Double siRNA-targeting of cIAP2 and LIVIN results in synergetic sensitization of HCT-116 cells to oxaliplatin treatment

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Purpose: Most colon cancers show low sensitivity to treatment with oxaliplatin and a specific strategy is needed to overcome this problem. Our approach uses RNA interference to silence the expression of target genes responsible for the development of oxaliplatin resistance. Profile analysis of genes related to the regulation of apoptosis allowed identification of target genes showing the greatest degree of upregulation in response to oxaliplatin exposure.

Methods: We designed a panel of genes with functions closely related to inactivation of the caspase cascade, endoplasmic reticulum stress reduction, and drug metabolism. The candidate genes were silenced by means of specific small interfering RNA (siRNA) oligonucleotides.

Results: The caspase 3 and 9 inhibitors of apoptosis 2 (cIAP2) and LIVIN were found to be the most dose-responsive genes during the period of oxaliplatin treatment. Two-fold sensitization of cells to oxaliplatin was observed with independent knockdown of either cIAP2 or LIVIN expression. siRNA-silencing of both targets produced a five-fold increase in oxaliplatin sensitivity of HCT-116 cells.

Conclusion: A dose-dependent approach revealed reliable targets for siRNA-silencing under low doses of oxaliplatin. Targeting the key proapoptotic chain with several specific siRNAs resulted in synergetic sensitization of HCT-116 cells to oxaliplatin treatment.

Keywords: drug resistance, inhibition of apoptosis, RNA interference

Introduction
Following the introduction of the drug oxaliplatin in colorectal cancer therapy, 40%–50% of patients have shown improved response rates and prolonged survival. However, more than half of patients experience side effects and drug resistance and new therapeutic approaches are needed.

Many studies¹–⁴ have searched for candidate target genes that express response to oxaliplatin treatment. Recent investigations using array technology have revealed a number of candidate gene clusters involved in the cell cycle, DNA replication, DNA transcription, and apoptosis.⁵,⁶ We focused on the caspase pathways in order to identify the most responsive markers related to oxaliplatin treatment. The activity of caspsases is regulated through a variety of apoptotic inhibitors, including such protein families as baculoviral IAP repeat-containing (BIRC) proteins, Bcl-2, heat-shock proteins (HSPs), glucose-regulated proteins (GRPs), tumor necrosis factor receptor (TNFR)-related proteins (such as TRAP1), etc. The expression panel used in our investigation contained direct inhibitors of caspsases (cIAP1/2, XIAP, Survivin, and LIVIN), CFLAR inhibitors of reactive oxygen species (ROS)-induced apoptosis (TRAP1, Bcl-xI, Bcl2, and Grp78), and several genes involved in drug metabolism (Gsp1, Gstm2,
and endonuclease excision repair cross-complementing 1 \([\text{ERCC}1]\) as representative of the DNA reparation system. Together, these genes are involved in the mechanism that prevents apoptosis of cancer cells caused by either drug treatment or other extrinsic stress factors. Although several of these proteins have been previously used as the targets of RNA interference (i) knockdown in different cancer cell types, \(2,3,7,8\) their role in chemotheraphy resistance is still not clearly understood. Our approach was to study time- and dose-dependent responses of the HCT-116 cell line to oxaliplatin exposure by means of an expression panel of apoptotic inhibitors consisting of different protein families. Our study has revealed at least four genes that are upregulated by oxaliplatin doses of 5 and 10 \(\mu\)M, of which \(\text{c-IAP}2\) and \(\text{LIVIN}\) appeared to be the most dose-responsive during incubation with oxaliplatin. The caspase 3 and 9 inhibitor \(\text{c-IAP}2\) is a well-known \(c\)-\text{Myc} target and is frequently elevated in lung, colon, and pancreatic cancers, while there is relatively no information about \(\text{Birc}7/\text{LIVIN}\). Knockdown of both \(\text{LIVIN}\) and \(\text{c-IAP}2\) resulted in dramatic levels of apoptosis at 10 \(\mu\)M of oxaliplatin. This observation suggests a novel synergistic role of these genes in the regulation of apoptosis and the development of oxaliplatin resistance.

