxaliplatin on p53-mutated Isreco1, HT29, and SW480 cells (Fig. 2A). Finally, we investigated the role of the cyclin-dependent kinase inhibitor p21, an important target of p53 and a key protein governing cell cycle arrest in G1 and G2 phases after DNA damage [9,11,20]. Oxaliplatin exposure of HCT116 cells increased p21 expression, from 4 to 120 h after treatment as detected by immunoblotting, with a maximal increase at 24–48 h (Fig. 5A). In contrast, expression of p21 remained undetectable by immunoblotting both in control and oxaliplatin-treated p53-mutated Isreco1 and V9P cells (Fig. 5B). Depletion of p53 using siRNA assay abolished the expression of p21 in untreated (data not shown) and oxaliplatin-treated (Fig. 5C) HCT116 cells. Moreover, isogenic HCT116 $p21^{-/-}$ cells (Fig. 5D) exhibited a significantly increased IC50 to oxaliplatin (90 ± 30 μM), in the same order of magnitude as HCT116 $p53^{-/-}$ cells, as compared to parental HCT116 cells. Our results thus confirm and extend the role of the p53–p21 pathway in oxaliplatin sensitivity of the p53 wild-type HCT116 colon cancer cell line.

3.4. *Mutant p53 has a residual effect on oxaliplatin sensitivity of Isreco1 and V9P colon cancer cell lines, independently of the p53 transcriptional activity*

We used siRNA invalidation of p53 to test the role of the constitutively expressed p53 protein on the sensitivity to

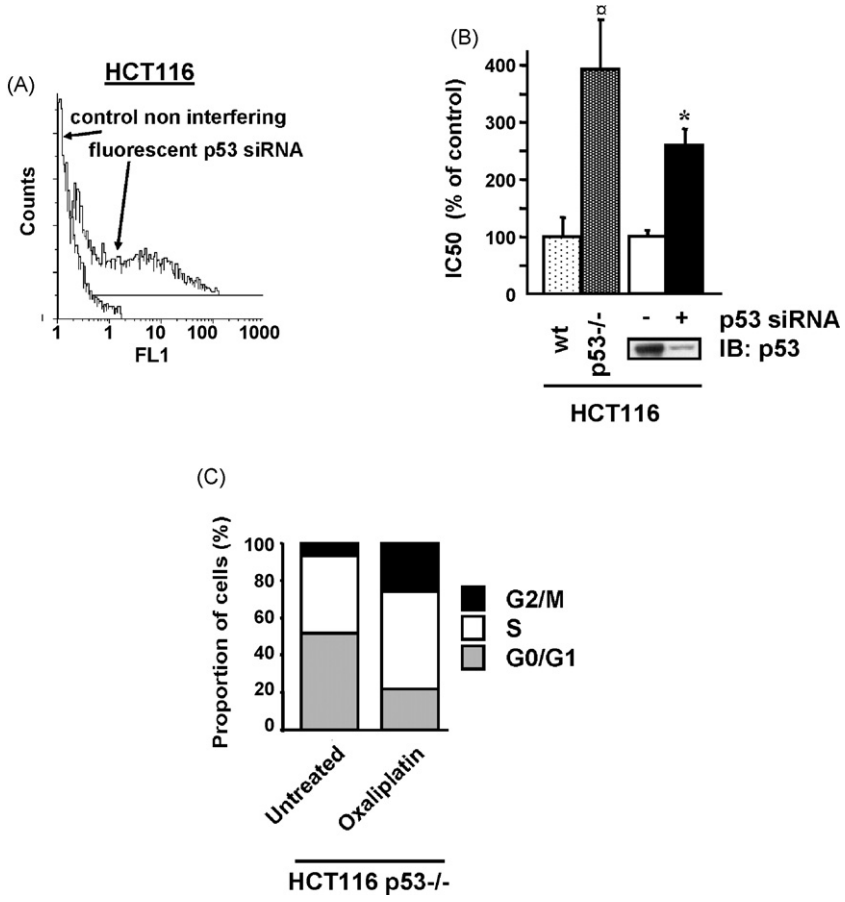


Fig. 4 – Role of p53 in the sensitivity to oxaliplatin of HCT116 cell line. (A) p53 siRNA transfection efficiency in HCT116 cell line. Cells were transfected with 200 nM of fluorescent p53 siRNA or control non-silencing siRNA as described in Section 2, and fluorescence was measured by flow cytometry 24 h after transfection. (B) Effect of p53 siRNA on cell sensitivity to oxaliplatin. Cells were transfected with 200 nM of p53 (+) or control non-silencing (−) siRNA, 48 h before a 2-h exposure to serial concentrations of oxaliplatin, and number of viable cells was determined 72 h later using MTT assay. Results are expressed in percent of IC50 from cells transfected with control non-silencing siRNA. *$P < 0.05$ as compared to values obtained from cells transfected with control non-silencing siRNA. Representative immunoblot of p53 expression is shown. The number of viable cells was also compared in wild-type (wt) and HCT116 p53$^{-/-}$ cell lines treated or not with oxaliplatin. ¤$P < 0.05$ as compared to the IC50 of the parental HCT116 cell line. Results are the mean ± S.E.M. of three experiments each performed in triplicate. (C) Effect of oxaliplatin on cell cycle distribution in HCT116 p53$^{-/-}$ cells. After propidium iodide staining, cell cycle distribution was determined by flow cytometry 24 h after the beginning of oxaliplatin exposure. Results are representative of at least three independent experiments; usual error magnitude for each point never exceeded 6%.

