Repression of cell cycle–related proteins by oxaliplatin but not cisplatin in human colon cancer cells

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Abstract

Oxaliplatin (Eloxatin) is a third-generation platinum derivative with an *in vitro* and *in vivo* spectrum of activity distinct from that of cisplatin, especially in colon cancer cells. Here, we studied the molecular basis of this difference on the HCT-116 human colon carcinoma cell line (mismatch repair-deficient, wild-type functional p53). Oxaliplatin inhibited HCT-116 cell proliferation with greater efficacy than cisplatin. At comparable concentrations, cisplatin slowed down the replication phase and activated the G_2-M checkpoint, whereas oxaliplatin activated the G_1-S checkpoint and completely blocked the G_2-M transition. With the aim of finding oxaliplatin-specific target genes and mechanisms differing from those of cisplatin, we established the transcriptional signatures of both products on HCT-116 cells using microarray technology. Based on hierarchical clustering, we found that (*a*) many more genes were modulated by oxaliplatin compared with cisplatin and (*b*) among the 117 modulated genes, 79 were regulated similarly by both drugs and, in sharp contrast, 38 genes were dose dependently down-regulated by oxaliplatin and, conversely, up-regulated or unaffected by cisplatin. Interestingly, several cell cycle–related genes encoding proteins involved in DNA replication and G_2-M progression belong to this latter group. RNA modulations, confirmed at the protein level, were in accordance with oxaliplatin- and cisplatin-induced cell cycle variations. Beyond the identification of genes affected by both drugs, the identified oxaliplatin-specific target genes could be useful as predictive markers for evaluating and comparing the efficacy and molecular pharmacology of platinum drugs. [Mol Cancer Ther 2006;5(9):2149–57]

Received 6/27/05; revised 6/30/06; accepted 7/12/06.

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doi:10.1158/1535-7163.MCT-05-0212

Introduction

Platinum drugs are among the most widely used compounds in cancer chemotherapy. The cytotoxicity of platinum derivatives is characterized by the formation of platinum adducts on DNA, with Pt-GG and Pt-AG intrastrand cross-links being the major lesions (1, 2). Oxaliplatin (*cis*-[(1*R*,2*R*)-1,2-cyclohexanediamine-*N*,*N*′] oxalato (2-)-*O*,*O*′ platinum; Eloxatin) is a third-generation platinum derivative bearing a 1,2-diaminocyclohexane carrier ligand and is now approved, in combination with 5-fluorouracil, for the treatment of advanced colorectal cancer (3). *In vitro* comparisons of oxaliplatin with cisplatin showed that oxaliplatin requires fewer DNA lesions than cisplatin to inhibit cell growth (1, 2, 4) and it has a broad potent antiproliferative activity, especially in cisplatin-resistant colon cancer cell lines (5, 6). In line with this, preclinical studies using tumor models and clinical data showed that oxaliplatin is therapeutically beneficial in cisplatin resistant tumors that are common in colorectal cancer (7, 8). Altogether, these data suggest that oxaliplatin might have different cellular targets and mechanisms of action compared with those of cisplatin, which have yet to be established.

DNA damage resulting from exposure to cytotoxic agents induces cell cycle arrest, which mainly occurs at the G_1-S or/and G_2-M transition via activation of checkpoint proteins that inhibit cyclin-dependent kinase (CDK) activities (9). For example, inhibition of CDK4/cyclin D and/or CDK2/cyclin E activity leads to G_1-phase arrest, inhibition of CDK2/cyclin A activity delays S-phase progression, and the inhibition of CDK1/cyclin B, a key regulator of the G_2-M transition, induces G_2 arrest. CDK1 activation is controlled by dephosphorylation at Tyr^{15} by CDC25C phosphatase and CDC25C phosphatase is inactivated by phosphorylation via Chk1 after DNA damage. Most studies on the effects of platinum drugs on cell cycle progression have been done with cisplatin and showed that it reduces the DNA synthesis rate (10, 11) with a subsequent S-phase slowdown (12) followed by G_2-M arrest (12, 13). Although it has not yet been clearly established whether cisplatin-treated cells are arrested in the G_2 or M phase, cisplatin-induced G_2-M arrest is consistent with the inhibition of the CDK1/cyclin B activity by cisplatin (13).

The effects of oxaliplatin on the cell cycle have been reported similar to the effects of cisplatin. However, contrasting results on the effects of 1,2-diaminocyclohexane-platinum compounds on cell cycle progression have been reported (14). For example, on OVCA-429 ovarian cancer cells, cisplatin arrests both wild-type p53 and

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mutant p53 cells in G_2-M, whereas 1,2-diaminocyclohexane-acetato-Pt arrests wild-type p53 cells in G_1 and mutant p53 cells in G_2-M (14).

In this context, further investigations on the effects of oxaliplatin on cell cycle progression and on identification of cell cycle–related target genes and mechanisms of action are essential.

The aim of this study was to investigate the effects of oxaliplatin on cell cycle progression and to identify cell cycle–related oxaliplatin-specific target genes and mechanisms that would explain the higher efficacy of oxaliplatin compared with cisplatin, especially in colon carcinoma cells. To this aim, the effects of both oxaliplatin and cisplatin were investigated on the HCT-116 colon carcinoma cell cycle. HCT-116 cells are deficient in DNA mismatch repair because of a genetic defect in the *hMLH1* gene, which is located on chromosome 3 (15). This deficiency is a common etiologic factor for colon cancer (16) and, interestingly, is a key factor for cisplatin and carboplatin resistance (6). HCT-116 cells express a functional p53 protein and have mostly intact DNA damage–dependent checkpoints, thus allowing cell cycle investigations. Furthermore, HCT-116 cells have already been used in analyses on the mechanism of action of platinum compounds (6, 17). Until recently, the only key differentiating variable between 1,2-diaminocyclohexane platinum and other platinum compounds [which present a very different profile in the National Cancer Institute COMPARE program (6) and a differential clinical activity profile], both as a single agent and notably in combination (7) was mismatch repair status (18).

