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Gene Expression Signature Predicting High-Grade Prostate Cancer Responses to Oxaliplatin[S]

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ABSTRACT

Prostate cancer is one of the leading causes of cancer-related deaths among men. Several prognostic factors allow differentiation of low-grade tumors from high-grade tumors with high metastatic potential. High-grade tumors are currently treated with hormone therapy, to which taxanes are added when the tumors become resistant to castration. Clinical trials with other anticancer agents did not take into account the genetic backgrounds of the tumors, and most trials demonstrated low response rates. Here we used an in silico approach to screen for drug candidates that might be used as alternatives to taxanes, on the basis of a published expression signature involving 86 genes that could distinguish high-grade and low-grade tumors (*Proc Natl Acad Sci USA* **103:**10991–10996, 2006). We explored the National Cancer Institute databases, which include data on the gene expression profiles of 60 human tumor cell lines and the in vitro sensitivities of the cell lines to anticancer drugs, and we identified several genes in the signature for which expression levels were correlated with chemosensitivity. As an example of the validation of this in silico approach, we identified a set of six genes for which expression levels could predict cell sensitivity to oxaliplatin but not cisplatin. This signature was validated in vitro through silencing of the genes in DU145, LNCaP, and C4-2B prostate cancer cells, which was accompanied by changes in oxaliplatin but not cisplatin cytotoxicity. These results demonstrate the relevance of our approach for the identification of both alternative treatments for high-grade prostate cancers and new biomarkers to predict clinical tumor responses.

Introduction

With more than 240,000 new cases in the United States in 2011 (http://seer.cancer.gov) and an incidence rate of 214 cases/100,000 men in Europe (Bosetti et al., 2011), prostate cancer is the second leading cause of death attributable to cancer throughout the world. Prostate cancer includes various disease types, ranging from indolent asymptomatic cancer characterized by slow-growing tumors to advanced prostate cancer characterized by its aggressiveness and its high metastatic potential. This heterogeneity is evidenced by three main clinical/biological parameters that can be observed at the time of diagnosis, i.e., serum prostate-specific antigen (PSA) levels, clinical stage, and Gleason score, which have been used to predict disease outcomes with relatively good accuracy (Stephenson et al., 2005). The Gleason grading system is based on microscopic features of tumor architecture, and scores reflect the degree of differentiation of tumor cells (Gleason, 1992). Five histological patterns can be assigned, from pattern 1 with well differentiated cells to pattern 5 with poorly differentiated cells, to distinguish between low-grade (grades 1–3) and high-grade (grades 4 and 5) tumors. The sum of the two most-represented patterns within a tumor corresponds to the Gleason score; scores range from 2 to 10. For more than 35 years, this score has remained one of the most-powerful prognostic predictors of disease outcomes, including death (Albertsen et al., 1995). In addition to clinical stage, the Gleason score represents a crucial parameter for the choice of therapy. Low-grade prostate cancers (scores below 3 + 4) are usually sensitive to hormone deprivation, which can be achieved through surgical castration or pharmacological treatment with antiandrogens, with median response durations exceeding 3 years. In contrast, high-grade prostate cancers (scores of at least 4 + 3) are intrinsically or rapidly become resistant to hormone therapy because of DNA-based alterations of androgen receptors, such as mutations or amplifications (Brooke and Bevan, 2009). Such can-

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ABBREVIATIONS: PSA, prostate-specific antigen; RT, reverse transcription; PCR, polymerase chain reaction; BPH, benign prostatic hyperplasia; NCI, National Cancer Institute; siRNA, small interfering RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

cers are referred to as castration-resistant prostate cancers (Bonkhoff and Berges, 2010) but are still treated with hormone deprivation, to which the first-line chemotherapeutic agent docetaxel is added when they become resistant to castration (Mancuso et al., 2007). Despite the recent approval of the new taxane cabazitaxel (Paller and Antonarakis, 2011), survival rates remain poor, which points toward the need for more-efficient drug alternatives.