oxaliplatin of p53-mutated colon cancer cells. Transfection of a fluorescent p53 siRNA, followed by flow cytometry analysis, resulted in a mean transfection rate of 73% in Isreco1 and only 37% in V9P cells (Fig. 6A), but immunoblot analysis revealed that this was sufficient to notably reduce p53 protein expression (Fig. 6C). Knock-down of p53 in Isreco1 cells was effective at 24 h, maximal at 72 h, before a slight re-expression of the protein at 96–120 h (Fig. 6B). Similar results were obtained for V9P cells (not shown). Knock-down of the p53 protein led to a significant increase of oxaliplatin IC50, of 2.2 fold (310 ± 10 μM versus 140 ± 41 μM) and 1.9-fold (23.1 ± 9.6 μM versus 12.2 ± 5.9 μM) for Isreco1 and V9P cells, respectively, as compared to cells transfected with a non-silencing siRNA (Fig. 6C). We then checked whether these p53 mutants had maintained any transactivation properties, using a yeast functional assay, as described in Section 2. As shown in Fig. 6D, the yeast functional assay in Isreco1 and V9P cell lines resulted in 100% red colonies, meaning that neither V9P nor Isreco1 cell p53 mutant proteins had maintained transactivation properties on these four reporter genes. We can conclude that the transactivation abilities of the mutant p53 proteins explain neither the different sensitivity to oxaliplatin of Isreco1 and V9P cell lines, nor the residual effect of mutant p53 protein on oxaliplatin sensitivity.

3.5. *The p53-dependent sensitivity of colon cancer cells to platinum compounds is restricted to oxaliplatin*

We then aimed to determinate if the p53-dependent sensitivity of colon cancer cells to oxaliplatin was also observed with other platinum compounds. For this purpose, we used cisplatin and transplatin, although these drugs did not prove

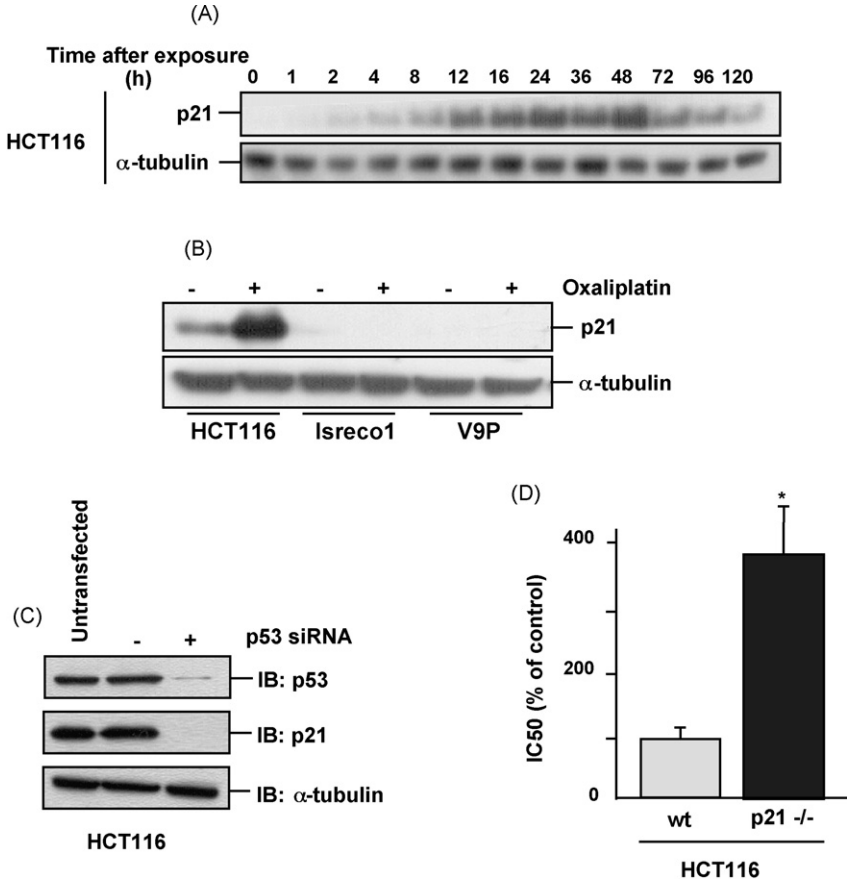
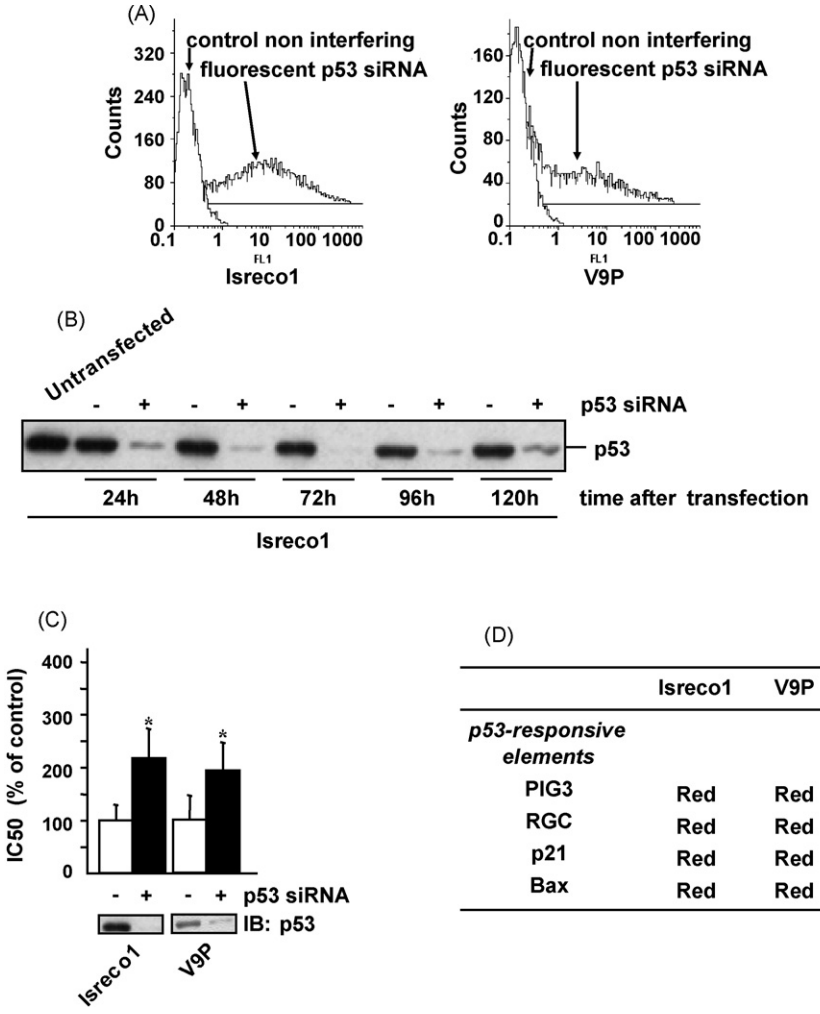