Using a global gene expression approach, we examined transcriptional modulations induced by both drugs and identified cell cycle–related genes that are inversely regulated by oxaliplatin and cisplatin. RNA modulations were confirmed at the protein level. These target genes that we identified here may be important mediators of specific tumor cell response to oxaliplatin treatment.

Materials and Methods

Cell Culture

The HCT-116 human colon cancer cell line was from the American Type Culture Collection (Rockville, MD). Cells were grown as monolayers in McCoy's medium supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2.

Proliferation Assays

HCT-116 cells were plated (5,000 per well) in 96-well Cytostar-T scintillating microplates (Amersham Pharmacia, Little Chalfont, United Kingdom) in the presence of oxaliplatin (supplied by Sanofi-Synthelabo Recherche, Montpellier, France), cisplatin [commercially available from Sigma (St. Louis, MO) as a lyophilized powder in 50-mg vial], or solvent. Proliferation was measured by the incorporation of [^{14}C]thymidine (Amersham Pharmacia). Cells were pulsed with [^{14}C]thymidine (0.5 μCi/mL) for 12 hours and then counted using a Microbeta 1450 Trilux scintillation counter (Wallac, Wellesley, MA). Each experimental point is done in quadruplicate. The proliferation assay has been done thrice.

Cell Cycle Analysis

HCT-116 cells were plated (0.5×10^6 per well) in six-well plates. Twenty-four hours later, cells were cultured for 24 hours in the presence of drug or solvent. Cells were harvested by trypsinization, washed twice with PBS, and resuspended in 70% ethanol in PBS and kept at 4°C overnight. After washing, cells were incubated for 5 minutes with PBS containing 0.25% Triton X-100, washed twice, resuspended in PBS-fetal bovine serum (1%), and labeled with MPM-2 antibody (1 μg final concentration; Upstate Biotechnology, Charlottesville, VA) for 45 minutes at room temperature. Cells were rinsed twice with PBS and incubated with Alexa Fluor 633–conjugated goat anti-mouse antibody (10 μg/mL final concentration; Molecular Probes, Eugene, OR) for 30 minutes at room temperature. After washing twice with PBS, cells were resuspended in FITC-conjugated mouse IgG anti-cyclin B1 antibody solution (20 μL/test; BD PharMingen, San Diego, CA). Before analysis, cells were washed twice and stained with propidium iodide (PI) solution (5 μg/mL PI in PBS containing 0.1% Triton X-100 and 0.2 mg/mL RNase A). Samples were analyzed on a Becton Dickinson (Heidelberg, Germany) FACScan, and the data were analyzed using CellQuest software. Each experimental point is done in duplicate and the results have been confirmed at least in three independent experiments.

RNA Preparation

For each collection point, the cell monolayer (15×10^6 cells) was washed twice with PBS at 4°C and total RNAs were extracted using the RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Microarray Assay

RNA targets (biotin-labeled RNA fragments) were produced from 5 μg total RNAs by first synthesizing double-stranded cDNA followed by an *in vitro* transcription reaction and a fragmentation reaction. An hybridization mixture containing the cRNA fragments, probe array controls (Affymetrix, Inc., Santa Clara, CA), bovine serum albumin, and herring sperm DNA was prepared and hybridized to human cancer G110 arrays, containing 1,700 cancer-associated genes (Affymetrix) at 45°C for 16 hours. The hybridized probe array was then washed. Bound biotin-labeled cRNA was detected with a streptavidin-phycoerythrin conjugate. Subsequent signal amplification was done with a biotinylated anti-streptavidin antibody. The washing and staining procedures were automated using the Affymetrix fluidics station. Each human cancer G110 array was scanned once using a Hewlett-Packard (Palo Alto, CA) GeneArray scanner. Each RNA preparation was hybridized to duplicate chips.

Microarray Data Analysis

Low-level data analysis was done using MAS version 5.0 software (Affymetrix.) by running absolute analyses on each array and comparative analyses between pairs of arrays hybridized with control and treated cells, respectively.

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All arrays were scaled to a mean gene expression intensity of 300. Detailed descriptions of the analysis algorithms have been published (19, 20). Briefly, absolute analyses provide an estimate of the expression intensity (''signal'') of each gene as well as a variable, called ''detection,'' which determines whether the gene can be considered as expressed (''P'' for ''present'' and ''M'' for ''marginal'') or not (''A'' for ''absent''). Comparative analyses identify modulated genes (''change'' variable set at ''I'' or ''MI'' for up-regulation and at ''D'' or ''MD'' for down-regulation associated with significant ''change *P*s''). Comparative analyses also provide estimates of the expression intensity ratios for each gene studied between the pairs of hybridized arrays being compared. The ratios are expressed in base 2 logarithmic scale and are called ''signal log ratios.''

For cluster analysis, only genes whose expression was significantly modulated in at least one of the conditions studied were retained. The following criteria were used: expression modulations (as determined by the ''change'' variable) had to be reproduced in at least one comparative analysis, and the signal log ratios had to be above 1 in at least one comparison. In addition, only genes whose normalized expression intensity was at least 20 in one sample were considered. Finally, genes whose expression was undetected (''detection'' equal to ''A'') in both treated and control samples in >75% of the comparisons were excluded. Cluster analysis was done on signal log ratios values weighed by the associated ''change *P*.'' Hierarchical clustering was obtained by calculating similarity between genes as the Jaccard correlation and grouping following the average linkage algorithm (unweighted pair group method with arithmetic mean) using the GeneMaths software package (Applied Maths, Inc., Austin, TX).