Clinical trials have been performed with mitoxantrone, epirubicin, mitomycin C, methotrexate, cyclophosphamide, 5-fluorouracil, vinorelbine, and platinum derivatives, alone or in combination, but have not yielded convincing results, despite some interesting responses (Mike et al., 2006). New targeted therapies such as antiangiogenic compounds, endothelin receptor inhibitors, receptor activator of nuclear factor κ-B ligand inhibitors, and CYP17 inhibitors also have been investigated, with encouraging results (Carducci and Jimeno, 2006; Dror Michaelson et al., 2009; de Bono et al., 2011). However, the patient populations included in those trials were heterogeneous and patient selection did not take into account tumor grades, despite the fact that several studies identified genes for which expression levels were correlated with Gleason grades (Lapointe et al., 2004; True et al., 2006; Wang et al., 2009; Cuzick et al., 2011; Penney et al., 2011). The study by True et al. (2006) identified a signature involving 86 genes the expression levels of which could be used to discriminate between low-grade and high-grade prostate tumor samples with 76% accuracy, in a validation set of 32 tumors.

On the basis of this expression classifier, we used an in silico approach analogous to the coexpression extrapolation principle, which translates in vitro sensitivity signatures into tools for predicting drug sensitivities (Lee et al., 2007), to identify anticancer drugs other than taxanes that might be proposed as potential alternatives for the treatment of high-grade prostate cancers. We extracted, from the publicly available, 60-cell line, NCI screening database of the Developmental Therapeutics Program, the expression levels of the 86 genes in the signature described by True et al. (2006), and we searched for correlations between the expression levels of the genes in the panel and the sensitivities of the 60 cell lines to 152 anticancer agents. Among the extensive number of correlations, we could identify a gene signature that predicted the selective sensitivity of prostate cancer cell lines to oxaliplatin. We validated this signature at the functional level by showing that down-regulation of these genes altered prostate cancer cell sensitivity to oxaliplatin but not cisplatin, which suggests that this platinum derivative might be a potential alternative for the treatment of high-grade prostate cancers. Our results also confirm the potential of gene expression models to identify drug candidates as well as new predictive markers of drug sensitivity.

Materials and Methods

In Silico Approach. The different steps of the in silico study are presented in Fig. 1. First, data extractions were performed by using the NCI-60 panel database, which is freely available from the Developmental Therapeutics Program (http://dtp.nci.nih.gov). We extracted the expression profiles of the 86 genes identified by True et al. (2006) across the 60 cell lines. The expression profiles of *TOP2A*,