Fig. 5 – Expression and role of p21 in the sensitivity to oxaliplatin. (A) HCT116 cells were exposed to oxaliplatin at their IC50s during 2 h, and incubated for several times prior to p21 determination using Western blotting. (B) HCT116, Isreco1 and V9P cells were treated (+) or not (−) for 2 h with oxaliplatin at their respective IC50s. Immunoblot experiments were performed 48 h after oxaliplatin exposure. (C) Expression of p21 in oxaliplatin-treated HCT116 cells transfected with control non-silencing siRNA (ct) or with p53 siRNA. Cells were transfected with 200 nM of siRNA 48 h before immunoblot experiments. Results (A and C) are representative of three independent experiments. (D) Effect of oxaliplatin on the number of viable cells in wild-type and HCT116 $p21^{-/-}$ cells. Cells were treated during 2 h with serial concentrations of oxaliplatin, and incubated for 72 h prior to MTT assay. *$P < 0.05$ as compared to the IC50 of the parental HCT116 cell line. Results are mean ± S.E.M. of at least three experiments each performed in triplicate.

to be efficient in human colon tumors [21]. Fig. 7A and B show the IC50s of the three selected cell lines for cisplatin and transplatin. We observed a very high IC50 for cisplatin in the three colon cancer cell lines, including the oxaliplatin highly sensitive V9P cell line (oxaliplatin and cisplatin IC50s: 14.8 ± 6.3 and 227.5 ± 47.7 μM, respectively), and almost no effect of transplatin. We then determined cell cycle distribution by flow cytometry in the three cell lines before and 24 h after cisplatin treatment (Fig. 7C). In contrast with the situation after oxaliplatin treatment, we did not observe any S phase accumulation of Isreco1 or V9P cells after cisplatin treatment, but in contrast a significant reduction of the S phase compartment in V9P cells (from 32.2% to 17.2%; $p = 0.02$). In addition, there was no cell cycle difference after cisplatin treatment between HCT116 $p53^{-/-}$ and parental cells. We then used siRNA to knock-down the p53 protein in the three selected cell lines. In contrast to the situation with oxaliplatin, we observed no significant modification of the IC50 neither in Isreco1, nor in V9P cells. When comparing wild-type and $p53^{-/-}$ HCT116 cells, we observed a low, non-significant, IC50 increase in $p53^{-/-}$ cells (Fig. 7D). In conclusion, our results suggest that the low sensitivity to cisplatin of colon cancer cells is not p53-dependent, and that transplatin has no effect on colon cancer cell viability in vitro.

3.6. *Oxaliplatin induces apoptosis only in the sensitive V9P cell line*

It has already been reported that oxaliplatin could induce apoptosis in colon cancer cells [4,13–15]. We thus decided to determine in our experimental conditions the proportion of apoptotic cells after oxaliplatin treatment using propidium iodide incorporation, as described in Section 2. We observed no sub G1 accumulation from 8 to 72 h after oxaliplatin treatment in HCT116 and Isreco1 cells (Fig. 8A, left panel). In contrast, oxaliplatin treatment induced apoptotic death in the p53-mutated sensitive cell line V9P. In contrary, as shown in Fig. 8A (right panel), cisplatin treatment of the three cell lines



	Isreco1	V9P
p53-responsive elements		
PIG3	Red	Red
RGC	Red	Red
p21	Red	Red
Bax	Red	Red