Western Blot Analysis

Cells (5×10^6) were washed with ice-cold PBS and incubated with 750 μL cold lysis buffer (50 mmol/L Tris, 120 mmol/L NaCl, 0.5% NP40, protease inhibitor cocktail) for 10 minutes on ice. Whole-cell lysates were clarified by centrifugation at 4,000 rpm for 15 minutes at 4°C. Samples (50 μg/condition) were heated in SDS sample buffer for 5 minutes at 95°C, run on 4% to 20% Tris-glycine gel (Novex, Invitrogen, Carlsbad, CA), and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, United Kingdom) using the Mini-Gel blot module (Invitrogen). Membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin in TBS [10 mmol/L Tris (pH 7.6) and 150 mmol/L NaCl] and then probed with anti-cyclin A (Neomarkers, Fremont, CA), anti-cyclin B1 (Neomarkers), anti-CDK1 (Cell Signaling, Beverly, MA), anti-phosphorylated CDK1 (Cell Signaling), anti-CDC25C (Cell Signaling), or anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight at 4°C. Incubation with the appropriate peroxidase-coupled secondary antibody and subsequent detection by enhanced chemiluminescence were done as recommended by the manufacturer (New England Biolabs, Ipswich, MA). Protein levels were quantified using GeneTools analysis software (Syngene, Inc., Frederick, MD).

Immunofluorescence Staining

Cells were fixed in 70% ethanol for 15 minutes at 4°C and rinsed twice with PBS. Cells were then permeabilized for 10 minutes at 4°C in PBS containing 0.25% Triton X-100, washed twice with PBS, and incubated with PBS containing 1% bovine serum albumin. Primary antibodies were added for 1 hour at room temperature. After washing twice with PBS, cells were incubated with Alexa Fluor 633–conjugated goat anti-mouse antibody (10 μg/mL final concentration) for 30 minutes at room temperature. Before analysis, cells were washed twice and stained for 20 minutes at room temperature with PI solution (5 μg/mL PI in PBS containing 0.1% Triton X-100 and 0.2 mg/mL RNase A). After washing twice with PBS, slides were analyzed with a Zeiss (Thornwood, NY) LSM410 confocal laser scanning microscope.

Results

Cell Cycle Phase Distribution following Oxaliplatin or Cisplatin Treatment

To determine the effective drug concentrations, HCT-116 cells were treated with various concentrations of oxaliplatin and cisplatin for 24 hours before a 12 h-thymidine pulse. In agreement with previously published data (6), both drugs inhibited HCT-116 proliferation with oxaliplatin more potent than cisplatin (i.e., its IC_{50} was 0.4 versus 1.2 μmol/L for cisplatin). Cell growth inhibition by oxaliplatin remained almost the same from 1 to 5 μmol/L (90% of inhibition), whereas the inhibition induced by cisplatin only reached that high level at 5 μmol/L (data not shown). This indicated that oxaliplatin was ~5-fold more potent in inhibiting DNA synthesis than was cisplatin. Doses from 1 to 10 μmol/L of both drugs have been used to study the effects of cisplatin and oxaliplatin on the cell cycle and the comparison between the two drugs has been done with 1 μmol/L oxaliplatin and 5 μmol/L cisplatin.

To investigate the effects of oxaliplatin and cisplatin on cell cycle progression, HCT-116 cells were treated for 24 hours and analyzed by flow cytometry. The profiles (Fig. 1A) show correlated dual-variable plots of cell count versus PI uptake after 24 h of treatment with 1 and 5 μmol/L of each drug. They indicated that 1 μmol/L oxaliplatin slightly increased the relative number of cells in the G_2-M phase (28% versus 22% for control cells) and decreased the relative number of cells in the S phase (4% for 1 μmol/L oxaliplatin versus 20% for control cells), although it had little effect on the cell population in G_1 (68% for 1 μmol/L oxaliplatin versus 58% for control cells). Under the same conditions, 5 μmol/L cisplatin increased the relative number of cells in G_2-M (43% for 5 μmol/L cisplatin versus 22% for control cells) but, contrary to oxaliplatin, it increased the relative number of cells in the S phase (36% for 5 μmol/L cisplatin versus 20% for control cells) and decreased the number of cells in G_1 (19% for 5 μmol/L cisplatin versus 58% for control cells).

G_2 and M phases were further analyzed using the MPM-2 labeling of mitotic cells. The MPM-2 antibody specifically