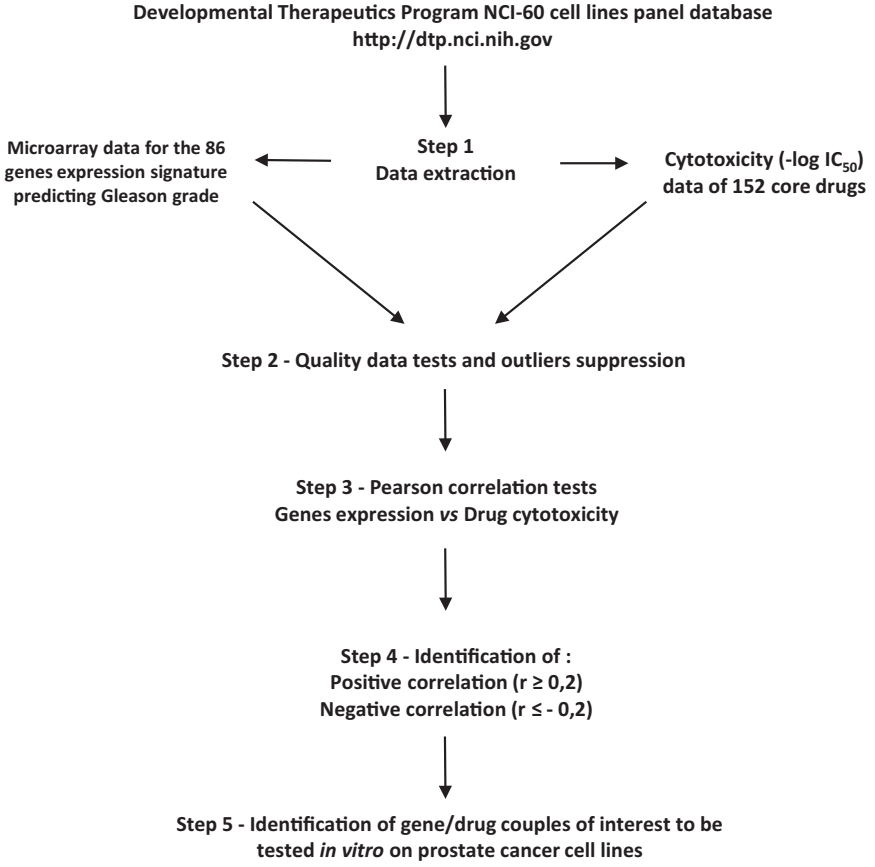

Fig. 1. Overview of the steps in the in silico approach.

TOP1, *MGMT*, and *ABCB1* genes were extracted as independent controls, because they were shown in numerous models to be associated with the activity of several anticancer drugs. The expression data were obtained with human U95 (A–E) and U133 (A and B) Affymetrix (Santa Clara, CA) microarrays, as described previously (Scherf et al., 2000). Data sets were imported into Bioconductor by using the ArrayExpress package (Kauffmann et al., 2009). The robust multiarray average function from the affy package (Gautier et al., 2004) was used for background correction, quantile normalization, and probe set summarization. Data quality assessment was performed by using the Bioconductor package arrayQualityMetrics to remove outliers from the data sets (Kauffmann and Huber, 2010). An array was considered an outlier if it was identified as such with at least two of the three quality metrics evaluated by the package (Supplemental Fig. 1, A and B) (Kauffmann and Huber, 2010). Microarrays that were considered outliers are presented in Supplemental Fig. 1C.

Differentially expressed genes were identified by using the moderated *t* test from the limma package (Smyth, 2004). The *p* values were adjusted for multiple testing by using the Benjamini-Hochberg method to control the false-discovery rate, and genes were considered significant when adjusted *p* values were <0.01. When several expression data sets were available for a single gene, we determined whether the data sets were correlated with each another by using Pearson's correlation test. Expression data sets (microarrays) with Pearson coefficients of ≥ 0.5 were retained for further analyses.

We extracted cytotoxicity data, expressed as $-\log IC_{50}$ values, for 152 drugs that are representative of the major classes of antiproliferative compounds that have been screened by the NCI. Associations between chemosensitivity and gene expression levels in the NCI cell line panel were established through the calculation of Pearson correlation coefficients, as performed previously by Scherf et al. (2000). With 58 degrees of freedom, Pearson coefficients less than -0.2 or more than $+0.2$ corresponded to *p* values of <0.05 (Fig. 1). As discussed below, such *p* values, which did not take into account multiple testing, allowed us to perform a first screen to provide a reasonable number of hints regarding potential correlations between gene expression levels and sensitivity to drugs that could be tested in vitro with a functional approach.