Fig. 6 – Role of p53 in the sensitivity to oxaliplatin of Isreco1 and V9P cell lines. (A) p53 siRNA transfection efficiency in Isreco1 and V9P cell lines. Cells were transfected with 200 nM of fluorescent p53 siRNA or control non-silencing siRNA as described in Section 2, and fluorescence was measured by flow cytometry 24 h after transfection. (B) Expression of p53 in Isreco1 cells after siRNA transfection. Cells were transfected with 200 nM of p53 (+) or control non-interfering (−) siRNA, and p53 expression was determined by immunoblotting for a range of times. Results (A and B) are representative of at least three independent experiments. (C) Cells were transfected with 200 nM of p53 (+) or control non-silencing (−) siRNA, 48 h before a 2-h exposure to serial concentrations of oxaliplatin, and the number of viable cells was determined 72 h later using MTT assay. Results are expressed in percent of IC50 from cells transfected with control non-silencing siRNA. *$P < 0.05$ as compared to values obtained from cells transfected with non-silencing siRNA. Representative immunoblot of p53 expression is shown. (D) Activation by mutant p53 from Isreco1 and V9P cells of the ADE2 reporter gene under control of different p53-responsive elements. Transcription of the ADE2 gene, under the control of the promoter elements PIG3, RGC, p21 or Bax was scored in Trp+ transformants expressing p53 mutants. Trp+ transformants were selected for 3 days at 30 °C on plates lacking tryptophan but containing limited amount of adenine. White: complete ADE2 transactivation; red: no ADE2 transactivation.

resulted in a significant pro-apoptotic effect, more pronounced in HCT116 and V9P cells. This pro-apoptotic effect lasted for 72 h. In order to confirm the absence of oxaliplatin-induced apoptosis in Isreco1 and HCT116 cells, we then studied the expression of cleaved PARP (poly(ADP-ribose) polymerase) and the activation of caspase-3, two major proteins in the cascade of molecular events leading to apoptosis. The cleavage of PARP was evaluated by Western blot 4 and 24 h after a 2-h oxaliplatin treatment at the IC50 specific of each cell line (Fig. 8B). No PARP cleavage was observed in the p53 wild-type HCT116 cell line, nor in the p53-mutated Isreco1 and V9P cell lines. As a positive control, cell treatment in the presence of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL, 50 ng/ml for 4 h) induced a marked PARP cleavage, as well as the treatment with cisplatin (not shown), confirming apoptosis induced by this drug. Only high concentrations of oxaliplatin, corresponding to ten times the IC50 of each cell line, resulted in a faint cleavage of PARP (Fig. 8C). Measured by colorimetric assay, no significant caspase-3 activation was detected in any cell line,