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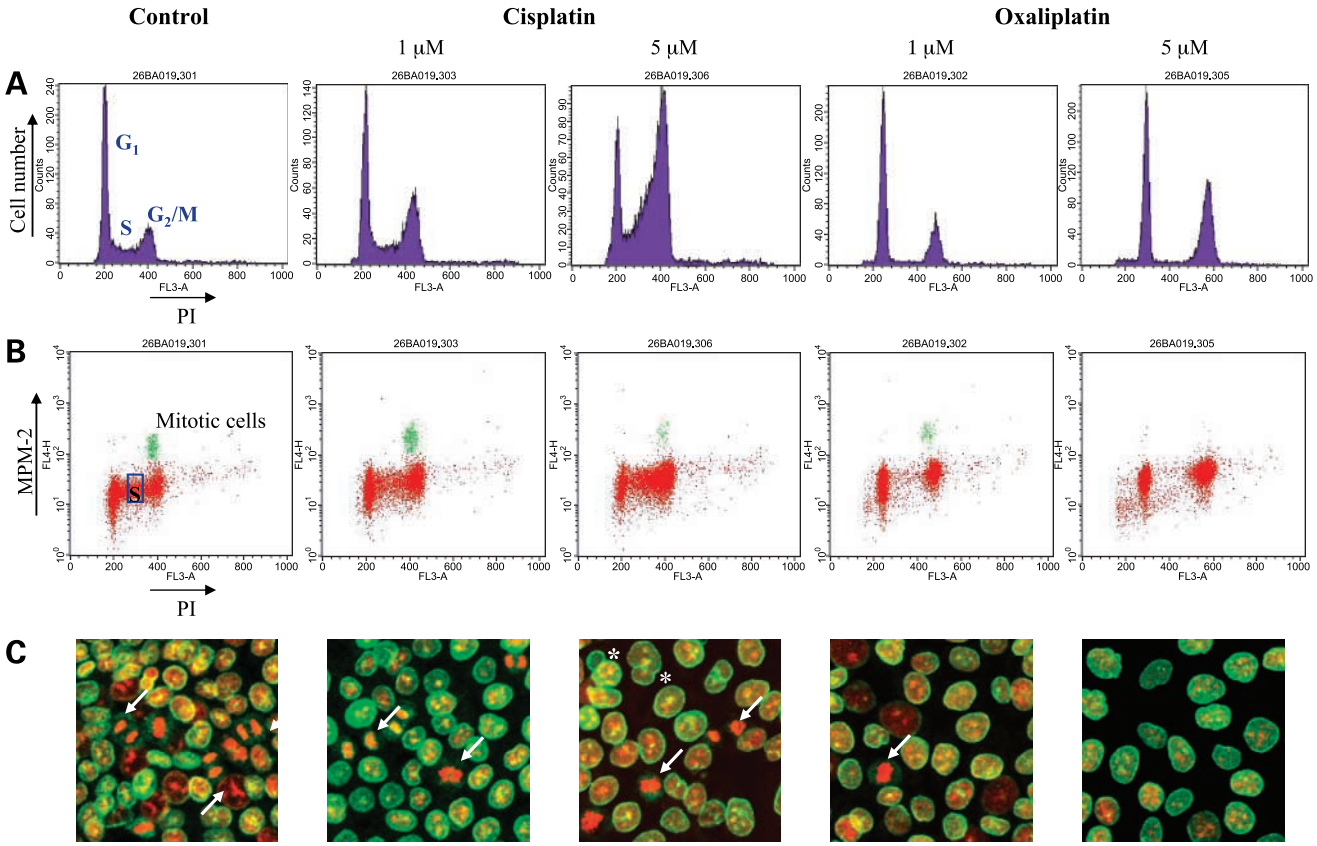

Figure 1. Effects of 24 h of oxaliplatin and cisplatin (1 and 5 μmol/L) treatment on the cell cycle of asynchronously growing HCT-116 cells assessed by fluorescence-activated cell sorting analysis. **A,** correlated dual-variable plots of cell counts versus PI uptake. G_1, S, and G_2-M cell populations. **B,** correlated dual-variable plots of MPM-2 versus PI uptake. *Green,* mitotic cells; *framed,* cells in the S phase. **C,** immunofluorescence staining of the nuclear membrane after 24 h of oxaliplatin or cisplatin (1 and 5 μmol/L) treatment. After treatment, cells were stained with PI (red fluorescence) and lamin B2 antibody plus Alexa Fluor 633–conjugated secondary antibody (green fluorescence) to visualize the nuclear integrity and nuclear membrane, respectively. *Arrows,* mitotic cells; *asterisks,* abnormal G_1 cells.

recognizes epitopes on phosphoproteins expressed at an early stage of mitosis (21). Correlated dot plots of MPM-2 labeling versus PI uptake are shown in Fig. 1B. Both 5 μmol/L cisplatin and 1 μmol/L oxaliplatin blocked cells in the G_2 phase, thus reducing the number of mitotic cells compared with control cells. With cisplatin (10 μmol/L) treatment, few cells still bypassed the G_2 checkpoint and entered mitosis (data not shown), whereas oxaliplatin (5 and 10 μmol/L)–treated cells did not evade the G_2 checkpoint (Fig. 1B; data not shown). To further document the effects of oxaliplatin and cisplatin on the M-phase entry, we assessed the nuclear membrane integrity, as its breakdown is a common feature of mitotic cells. The results of fluorescence microscopy experiments with PI labeling (DNA labeling) and lamin B2 antibody staining (nuclear membrane labeling), shown in Fig. 1C, confirmed the reduction of the number of mitotic cells following 24 hours of treatment with 1 μmol/L oxaliplatin or 5 μmol/L cisplatin compared with the control. Abnormal G_1 cells were observed among the cisplatin-treated cells (Fig. 1C). Their shapes suggested that they were G_1 cells issued from abnormal mitosis. Experiments done with 1 to 10 μmol/L cisplatin and oxaliplatin showed that the effect of the drugs on cell distribution was concentration dependent from 1 to 5 μmol/L drug concentration and plateaued at 5 μmol/L in both cases (data not shown).

Taken together, these data showed that cisplatin slowed down the replication phase and partially blocked cells in the G_2 phase, probably allowing aberrant mitosis. More interestingly, they showed that oxaliplatin strongly blocked cells in the G_1 and G_2 phases. Although they are close compounds, cisplatin and oxaliplatin did not alter cell cycle progression to the same extent, which suggests that oxaliplatin and cisplatin may have common and distinct mechanisms of action.

Oxaliplatin and Cisplatin Gene Expression Profiles

A global gene expression approach was used to identify cisplatin- and oxaliplatin-specific target genes. Using DNA chips containing 1,700 human cancer-related genes, we identified genes whose expression was dose dependently affected by oxaliplatin and cisplatin after 48 hours of treatment (shorter treatments did not give significant results). We compared gene expression profiles of HCT-116 cells treated for 48 hours with oxaliplatin and cisplatin