Cell Lines and Drugs. The human DU145 and LNCaP prostate cancer cell lines, the normal benign prostatic hyperplasia (BPH) cell line, and the human MDA-MB-468 breast adenocarcinoma cell line were obtained from the National Cancer Institute (Bethesda, MD). The hormone-insensitive, bone-metastatic, LNCaP-derivative, C4-2B prostate cancer cell line was a kind gift from Dr. O. Cuvillier (CNRS UMR 5089, Toulouse, France). DU145 cells were routinely grown in minimal essential medium, and C4-2B and MDA-MB-468 cells were grown in RPMI 1640 medium. Media were supplemented with 10% fetal bovine serum. BPH and LNCaP cells were grown in Quantum 263 medium containing 10% fetal bovine serum (PAA Laboratories, Pasching, Austria). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cisplatin and oxaliplatin were purchased from Sigma-Aldrich (St. Louis, MO). Fresh 10 mM stock solutions were prepared and diluted in dimethylsulfoxide before use.

siRNA Transfections. Control (nontargeting) siRNA and siRNA cocktails targeting the different genes were purchased from Dharmacon RNA Technologies (Lafayette, CO). The specific sequences are presented in Supplemental Table 1. Cells (150,000 cells/well) were seeded in six-well plates, and transfections were performed 24 h later with 100 pmol of control or targeting siRNA and Oligofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. At the time of drug treatment, a fraction of the cells were rinsed with ice-cold phosphate-buffered saline and harvested. After centrifugation, dry cell pellets were frozen at −80°C for quantitative RT-PCR and Western blot analyses, to verify the efficacy of gene repression.

Quantitative RT-PCR Assays. Total RNA from dry cells pellets was extracted and purified by using an RNeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol, with a final volume of 30 μl of RNase-free water. RNAs (500 ng) were reverse-transcribed by using a SuperScript VILO cDNA synthesis kit (Invitrogen), according to the manufacturer's instructions, with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA), as follows: 10 min at 25°C, 60 min at 42°C, and 5 min at 85°C.

Real-time quantitative PCR assays were performed by using 10 ng of cDNAs in a final volume of 25 μl containing 1× SYBR Green, 1.5 mM $MgCl_2$, 0.2 μM concentrations of each primer (Supplemental Table 2), 0.2 mM dNTP mixture, and 1 unit of Taq DNA polymerase (Invitrogen). Reactions were performed by using a Corbett Research Rotor-Gene 3000 thermal cycler (QIAGEN), as follows: 5 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final elongation step of 2 min at 72°C. Three independent reactions were performed, and expression levels were normalized with respect to glyceraldehyde 3-phosphate dehydrogenase and 18S RNA levels. Results were analyzed by using the cycle threshold method, by comparing the cycle threshold values obtained for targeted siRNA samples and control siRNA samples, and are expressed as mRNA levels relative to control values (mean ± S.D.).

Cytotoxicity Assays. The effects of siRNA on growth inhibition induced by platinum derivatives were evaluated by using MTT assays or crystal violet staining. The day after siRNA transfection, cells were detached with trypsin and were seeded into 96-well plates at the following densities: 1000 cells/well for BPH and C4-2B cells, 1500 cells/well for DU145 cells, 2000 cells/well for MDA-MB-468 cells, and 5000 cells/well for LNCaP cells. On the following day, cells were treated with cisplatin or oxaliplatin continuously for 72 h. Cells were rinsed with 1× phosphate-buffered saline and were incubated for 4 h at 37°C with fresh medium containing 5 mg/ml MTT (Sigma-Aldrich). The medium was then replaced with dimethylsulfoxide to dissolve formazan crystals, which are produced through the reduction of MTT by living cells. Absorbance was measured through spectrophotometry at 570 nm by using a PowerWave X reader (BioTek Instruments, Winooski, VT). In the case of LNCaP cells, cells were fixed with 70% ethanol and living cells were stained through incubation with crystal violet (2.3%, w/v) for 10 min. Cells were rinsed with water and lysed with 1% SDS, and absorbance was measured through spectrophotometry at 600 nm.

Statistical Analyses. Prism software (GraphPad Software Inc., San Diego, CA) was used to calculate IC_{50} values from growth inhibition assay data and to perform statistical comparisons between samples. Data are expressed as mean ± S.D. Statistical significance was determined with Student's unpaired *t* tests. Differences with *p* values of <0.05 were considered significant.