### Materials and methods

#### Cells

Colon cancer HCT-116 \(p53^{-/-}\) cells were a gift from Professor AA Sh till, N.N. Blokhin Cancer Research Center, Moscow, Russian Federation and were maintained in Dulbecco’s Modified Eagle’s Medium (\(\text{DMEM}\)) Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (\(\text{FBS}\)) and 50 \(\mu\)g of penicillin-streptomycin at 37 °C in 5% CO\(_2\).

#### Oxaliplatin treatment and real-time-PCR

The cells were seeded in 6-well plates in \(\text{DMEM}\) and 5% FBS without antibiotics, so that they will give 50% confluence on the next day. Cells were exposed to 5 and 10 \(\mu\)M oxaliplatin (Pharmachemie BV, Haarlem, the Netherlands) for 24 and 48 hours and real-time-PCR was performed to analyze gene expression. Untreated cells served as a negative control.

#### Cell viability and apoptosis

Cell viability was determined by Trypan blue and apoptosis assay staining. Staining for the apoptosis analysis was performed using a Vybrant Apoptosis Assay Kit \#5 (Life Technologies) with Hoechst 33342/propidium iodide, according to the manufacturer’s instructions. Cells were viewed and counted using an Axio Observer D1 microscope (Carl Zeiss Meditec AG, Jena, Germany) with 10x/20x objective lenses. Images were captured using a Carl Zeiss AxioCam MRc camera. Tests were performed in triplicate, counting a minimum of 600 cells total in each.

### RNA isolation, reverse transcription

In order to perform reverse-transcription-PCR for all samples in equivalent conditions, RNA was isolated from each experimental well using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and cells from triplicate wells were combined. Lysis buffer was added to the wells with the cells and incubated for 15–20 minutes until all cells were lysed. Isolation was performed according to the manufacturer’s instructions and the RNA concentration was measured on a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). One hundred to five hundred nanograms of total RNA was applied for RT in a total volume of 20 \(\mu\)L using 1–5 U of Promt II reverse transcriptase (Promega Corporation, Fitchburg, WI, USA) at 42°C for 1 hour. The reaction was stopped by heating at 70°C for 10 minutes.

### Real-time PCR

Expression profiles of the genes \(\text{c-IAP}1, \text{c-IAP}2, \text{XIAP}, \text{Survivin}, \text{LIVIN}, \text{FLIP}\) (\(L\)-long chain splice variant), \(c\)-\text{Myc}, \(\text{Grp}78, 1\)-\text{Bcl}-2, \(1\)-\text{Bcl}-xl, \(\text{Gsp}1, \text{Gst}m2, \text{Gst}m4, \text{ERCC}1, \text{TRAP}1\) were determined real-time PCR using a StepOne Real-Time PCR System (Life Technologies) with EveGreen (Biotium, Hayward, CA, USA). Primers were designed according to standard rules in order to amplify 200–220 base-pair fragments of all analyzed genes. The primer sequences used for amplification were as follows:

- **IAP-1F** (\(c\)-\text{IAP}1 forward): \(\text{AGGGTGTAGTCTGGATAACGAA}\)
- **IAP-1R** (\(c\)-\text{IAP}1 reverse): \(\text{TTGTTCACCAAGGTCCTATTA}\)
- **Bir3-F** (\(c\)-\text{IAP}2 forward): \(\text{AGGGTTGGAGAACTCCTGAGAT}\)
- **Bir3-R** (\(c\)-\text{IAP}2 reverse): \(\text{GCACATTAATCACAAGGAGTA}\)
- **XF** (\(\text{XIAP}\) forward): \(\text{TAGGTTAGGTGATAAGTAA}\)
- **XR** (\(\text{XIAP}\) reverse): \(\text{TTCCTAGTTGAGTCTGTT}\)
- **Bir5F** (\(\text{Birc} 5\) forward): \(\text{CCACGTTGTTCCCTGTT}\)
- **Bir5R** (\(\text{Birc} 5\) reverse): \(\text{GGCTTCTTCTGTGTAGAT}\)
- **Liv-F** (\(\text{LIVIN}\) forward): \(\text{TGCCTGTTCGGACTGTT}\)
- **Liv-R** (\(\text{LIVIN}\) reverse): \(\text{GCATCAGGTCCTGTT}\)
- **LF1** (\(\text{FLIP}\) forward): \(\text{TTTGAGAAGTAAAGTAA}\)
- **LF2** (\(\text{FLIP}\) reverse): \(\text{GGGGTGTGTTCCCTGTT}\)
- **CMF** (\(c\)-\text{Myc} forward): \(\text{AGGCAGATCGAACCGGAT}\)
- **CMR** (\(c\)-\text{Myc} reverse): \(\text{TCCCTTGCGGACTGTT}\)
- **Grp-F** (\(\text{Grp}78\) forward): \(\text{AGGTGGCGAAACAAAGACAT}\)
Small interfering RNA (siRNA) design

siRNA oligonucleotides that knock down messenger (m) RNAs of the genes cIAP2 and LIVIN and non-targeting (scrambled) siRNAs were designed using the program BLOCK-iT™ RNAi Designer (Life Technologies). Tuschl’s motif was used as the criterion for the target sequence search. We used an “AA(N19)TT” pattern, so that the tool searched for an area of the sequence that begins with “AA” and ends with “TT,” with 19 bases in-between. Several variants of each target were designed for each degree. The GAPDH gene was amplified as a control with each group of genes at the corresponding annealing temperature. All experiments were repeated at least three times.

Results

Gene expression in HCT-116 cells in response to dosage and timing of oxaliplatin treatment

To determine the level of the expression response to different doses of oxaliplatin, HCT-116 cells were plated (2 × 10^4 per well on a 48-well plate) and grown with the addition of oxaliplatin. In most cases, the effect of siRNA inhibition appeared after 48–72 hours or more. However, some cell lines did not survive during the long period of treatment with oxaliplatin. We incubated the HCT-116 cells with oxaliplatin for 24, 48, 72, and 96 hours (Figure 1). We observed that 48 hours of treatment with oxaliplatin is the optimal period for evaluating the apoptotic effect produced by RNA interference; a longer period results in more than 30% cell death, which is equivalent to the effect of a single siRNA. Furthermore, a strong apoptotic response makes it difficult to perform accurate measurements of mRNA expression. Oxaliplatin at 5 µM and 10 µM was added to each

• cIAP2: sense, UAAGGGAAGAGGAGAGAAdTdT; antisense, UUCUCUCUCUCUCUCUCUCUAdTdT
• LIVIN: sense, CUGUACCUGUUUGGAGUGCdTdT; antisense, AGCAUCCAAACAGGUACAGdTdT

Statistical analysis

Statistical analysis was carried out using an unpaired Student’s t-test (GraphPad Software Inc, La Jolla, CA, USA). Results were considered statistically significant where P < 0.05.

Transfection, siRNA interference, and oxaliplatin treatment

Cells were seeded for transfection in 48-well plates in DMEM without antibiotics 1 day before transfection so that they were 55%–60% confluent on the day of transfection. For transfection, the regular medium was replaced with low-serum medium with 1% FBS without antibiotics. The cells were transfected with siRNA in a forward manner using Lipofectamine RNAiMAX (Invitrogen; Life Technology). The final concentrations of the siRNA mixtures contained two equimolar siRNAs in concentrations up to 50 nM, as recommended in the RNAiMAX instruction protocol. Knockdown efficiency was examined using real time-PCR, performed as previously stated. Oxaliplatin (10 µM) was added to each well containing the siRNA transfected cells. Untreated cells with no siRNA or oxaliplatin were used as negative controls; cells treated with oxaliplatin only were used as an siRNA negative control; and cells treated with scrambled siRNA were used as a scrambled control. The cells were incubated with the siRNA–Lipofectamine RNAiMAX complexes and oxaliplatin for 48 hours, and apoptosis was analyzed by fluorescent microscopic examination.
well, with triplicate wells for each concentration. An incubation period of 48 hours is commonly believed to be sufficient, as by this point all plated cells will have passed through the division cycle, and most would be able to express early (24 hours of oxaliplatin treatment) and late (48 hours) genes in response to oxaliplatin treatment. After 24 and 48 hours of incubation, real time-PCR analysis was performed. The profile expression results are described in Figure 2 and correspond to the classic apoptosis network shown in Figure 3.