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concentrations ranging from 0.5 to 10 μmol/L. Below 0.5 μmol/L drug, we did not detect any RNA modulations. Gene expression profiles were analyzed using hierarchical clustering (Fig. 2). Of these genes, 117 were significantly modulated (see Materials and Methods for the selection criteria). Modulated genes can be subgrouped within three subclusters: (A) 37 genes dose dependently up-regulated by both drugs, (B) 42 genes dose-dependently down-regulated by both drugs, (C) and 38 drug-specific gene modulations. Tables 1, 2 and 3 show the major significantly modulated genes from subclusters A, B, and C, respectively, and their functional assignments. Subcluster A contained proapoptotic genes, such as *Bax, Bak*, and *Fas/Apo-1*. Among these genes, oxaliplatin-induced modulations of *Bax* and *Bak* have already been reported (17). This subcluster also contained the *WAF-1/p21* gene, which is involved in cell cycle progression and has already been described as up-regulated in response to cisplatin treatment (22). Subcluster B included *histone H1, retinoblastoma 1*, and *catenin α1*. Histone H1 is necessary for the condensation of nucleosome chains into higher order structures; retinoblastoma 1, a nuclear protein, is a potent inhibitor of E2F-mediated transactivation; and catenin α1 associates with the cytoplasmic domain of a variety of cadherins. Subcluster C revealed that cisplatin and oxaliplatin induced distinct gene modulation patterns. It contained genes strongly down-regulated by oxaliplatin, whereas they were either up-regulated or unaffected by cisplatin. What really matters is that the pattern of inverse response remained valid for the 1 μmol/L oxaliplatin versus 5 μmol/L cisplatin comparison (Fig. 2, *framed*). From this subcluster C, some cell cycle–related genes were involved in the G_2-M transition (i.e., *cyclin B, CDC25C*, and *CDK1*) or in the duplication phase (i.e., *CDC6, cyclin A, replication protein A, replication factor C*, and *primase*). Ki-67, the nucleolar proliferation marker, also belonged to subcluster C, such as B-Myb, another nuclear protein involved in cell proliferation. Several genes involved in the DNA replication (i.e., *P1-Cdc21, Cdc7-related kinase*, and *thymidine kinase*) were also inversely regulated by cisplatin and oxaliplatin. In addition, we observed modulation of centromere protein A and nucleosome assembly protein 1. Centromere protein A is a histone H3-like protein that is an essential component of centromeres and specifies the mitotic behavior of chromosomes, and the nucleosome assembly protein 1, a histone chaperone that mediates assembly proteins, participates in DNA replication and contributes to regulation of cell proliferation.

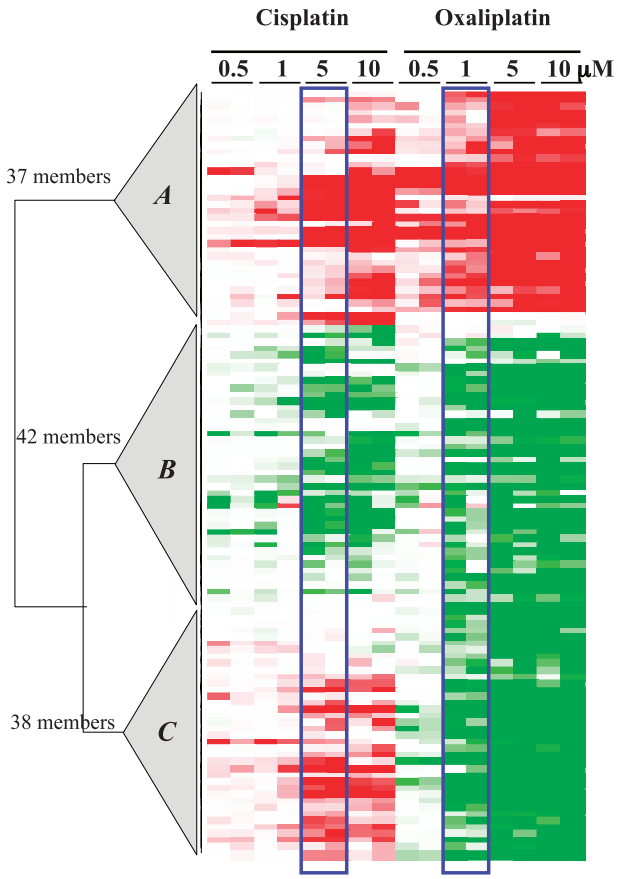

Figure 2. Hierarchical clustering of gene expression data measured after 48 h of oxaliplatin and cisplatin treatment. The single-dimension clustered gene expression measured from G110 array analyses using Jaccard correlation and algorithm unweighted pair group method with arithmetic mean. *Row,* a separate gene; *column,* a treated sample hybridized onto an array. *Green scale,* down-regulation; *red scale,* up-regulation; *white scale,* no change. Data are relative to untreated cells. *Right,* subclusters A, B, and C. *Underlined,* comparison of 1 μmol/L oxaliplatin versus 5 μmol/L cisplatin.

We have tested two more cell lines: the HT-29 human colon cell line and the A2780 human ovary cell line. In the HT-29 cell line (data not shown), the genes from subcluster C, specific of the oxaliplatin transcriptional signature in the HCT-116 cell line, were unchanged or up-regulated by both cisplatin and oxaliplatin. No oxaliplatin-specific transcriptional signature appeared with this cell line. In the A2780 cell line (data not shown), the genes from subcluster C were down-regulated by oxaliplatin (0.5–10 μmol/L) as in the HCT-116 cell line.

Protein Expression in HCT-116 Cells following Oxaliplatin or Cisplatin Treatment

Cell cycle–related gene modulations were further investigated at the protein level. As shown in Fig. 3A and Table 4, the protein levels were correlated with the mRNA modulations: the cyclin A, cyclin B, CDK1, and CDC25C protein expressions were unchanged or slightly repressed by 24 hours of oxaliplatin (1 μmol/L) treatment, strongly repressed after 48 hours of oxaliplatin (1 μmol/L) treatment, and either unaffected or slightly enhanced by 24 and 48 hours of cisplatin (5 μmol/L) treatment. As CDK1 activity is regulated by phosphorylation, we used an antibody targeting the CDK1 phosphorylated form (Tyr^{15}). The results showed that expression of the phosphorylated form was down-regulated by 48 hours of oxaliplatin treatment (P-CDK1/actin ratio of 0.16 for 1 μmol/L oxaliplatin; Table 4) and slightly up-regulated by 48 hours of cisplatin treatment (P-CDK1/actin ratio of 1.59 for 5 μmol/L cisplatin; Table 4). As cyclin B is one of the key