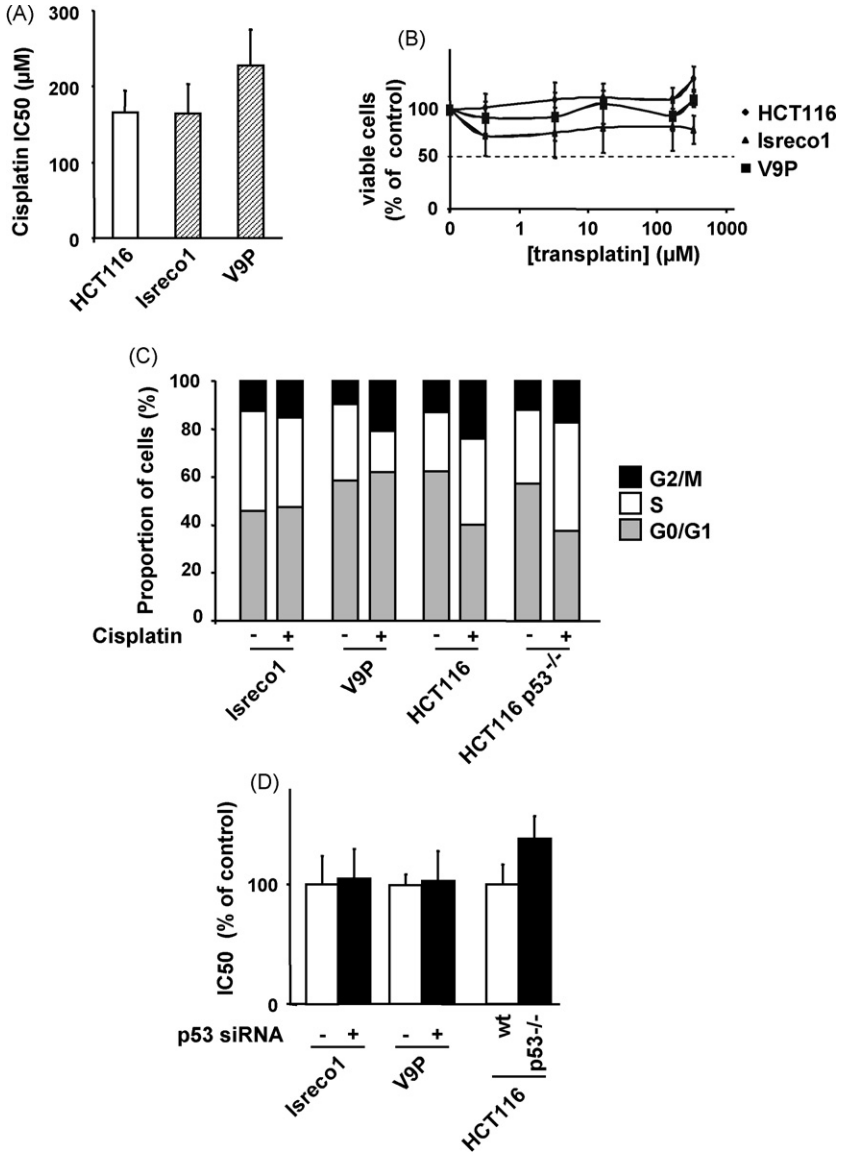


Fig. 7 – Role of p53 in sensitivity to platinum compounds of colon cancer cells. (A) Cells were treated for a 2-h period with increasing concentrations of cisplatin, and IC50 was determined 72 h later using MTT assay as described in Section 2. Results are the mean ± S.E.M. of at least three independent experiments, each performed in triplicate. (B) Survival curves for colon cancer cell lines treated with transplatin (derived from MTT assays). Graph represents the average of values from at least three independent experiments in triplicates. (C) The selected cell lines were treated during 2 h with cisplatin used at their respective IC50s. After propidium iodide staining, cell cycle distribution was determined by flow cytometry 24 h after the beginning of cisplatin exposure. Results are representative of at least three independent experiments; usual error magnitude for each point never exceeded 10%. (D) Effect of p53 siRNA on cell sensitivity to cisplatin. Cells were transfected with 200 nM of p53 (+) or control non-silencing (−) siRNA, 48 h before a 2-h exposure to serial concentrations of cisplatin, and number of viable cells was determined 72 h later using MTT assay. Results are expressed in percent of IC50 from cells transfected with control non-silencing siRNA. The number of viable cells was also compared in wild-type (wt) and HCT116 $p53^{-/-}$ cell lines treated or not with cisplatin. Results are the mean ± S.E.M. of three experiments each performed in triplicate.

while TRAIL treatment resulted in enzyme activation in all (Fig. 8D). These results suggest that, in our experimental conditions, oxaliplatin exerts a major effect on cell cycle but has no or very low effect on the apoptotic death pathway. Interestingly, only the p53-mutated sensitive cell line (V9P) responded to oxaliplatin by activating an atypical, possible caspase-independent, apoptotic pathway.

3.7. *TAp73 activation does not explain sensitivity to oxaliplatin in the V9P cell line*

The p53 family member p73 protein could be an explanation for the p53 independent apoptosis in the V9P cell line. It has been shown that p73 is increased by cisplatin in colon cancer cell lines [22], and gives rise to TAp73 and ΔNp73 isoforms due