**Early genes**

*TRAP1, XIAP, and ERCC1* showed the strongest early increase in expression (more than two-fold) at both concentrations of

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**Figure 1** Viability of HCT-116 cells that were treated with 10 µM of oxaliplatin.

**Notes:** HCT-116 cells were treated with 10 µM of oxaliplatin and incubated for 24–96 hours (h) in Dulbecco’s Modified Eagle’s Medium with 1% fetal bovine serum. Viability was identified by Trypan blue.

**Figure 2** Dose- and time-dependent gene expression profiles.

**Notes:** HCT-116 cells were treated with 5 or 10 µM of oxaliplatin. Fold changes were determined by ∆∆Ct relative quantification method. Calculation of fold changes was performed using a formula $2^{-\Delta\Delta Ct}$, where “2” is a fold change in amplicon between cycles and ∆∆Ct the normalized cycle change between sample and reference. Samples are the genes from the cells that were treated with oxaliplatin, and reference are the genes from the untreated cells. Gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase human) was used as the endogenous control. (A) Gene expression in dose-dependent analysis during the period of 48 hours of oxaliplatin treatment. (B) Dose-dependent profiles of early genes (24 hours). (C) Dose-dependent profiles of late genes (48 hours). (D) LIVIN and cIAP-2 were chosen as the candidate genes for small interfering RNA (siRNA) knockdown.
oxaliplatin, and slight increases were observed for c-Myc and Gstm2 at 10 µM of oxaliplatin (Figures 2B and 3). Early expression of LIVIN and cIAP2 genes was intermediate (1.2–1.7×). Upregulation of TRAP1, ERCC1, and Gstm2 was found to be strongest at 10 µM of oxaliplatin; however, none of these genes were upregulated for the entire period of drug exposure. TRAP1 is believed to localize to the cytosolic and mitochondrial fractions. The functional role of TRAP1 in mitochondria has been shown by a number of studies,9,10 in which it has been described as a negative mediator of cytochrome c release (Figure 3) and downstream activation of caspase 9. Subsequent activation of the caspase 9 and 3 inhibitors LIVIN and cIAP2 seems to support the consistency of such events. Early activation of TRAP1 may occur as a response to drug metabolism that triggers activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway11 and subsequent activation of XIAP, which stops apoptotic cell death by direct inhibition of caspases 3, 7, and 9 (Figure 3).

Late genes
Among the late genes, the targets of NF-κB signaling, the caspase inhibitors cIAP1, cIAP2, XIAP, and FLIP,12,13 appeared to be upregulated, mostly at 10 µM of oxaliplatin (Figures 2C and 3). LIVIN and cIAP2 are believed to inhibit apoptosis downstream of the caspase cascade by direct inhibition of both caspases 3 and 9. FLIP was among the most highly upregulated late genes at 10 µM of oxaliplatin. This event correlated with upregulation of another apoptotic inhibitor, Bcl-xL, under the same time/dose conditions, and was likely due to extension of the ROS influence and NF-κB signaling because of increased concentrations of the drug.