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Table 1. Functional assignment of major genes that are induced by 0.5 to 10 μmol/L cisplatin and oxaliplatin (subcluster A)

Function	Gene name	Accession no.
Apoptosis	*Bax α*	2065_s_at
	Bax δ	1997_s_at
	Bax γ	2067_f_at
	Bak	846_s_at
	Fas/Apo-1	1440_s_at
	MDM-2	1880_at
Cell cycle	*p21$^{waf1/cip1}$*	2031_s_at
	Cyclin G_1	1920_s_at
	MDM-2	1880_at
	Proliferating cell nuclear antigen	1824_s_at
Transcription	*RNA polymerase II*	503_at
Signal transduction	*Ras*	1590_s_at
	Annexin II	757_at
	Inositol polyphosphate 1-phosphatase	656_at
	Inositol polyphosphate 5-phosphatase	172_at
DNA replication	*Proliferating cell nuclear antigen*	1824_s_at
Drug resistance	*Semaphorin E*	377_g_at

proteins in the G_2-M transition, we measured its expression by flow cytometry (Fig. 3B). Figure 3B shows a significant decrease in cyclin B expression following treatment with 1 μmol/L oxaliplatin compared with untreated cells. By contrast, in the same conditions, 5 μmol/L cisplatin increased cyclin B expression of cells in the G_2-M phase. These results are visible after 24 hours of treatment but stronger after 48 hours.

Discussion

It is widely accepted that DNA damage is largely responsible for the cytotoxic properties of platinum (23, 24). Interestingly, oxaliplatin, which is more cytotoxic than cisplatin (6), forms fewer DNA adducts than cisplatin does [e.g., 0.86 ± 0.04 versus 1.36 ± 0.01 adducts/10^6 bp/10 μmol/L drug/1 hour in CEM cells (4); refs. 1, 2, 4]. This suggests different mechanisms of action.

Activation of cell cycle checkpoints is a general cellular response following exposure to cytotoxic agents. These checkpoints arrest cells at the G_1-S or G_2-M transitions. Previous studies indicated that cisplatin and other platinum agents predominantly reduce the rate of DNA synthesis (10, 12) and inhibit cell cycle progression at the G_1-S and/or G_2-M phases (12, 25). Here, we show, in agreement with previously published data (10, 12, 25), that cisplatin slowed down cells in the S phase and disturbed the G_2-M transition. More interestingly, our results indicated that in HCT-116 cells, oxaliplatin strongly blocked cells in the G_1 and G_2 phases. The fact that cisplatin and oxaliplatin had different effects on cell cycle progression suggests that the two drugs do not have the same mechanism of action.

In this study, we analyzed the effects of cisplatin and oxaliplatin at the gene expression level. We have an anticancer drug transcriptional signature database with several cytotoxic agents, including antimetabolite agents (methothrexate and 5-fluorouracil), alkylating agents (melphalan, oxaliplatin, and cisplatin), and topoisomerase inhibitors (camptothecin, doxorubicin, and etoposide). Hierarchical clustering of anticancer drugs showed that cisplatin and oxaliplatin were grouped together, close to the alkylating agent melphalan (data not shown). This was in accordance with the fact that cisplatin and oxaliplatin belong to the same drug family and are also alkylating agents. As expected, the signatures of cisplatin and oxaliplatin had common features (Fig. 2). Modulations of genes involved in the apoptosis response, such as *Bax* and *Fas*, are part of this platinum signature, indicating a common cell death mechanism. The hierarchical clustering elicited a cluster of 38 genes that could help to differentiate the effects of oxaliplatin and cisplatin as they are specifically modulated by cisplatin or oxaliplatin. Cell cycle–related genes are included in this cluster. Previous studies showed that these cell cycle–related genes share common sequences in their promoters: the CDE and CHR promoter elements (26). Moreover, these sequences have already been described as common regulators of these genes and involved in G_2-M arrest (26). Considering that this sequence is very rich in GC bases, altogether these data highly suggested that this promoter sequence has a key role in oxaliplatin-specific transcriptional signature and that these genes may be modulated by oxaliplatin.

The transcriptional study results and their validation at the protein level suggested that two different mechanisms are responsible for oxaliplatin- and cisplatin-induced G_2 transition control. Oxaliplatin induced a strong inhibition of CDK1 and cyclin B protein expression. It is now widely accepted that the G_2-M transition is partly governed by the CDK1-cyclin B complex. G_2 arrest associated with cyclin B repression has already been reported on treatment with adriamycin, etoposide, and irinotecan (27), suggesting that cyclin B repression is involved in the G_2 arrest mechanism induced by anticancer drugs. p21$^{waf1/cip1}$ exerts a key role in controlling the G_2 arrest by inhibiting the CDK1-cyclin B complex. Microarray analysis showed that, under oxaliplatin treatment, p21$^{waf1/cip1}$ RNA expression was up-regulated by 4.8-fold (only 1.6-fold activation with

Table 2. Functional assignment of major genes that are repressed by 0.5 to 10 μmol/L cisplatin and oxaliplatin (subcluster B)

Function	Gene name	Accession no.
Cell cycle	*CDC25B*	1347_at
	CDKN2A	1713_s_at
	CDKN1C	1787_at
	Retinoblastoma 1	1937_at
Transcription	*Transcription elongation factor*	1073_at
Signal transduction	*Sky*	2086_s_at
	HER-3	1585_at
Cell adhesion	*Catenin α1*	2085_s_at
Nucleosome assembly	*Histone H1*	580_at

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cisplatin treatment) as early as 6 hours of treatment (data not shown). We hypothesize that the $p21^{waf1/cip1}$ pathway may be involved in early events of the oxaliplatin-induced G_2 block. Absence of cyclin B and CDK1 after 48 hours of oxaliplatin treatment may be involved as late events in the establishment of a very strong arrest of cells in G_2 phase by oxaliplatin. Cisplatin treatment did not decrease the CDK1 or cyclin B protein level, rather cisplatin-induced G_2 arrest was correlated with a slight accumulation of both proteins. It has been widely shown that the phosphorylation of CDK1 on Tyr^{15} by CDC25C (28) inactivates the CDK1-cyclin B complex, which blocks M-phase entry. Considering the total amount of CDK1 (P-CDK1 and CDK1) in our study, the CDK1 phosphorylated form was not increased by cisplatin, suggesting that the CDK1 activity is not responsible for the G_2 blockage induced by cisplatin.