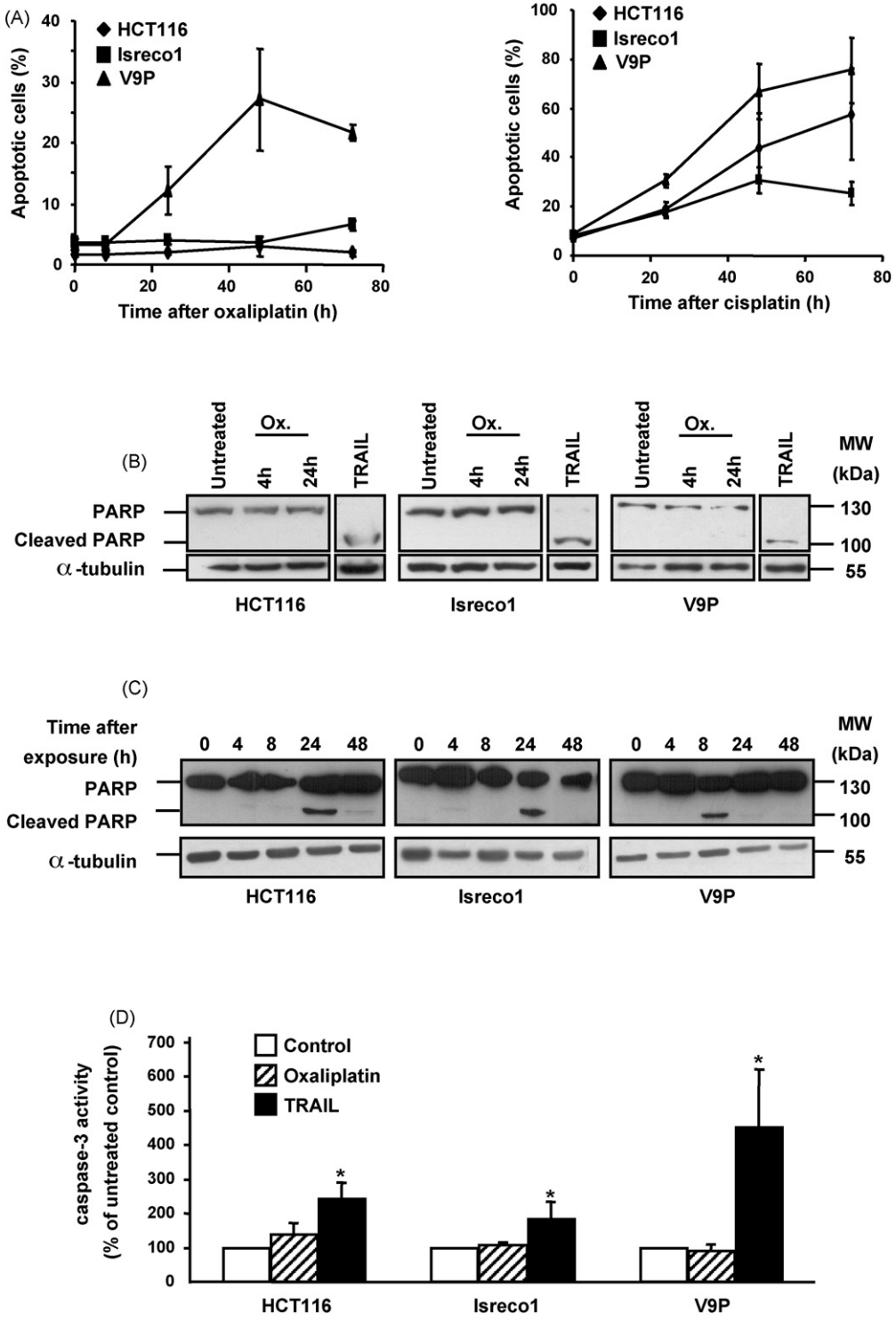


Fig. 8 – Effect of oxaliplatin and cisplatin on apoptosis in colorectal cancer cell lines. (A) Kinetics of the oxaliplatin- and cisplatin-induced apoptosis (left and right panel, respectively) in indicated colon cancer cell lines. Cells were treated with oxaliplatin or cisplatin at their respective IC50s for 2 h, and stained by PI 8–72 h after oxaliplatin treatment and 24–72 h after cisplatin treatment. After measurement by flow cytometry of genomic DNA fragmentation in HCT116, Isreco1 and V9P cell lines, cells displaying a sub-G1 hypodiploid DNA content are considered as apoptotic. Error bars represent mean ± S.E.M. from three independent experiments. (B) Western blot analysis of PARP cleavage in HCT116, Isreco1 and V9P cells treated with oxaliplatin. Cells were treated with corresponding IC50 of oxaliplatin during 2 h, or with TRAIL (50 ng/ml) as positive control of PARP cleavage, and PARP expression was determined by immunoblotting 4 and 24 h after oxaliplatin exposure, and 4 h after TRAIL exposure. Results are representative of at least three separate experiments. (C) Western blot analysis of PARP cleavage in HCT116, Isreco1 and V9P cells treated with oxaliplatin. Cells were treated with corresponding IC50 10× of

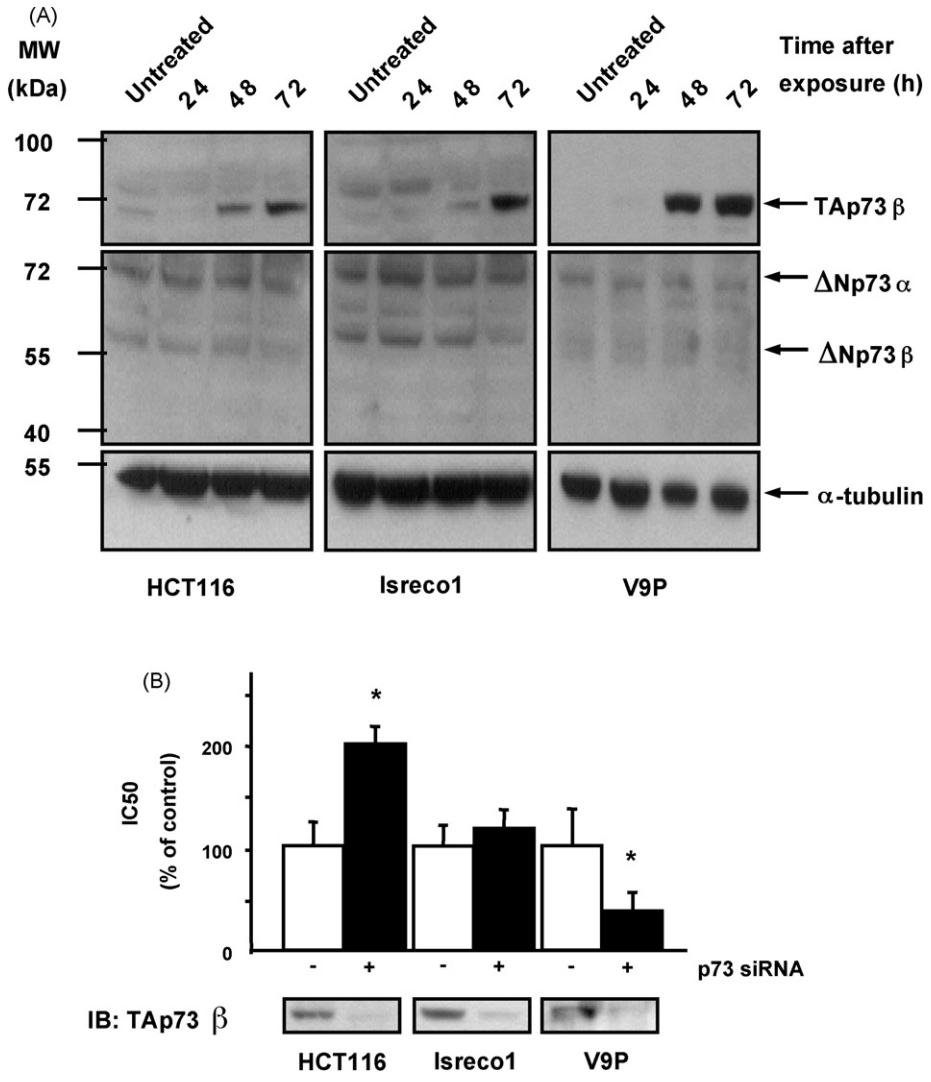


Fig. 9 – Expression and function of p73 in oxaliplatin-treated HCT116, Isreco1 and V9P cell lines. (A) Cells were treated with corresponding IC50 of oxaliplatin for 2 h, and the different TAp73 isoforms were revealed by Western blotting at different times after exposure. Results are representative of at least three separate experiments. (B) Cells were transfected with 50 nM p73 siRNA (+) or equivalent concentrations of control non-silencing siRNA (−), then exposed during 2 h to serial oxaliplatin concentrations, and the number of viable cells was determined 72 h later using MTT assay. Results are expressed in percent of IC50 from cells transfected with control non-silencing siRNA. *P < 0.05 compared to corresponding values obtained from cells transfected with control non-silencing siRNA. Representative blots of TAp73β are shown for each cell line. Results are mean ± S.E.M. of at least four experiments each performed in triplicate.