Oxaliplatin-dependent expression
Within 48 hours at 5 µM of oxaliplatin, elevated rates of expression were observed for LIVIN, Gstm2, and Gstp1. However, at 10 µM of oxaliplatin, we observed a twofold

Figure 3 Early and late phases of gene expression under 5 and 10 µM of oxaliplatin.
Notes: In small circles are the expressions of genes measured by means of real-time PCR (except cytochrome [Cyt] c [yellow]). Quantity of circles at all stages correlates with the measured level of gene expression. Large ellipses depict NF-κB signaling and caspase (casp 8, 9, 7, 3) cascades, that were implicated in either regulation of gene expression or targeting. Black and red arrows indicate activation, while blue blocking arrows indicate inhibition processes.
Abbreviations: PCR, polymerase chain reaction; ROS, reactive oxygen species; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.
reduction of Gstm2 and downregulation of Gsp1. Within 48 hours at 10 µM of oxaliplatin, increased rates of expression were observed for cIAP2 (four-fold increase), LIVIN (three-fold), and FLIP, Bcl-xl, and XIAP (two-fold). TRAP1 and Survivin were significantly downregulated during this period. Upregulation of FLIP and ERCC1 was found to be time-dependent at the higher dose of oxaliplatin, while at 5 µM, downregulation of these genes was observed in 48 hours.

Time- and dose-dependent genes

Birc7/LIVIN was the only gene that appeared to exhibit stable elevated expression within 48 hours at the lower concentration and was dose-dependent at the later stage. The expression of cIAP2 appeared to be both time- and dose-dependent (Figures 2D and 3). We chose to further investigate LIVIN and cIAP2 (Figure 2D), as two direct inhibitors of pro-caspase 3 and 9, in order to determine whether double siRNA knockdown of two key apoptosis regulators may influence cell resistance to oxaliplatin.

siRNA silencing of the candidate genes

Knockdown of target genes was performed at the concentration of oxaliplatin (10 µM) that caused the greatest elevation in their expression levels (Figure 2A). For cIAP2, the maximum rate of silencing was obtained in 48 hours by means of 15 nM of siRNA and was as much as 80% (P < 0.024) without oxaliplatin and 65% when 10 µM of oxaliplatin was added (P < 0.01) (Figure 4). Similar results were observed for LIVIN.

HCT-116 cells containing 10 µM of oxaliplatin were transfected with 15 nM of either anti-cIAP2 or LIVIN siRNAs or both. Scrambled siRNA was added to the cells treated with oxaliplatin in order to control nonspecific inhibition. After 48 hours of incubation, the cells were stained with either Hoechst 33342/propidium iodide (Figure 5A-1a and 2b, B, and C2) or Trypan blue (Figure 5A - 2a and 1b). Apoptotic cells were examined under a fluorescent microscope with ten fields of view per well (10×/20× magnification) (Figure 5). Silencing of each gene resulted in a two-fold increase in sensitivity to oxaliplatin (P < 0.01) (Figure 5B) compared to the cells treated with only oxaliplatin (Figure 5A - 2a and 2b).

A significant rate of apoptosis (P < 0.005) was achieved by

Figure 5 Cells treated with siRNA and oxaliplatin.

Notes: Trypan blue and Hoechst/PI cells were used to detect apoptotic or necrotic cells. (A) 1a: The untreated cells stained with Hoechst/PI. 1b: the untreated cells stained with Trypan blue. 2a and b: cells treated with 10 µM of oxaliplatin. (B) 1: Cells treated with cIAP2 + 10 µM of oxaliplatin. 2: Cells treated with LIVIN + 10 µM of oxaliplatin; (C) 1: LIVIN + cIAP2 + 10 µM of oxaliplatin treated cells before staining with Hoechst/PI. 2: Hoechst/PI stained cells treated both with LIVIN + cIAP2 + 10 µM of oxaliplatin.

All photos made under 10× magnification. Yellow arrows indicate live cells; blue arrow indicates necrotic red cells; red arrows indicate apoptotic bright green cells.

Abbreviations: PI, propidium iodide; sirna, small interfering RNA.
means of combination of cIAP2 (Birc3) and LIVIN (Birc7) (Figures 5C and 6).