The results of the gene and protein expression analysis also enabled us to gain insight into the mechanism of action involved in the oxaliplatin-induced G_1 checkpoint. It is well known that the inhibition of cyclin A/CDK2 activity delays or prevents S-phase progression. In our experiments, the expression of cyclin A and CDK2 RNAs drastically decreased only after 48 hours of treatment, suggesting that inhibition of cyclin A and CDK2 RNA expression may be a consequence of the G_1 arrest. The absence of cyclin A and CDK1 proteins after 48 hours of oxaliplatin treatment could contribute to maintain the G_1 arrest. Oxaliplatin also down-regulated genes encoding proteins involved in the initiation of DNA replication, such as *CDC6* and *primase*, or in the progression of the DNA replication process, such as *replication protein A* and *replication factor C*, and this may also contribute to late blockage at the S phase entry.

A checkpoint response induced by cytotoxic agents can have both positive and negative effects on apoptosis (9, 29). It has been shown that disruption of the G_2 checkpoint control response elicited by cytotoxic agents often increases the cellular sensitivity to these drugs (9, 29, 30) because unbridled cell cycle progression in the presence of DNA damage is usually lethal. For this reason, components of the G_2 checkpoint response are potential targets for new chemotherapeutic agents. The relationship between the checkpoint response of the other phases of the cell cycle and the induction of apoptosis is less established (9, 29). It has been shown that G_1 checkpoint abrogation decreases drug sensitivity (31). This was supported by our results that showed that the G_1 checkpoint response was associated with increased sensitivity. Further studies should be carried out to examine whether the effect of oxaliplatin on the G_1 checkpoint is linked with the ability of oxaliplatin to bypass cisplatin resistance and/or how does it may increase the apoptotic consequences of its combination with other agents.

The greater efficacy of oxaliplatin compared with cisplatin (i.e., isocytotoxicity with fewer DNA/platinum adducts) does not always translate into clinical settings. Indeed, oxaliplatin at recommended doses shows only marginal clinical activity as a single agent in most human cancers, and this is seriously in cancers with known prevalent somatic or epigenetic mismatch repair deficiency (e.g., colon, ovarian, or endometrial cancers). Interestingly, in such tumors, mismatch repair-deficient phenotype is increasingly prevalent with tumor progression, although prospective clinical translational work has never been implemented to completely validate this insight (7).

Table 3. Functional assignment of major genes that are induced by 0.5 to 10 μmol/L cisplatin and repressed by 0.5 to 10 μmol/L oxaliplatin (subcluster C)

Function	Gene name	Accession no.
Cell cycle	*Cyclin A*	1943_at
	Cyclin B	1945_at
	CDK1	1803_at
	CDC25C	1584_at
	mki67	418_at
	B-myb	1854_at
	CIP2	1599_at
	Mad2	1721_g_at
DNA replication	*Replication factor C*	1053_at
	DNA primase	798_at
	Replication protein A	527_at
	Cdc7-related kinase	1809_at
	P1-Cdc21	981_at
	Thymidine kinase	910_at
	NAP1	571_at
	CDC6-related protein	1536_at
Nucleosome assembly	*Centromere protein-A*	527_at

An intriguing and positive characteristic of oxaliplatin is its marked preclinical and clinical synergy with a wide variety of anticancer agents, mainly antimetabolites, such as fluorouracil (32) or gemcitabine (33), but also with cisplatin (6). In most cases, these oxaliplatin-based combinations are superior to cisplatin-based combinations. This superiority is seen above and beyond the mismatch repair prevalence as it is the case with taxanes (34) or *Vinca* alkaloids (35). Preliminary work suggests that DNA synthesis (via the thymidylate synthase) and repair mechanisms (via the excision repair cross complementing 1) are also implicated in the synergy observed with 5-fluorouracil (36). The current data have been obtained in a wild-type functional p53 cell line. Our study has been extended to the p53-proficient A2780 cell line and the p53-deficient HT-29 cell line. Our results on the p53-proficient A2780 cell line confirmed the oxaliplatin-specific transcriptional signature. Our results also indicated that there was no oxaliplatin-specific transcriptional signature with the p53-deficient HT-29 cell line. These data support the fact that the G_2 cell cycle arrest associated with a decrease in cyclin A and B level is p53 dependent (26). The G_2 checkpoint activity seems to be a new and major differentiating factor between 1,2-diaminocyclohexane platinums and the other DNA alkylators, and it is probably linked to the nonrepairability of transcriptional gene sites associated with the G_2-M-related cyclins cascade. It could also explain the enhanced