to alternative promoter utilization and alternative mRNA splicing [23]. However, whereas TA isoforms share many common target genes with p53, ΔN isoforms are dominant inhibitors of p53- and TAp73-responsive gene expression. Therefore, we investigated the role of p73 in oxaliplatin chemoresistance in the three selected cell lines, first by examining p73 isoforms expression by immunoblotting, before and after oxaliplatin exposure, in HCT116, Isreco1 and V9P cells. TAp73β protein expression was detectable 48 and 72 h after treatment in HCT116 and V9P cell lines, and only at 72 h in Isreco1 (Fig. 9A, upper panel). In contrast, expression of ΔNp73α and ΔNp73β was low in the 3 untreated cell lines, and appeared unmodified after oxaliplatin treatment (Fig. 9A, middle panel). We next tested the role of TAp73 in the

oxaliplatin during 2 h and PARP expression was determined by immunoblotting 4, 8, 24 and 48 h after oxaliplatin exposure. Results are representative of at least three separate experiments. (D) Caspase-3 activity induced by oxaliplatin. Cells were treated with corresponding IC50 of oxaliplatin during 2 h, or with TRAIL during 4 h as positive control of caspase-3 activation, and relative caspase-3 activity was determined by colorimetric assay 8 h after oxaliplatin or TRAIL exposure. *P < 0.05 as compared to untreated control cells. Results are the mean ± S.E.M. of at least three separate experiments.

sensitivity of colon cancer cells to oxaliplatin, using a siRNA silencing strategy. Doubled-strand RNA oligonucleotides homologous to 5′ specific TAp73 sequences absent from the ΔNp73 sequence were used to specifically knock-down TAp73 expression in tumour cells [22]. Seventy-two hours after transfection of p73 siRNA, TA-p73β expression became undetectable by immunoblotting, comparatively to non-interfering siRNA-transfected cells (Fig. 9B). Depletion of TAp73 led to a significant increase of the IC50 of HCT116 cells, but not Isreco1 cells, submitted to oxaliplatin (64.6 ± 16.5 μM versus 31.8 ± 7.1 μM). As for the V9P cell line, depletion of TAp73 paradoxally increased cell sensitivity to oxaliplatin (IC50: 10.9 ± 5.5 μM versus 33.6 ± 11.8 μM). Our results thus suggest that activation of the p73 pathway does not explain the sensitivity to oxaliplatin of the p53-mutated V9P colon cancer cell line.

4. Discussion

One important result of the present study is to clarify the major role of cell-cycle arrest as opposed to apoptosis in the sensitivity to oxaliplatin of colon cancer cells. Original points of our study are the oxaliplatin treatment schedule, corresponding to the short-term usual administration in human treatment, and the use of a pre-determined IC50 for each cell line which allowed us to compare the cellular consequences of oxaliplatin treatment in these different cell lines. The use of a panel of cell lines, and of time- and dose-limited exposure to oxaliplatin, may explain some discrepancies with previous studies based on a limited number of cell lines, regarding especially the induction of apoptosis by oxaliplatin. A very long (from 6 to 72 h) exposure to oxaliplatin, which appears rather different from the infusion protocol in human therapy, may explain the previously reported effects on apoptosis [4,13,14]. Interestingly, the half-life of oxaliplatin in blood after infusion is of only 14 min [24]. In our conditions, whereas cisplatin induced apoptosis in all the cell lines tested, oxaliplatin-induced apoptosis plays clearly a minor role in toxicity, excepted the possible caspase-3 independent apoptotic pathway in V9P cells, possibly responsible for the persistent sensitivity of this p53-mutated cell line. Currently, several cellular roles of caspases emerge, suggesting that these enzymes, thought to mediate apoptotic cellular event, might not be required for all types of programmed cell deaths. Genetic studies showed that tBID, BIM, BAD, BAX and BAK proteins, which have been shown to promote caspase activation and apoptosis, can also kill cells independently of *Apaf-1* and downstream caspases, including caspase-3. Another candidate mediator of caspase-independent programmed cell death is apoptosis-inducing factor (AIF), a conserved mitochondrial oxidoreductase [25]. Further studies using the V9P cell line are required to clarify these pathways.