**Discussion**

The separation of gene expression into early and late phases may at first appear to be approximate; however, no genes dependent on NF-κB signaling were expressed in the first 24 hours, and none (except TRAP1, c-Myc, Bcl-xl and Gstm2) were found to be dose-dependent during this period (Figure 2C). The increase in oxaliplatin dosage, from 5 to 10 μM, resulted in synergetic elevation of TRAP1 and c-Myc at the early stage. This is not surprising as long as TRAP1 is considered a direct c-Myc target. The two-fold elevation of Gstm2 and Bcl-xl may be explained by their lack of sensitivity to lower doses of oxaliplatin. Bcl-xl maintains its sensitivity only to higher doses of oxaliplatin. Figure 2C and D clearly show that TRAP1 is an early-expressed gene, while cIAP2, FLIP, and XIAP are the most dose-dependent late genes. Our observations concerning the oxaliplatin sensitivity of Bcl2 family members were interesting; a possible explanation for the inactivity of Bcl2, compared to the upregulation of Bcl-xl expression, is its lack of sensitivity to low concentrations of oxaliplatin.

Due to oxaliplatin’s ability to induce DNA damage, there has been much discussion of the role of DNA repair genes in oxaliplatin resistance. In our experiments, we found ERCCI, which has been shown to be inversely correlated with the response to oxaliplatin. However, these findings appear to be controversial, since at least two studies found no association between DNA repair proteins and oxaliplatin resistance. In our experiments, we found ERCCI to be downregulated in 48 hours at 5 μM of oxaliplatin exposure. Almost full knockdown of Survivin expression was observed at higher doses of oxaliplatin (Figure 2D). The ability of oxaliplatin to downregulate Survivin has been repeatedly described.

Our data suggest that cIAP2 and LIVIN play an essential role in the oxaliplatin-induced resistance of HCT-116 cells. Unlike cIAP2, LIVIN was less frequently found to be overexpressed in colon cancers. In one very recent study, anti-LIVIN siRNA apoptosis was observed in vivo and in vitro (in HCT-116 cells), and the authors describe a remarkable decrease in cell viability under different doses of siRNA. One year previously, another group of researchers constructed a vector containing short hairpin RNA-LIVIN that caused apoptosis of HCT-8/V colon cancer cells. Simultaneous siRNA knockdown of Survivin and LIVIN was performed on Lovo colon cancer cells; however, no synergic effect was observed. By means of accurate measurement of oxaliplatin-induced expression, we have revealed that cIAP2 and LIVIN were the only genes not downregulated over 48 hours of 5 and 10 μM of oxaliplatin exposure. Individual knockdown by means of either anti-LIVIN or cIAP2 resulted in 32% cell death for both siRNAs. In general, this level of apoptosis is common in cases where only one siRNA is used in order to overcome platinum-containing drug resistance, and has been previously described by several authors. A very representative result was obtained by the previously mentioned group who performed LIVIN silencing on HCT-116 cells. They observed an approximately 30% decrease in cell viability with an siRNA concentration of 25 nM, which is similar to the concentration used in this study.

Both LIVIN and cIAP2 inhibit caspases 3 and 9, and our study indicated that both are time- and dose-dependent. This finding led us to suppose a synergistic role of these genes in oxaliplatin resistance. As the result of double siRNA silencing, we observed massive apoptosis that made cancer cells so weak and sensitive to ethylenediaminetetraacetic acid (EDTA)–trypsin treatment that cell imaging by means of a microscope was the only way to determine cell viability.

**Conclusion**

The siRNA cocktail appears to be an effective tool with which to inhibit several targets that are essential for cancer cell proliferation or survival activity. Over the last 3 years, a number of authors have published results showing a successful cooperative effect in other types of cancers by employing two or three different siRNAs, including cIAP1, cIAP2, and XIAP for prostate cancer or XIAP, LIVIN, and Survivin.
for bladder cancer. Together, these findings provide new insight into approaches for the prevention of drug resistance in cancer cells.

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Disclosure
The authors report no conflicts of interest in this work.

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