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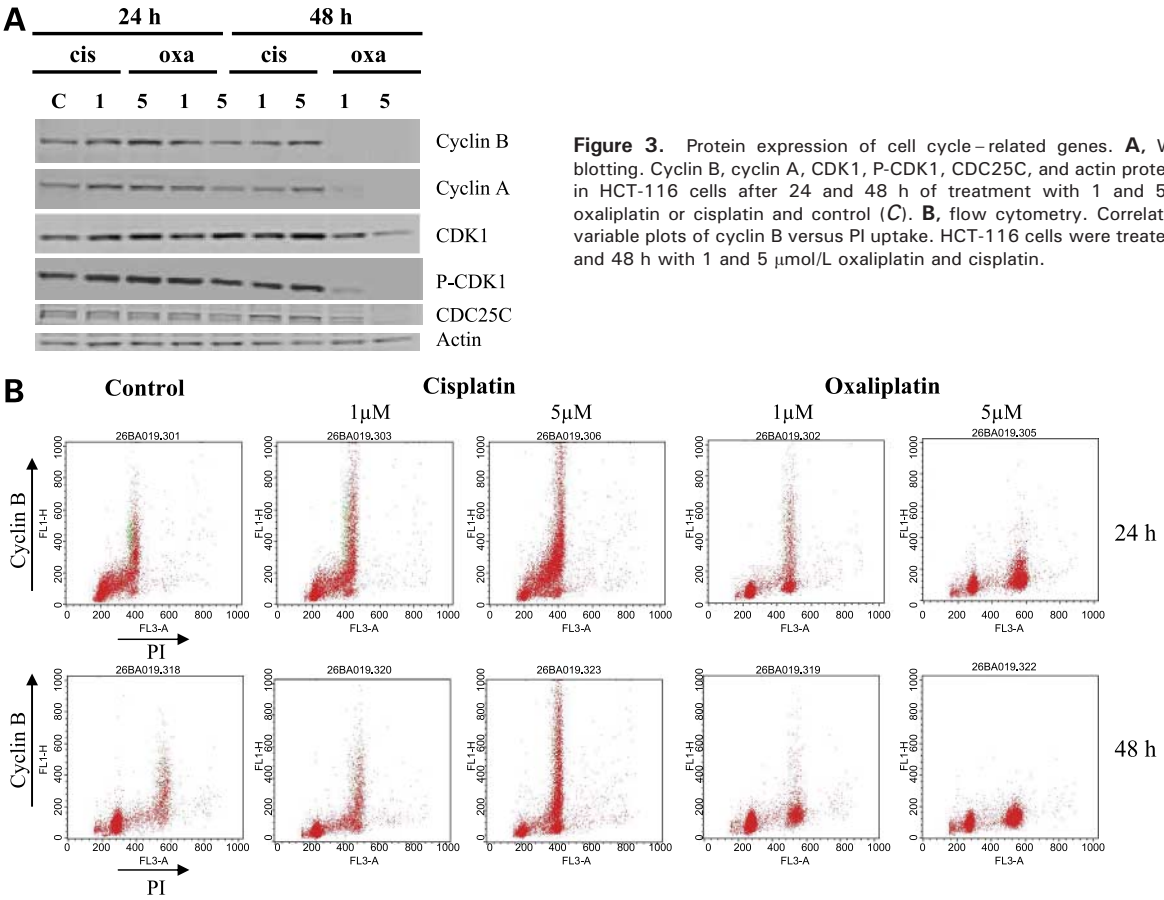

Figure 3. Protein expression of cell cycle–related genes. **A,** Western-blotting. Cyclin B, cyclin A, CDK1, P-CDK1, CDC25C, and actin protein levels in HCT-116 cells after 24 and 48 h of treatment with 1 and 5 μmol/L oxaliplatin or cisplatin and control (*C*). **B,** flow cytometry. Correlated dual-variable plots of cyclin B versus PI uptake. HCT-116 cells were treated for 24 and 48 h with 1 and 5 μmol/L oxaliplatin and cisplatin.

drug sensitivity to other anticancer agents acting on DNA or other targets when combined with oxaliplatin, such as 5-fluorouracil, gemcitabine, taxanes, etc., which act through cell cycle arrests at the G_1-S and/or M phases.

In summary, we showed for the first time that cells treated with cisplatin and oxaliplatin respond differently with regard to the genes that are modulated, suggesting two different mechanisms of action. We also showed that oxaliplatin was a strong and specific cyclin cascade G_2-M transition blocker and, to our knowledge, the first agent with such blocking specificity. Using a DNA array technology, we established the oxaliplatin and cisplatin transcriptional signatures in the HCT-116 human colon carcinoma cell line. We noted a platinum transcriptional signature and, in parallel and for the first time, observed cell cycle–related genes that were conversely regulated by oxaliplatin and cisplatin. These modulations were confirmed at the protein level. These results highly suggest that the cell cycle modifications induced by cisplatin and oxaliplatin are the result of distinct cellular mechanisms. Finally, the oxaliplatin-specific target genes that have been identified give clues for the mechanism of action of

Table 4. Protein/actin ratio (percentage of treated cells versus nontreated cells)

	Treatment (24 hours)				Treatment (48 hours)			
	Cisplatin		Oxaliplatin		Cisplatin		Oxaliplatin	
	1 μmol/L	5 μmol/L	1 μmol/L	5 μmol/L	1 μmol/L	5 μmol/L	1 μmol/L	5 μmol/L
Cyclin B	1.02	1.52	0.91	0.56	0.80	1.41	0.05	0.02
Cyclin A	1.03	0.97	0.78	0.48	0.67	1.15	0.16	0.03
CDK1	0.98	1.20	0.90	1.19	1.18	2.20	0.77	0.43
P-CDK1	0.92	1.21	1.09	0.75	0.89	1.59	0.16	0.02
CDC25C	1.53	0.91	1.11	0.66	2.05	2.35	0.61	0.38

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oxaliplatin. In addition, these genes could also be useful for evaluating and comparing the efficacy and molecular pharmacology of new platinum drugs. In this respect, the oxaliplatin-specific signature could be used to discriminate *in vitro* between cisplatin-like and oxaliplatin-like molecules. Further analysis are necessary to determine if these genes mediate the higher efficacy of oxaliplatin in combination with other agents compared with cisplatin based combinations.

Acknowledgments

We thank Vjekoslav Dulic (CRBM-CNRS FRE2593, Montpellier, France) for interesting discussion.

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Mol Cancer Ther 2006;5:2149-2157.

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