Cell cycle and apoptosis are intimately related and regulated by the p53 protein following DNA damage [11,15,26]. Cell cycle analysis in the 10 cell lines showed that oxaliplatin treatment led to a S phase arrest in p53-mutated cell lines, and to a G0/G1 and G2/M arrest in p53 wild-type ones, at the difference of cells treated with cisplatin. The close correlation between p53 status and cell cycle distribution after oxaliplatin treatment was confirmed using the HCT116 $p53^{-/-}$ cell line, in which cell cycle consequences of oxaliplatin treatment were modified accordingly to the pattern observed in p53-mutated cell lines. The observed balance between cell cycle arrest and apoptosis, in favor of the former, reminds on comparable results published with different chemotherapeutic agents: camptothecin treatment of colon cancer cells harbouring an intact p53 and p21 axis leads to cell cycle arrest without apoptosis [20]. Furthermore, camptothecin-induced DNA damage of cells with a mutant p53 led to a G2 cell cycle arrest [20], and it was also reported that p21-mediated cell cycle arrest inhibits apoptosis following p53 expression or DNA damage in colorectal cancer [27].

An important point of our study was the very close association between the p53-mutated status of colon cancer cells and a low sensitivity to oxaliplatin, a point that was still unclear considering previous studies [15]. The p53–p21 axis integrity appears as a crucial determinant of colon cancer cell sensitivity to oxaliplatin, what was confirmed by the present results on p21. A role of p53 mutations in resistance to chemotherapeutic agents is not a surprise, and has been demonstrated in several cell line models [3,9,10,28], including the resistance to 5-FU-induced apoptosis of p53-null colorectal cancer cells [5]. The V9P cell line was the exception in the panel of 10 colon cancer cell lines, with a high sensitivity to oxaliplatin despite the presence of a p53 mutation leading to a transactivation-deficient p53 mutant. This cell line, the only one where a possible caspase independent apoptotic death was detected, thus is a valuable model for the identification of alternative pathways for oxaliplatin toxicity in colon cancer cells.

The ‘‘gain-of-function’’ phenotype of some p53 mutants is now well known [12,29], and appears notably as a sign of chemoresistance as compared with p53 wild-type cells. When studying the effect of p53 depletion by RNA interference, we surprisingly observed an increased resistance to oxaliplatin (but not to cisplatin) not only in HCT116 cells, but also in Isreco1 and V9P cells harbouring a mutated, transcriptionally inactive, p53 protein. This oxaliplatin specific residual effect could be explained by an ability of the mutant protein to transactivate promoters different from those tested in this study. On the other hand, p53 transcription-independent activities have been described recently [30]. Genotoxic damage leads to translocation to the mitochondria of wild-type p53 and of some transactivation-deficient mutants, interaction with anti-apoptotic proteins of the Bcl 2 family, followed by a rapid apoptosis [31]. This however could not explain the observed residual effect of the mutant p53 in Isreco1 cells, as our results never identified an effect on apoptosis. Moreover, a transcription-independent effect of p53 on the cell cycle has never been reported. An other attractive hypothesis could be the modulation of DNA-damaging agents cytotoxicity by co-activating proteins such as the p53 family member p73, whose potential role in cancer cell chemoresistance has recently been outlined [22], but never studied in oxaliplatin resistance.

The p73 protein is a p53 homologue with important roles in human cancers, sharing several common target genes with p53 [32]. Differential transcription of the *p73* gene leads to several isoforms, including TAp73α, TAp73β, and ΔN isoforms. The ΔN isoforms of p73 are dominant negative inhibitors of the

TAp73 isoforms and of wild-type p53 [33], responsible for interaction loops between pro- and anti-apoptotic proteins [23]. Expression of the endogenous p73 protein has been shown to be rapidly increased by different cytotoxics such as camptothecin, etoposide, cisplatin and doxorubicin [22,34]. TAp73 accumulation leads to induction of apoptotic target genes [35], and TAp73β is known to be a more potent apoptosis inducer than p53 itself [36]. Interestingly, p73 induces apoptosis in wild-type p53 cells, e.g. mouse embryo fibroblasts [37], but also in cells lacking a functional p53 [22]. It thus represented an interesting pathway to explain the sensitivity of V9P.

In the present study, we observed a late induction of TAp73β after oxaliplatin treatment of the three representative colon cancer cell lines, independently of the p53 status. However, p73 depletion did not lead to an increased resistance in the p53-mutated cell line Isreco1, but was surprisingly responsible for a decreased resistance to oxaliplatin in V9P cells. These results suggest that the increase of p73 expression after oxaliplatin treatment did not result in p53-independent cytotoxicity in these colon cancer cell lines. In contrast, blocking TAp73 function in the HCT116 cell line, with an intact p53–p21 pathway, led to the expected enhanced resistance to oxaliplatin. Overexpression of antiapoptotic ΔNp73 variants occurs in many cancers, including colorectal cancer [33]. In cell culture, this protein can block chemotherapy-induced apoptosis in p53 wild-type tumour cells [33]. However, the presence of ΔNα and β isoforms did not explain the differences in response to oxaliplatin of the Isreco1 and V9P p53-mutated cell lines, since their expression was very low and remained so after oxaliplatin treatment in both cases. Interestingly, some p53 mutants can interact with p73 to block the protein function and then the chemotherapy-induced cytotoxicity [12,38]. This type of inhibitory interaction is notably dependent on the nature of the p53 mutation and on the Arg^{72} polymorphism of the *p53* gene [39]. Our results are thus consistent with the hypothesis that the role of p73 in the sensitivity to oxaliplatin in colon cancer cell lines is limited to wild-type p53 cells, with complex and yet unexplained effects in p53-mutated cell lines. Additional experiments are required to clarify the molecular mechanisms of differential chemosensitivity of p53-mutated colon cancer cell lines.

Conflict of interest statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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