Results

The general overview of the in silico approach that we used in our study is shown in Fig. 1 and is detailed under *Materials and Methods*. In the first step, we searched for available gene expression data sets that might be representative of high-grade prostate cancers; we selected an 86-gene classifier capable of distinguishing low-grade and high-grade cancers (True et al., 2006). We searched for correlations between the expression levels of these 86 genes and sensitivities to a panel of drugs by using the freely accessible NCI-60 database from the Developmental Therapeutics Program. From this database, we extracted data on the expression levels of the 86 genes in the 60 cancer cell lines in the panel and the sensitivities of the cell lines to 152 core compounds that represent the major classes of antiproliferative agents (expressed as $-\log IC_{50}$ values). Data quality assessments and identification of outlier arrays led to the elimination of 14 cell line data sets included in the U95 (A–E) and U133 (A and B) microar-

TABLE 1

Results of in silico study

For each class of anticancer agents, drugs for which correlations were found between sensitivities and expression levels of the indicated genes are listed. Correlations were considered significant for Pearson coefficients below −0.2 or above +0.2.

Mechanism of Action & Drug	NSC No.	Correlated Genes
AA A2/A6		
Mitomycin	26980	*PRDX5*
Porfiromycin	56410	*PRDX5*
Carmustine	409962	*EIF4A1*, *FTH1*, *HIRIP3*, *LTBR*, *NPC2*, *PRDX5*
Chlorozotocin	178248	*FLJ35093*, *FTH1*, *PRDX5*
Clomesone	338947	*PRDX5*
Irofulven	683863	*ATP5G3*, *HIRIP3*, *NEDD5*, *RHOT2*
Lomustine	683863	*EIF4A1*, *FTH1*, *RHOT2*
Mitozolamide	79037	*PRDX5*
PCNU	353451	*FTH1*, *LTBR*, *PRDX5*
Semustine	95466	*FTH1*, *HER2*, *PRDX5*
Trabectadin	648766	*NEDD5*
AA A7		
Asaley	167780	*FTH1*, *PCCB*
Busulfan	750	*EIF4A1*
Carboplatin	241240	*AZGP1*, *HMGB1*, *PRDX5*, *RAB6A*, *TMPRSS2*
Chlorambucil	3088	*FLJ35093*, *FTH1*, *PRDX5*
Cisplatin	119875	*AZGP1*, *HMGB1*, *PRDX5*, *RAB6A*, *TMPRSS2*
Cyclodisone	348948	*PRDX5*
Diaminocyclohexyl-platinum(II)	271674	*FLJ35093*, *PCCB*, *SHMT2*
Dianhydrogalactitol	132313	*EIF4A1*, *FLJ35093*
Diaziridinylbenzoquinone	182986	*NPC2*
Fluorodopan	73754	*CPE*, *EIF4A1*, *HIRIP3*, *FTH1*
Hepsulfam	329680	*FLJ35093*
Iproplatin	256927	*CD59*, *CPE*, *HIRIP3*
Mechlorethamine	762	*CD59*, *CPE*, *FLJ35093*, *HIRIP3*
Melphalan	8806	*EIF4A1*, *FLJ35093*, *HIRIP3*
Oxaliplatin	266046	*ATP5G3*, *CD59*, *CDKN2C*, *DPM1*, *EIF4A1*, *FLJ35093*, *HMGB1*, *JUN*, *PCCB*, *RHOT2*, *RPL13*, *SHMT2*
Piperazine mustard	344007	*FLJ35093*, *PRDX5*, *RAB6A*
Piperazine dione	135758	*FLJ35093*
Pipobroman	25154	*EIF4A1*, *HIRIP3*
Spiromustine	172112	*CPE*, *FTH1*
Teroxirone	296934	*EIF4A1*, *FLJ35093*, *HIRIP3*, *PRDX5*
Tetraplatin	363812	*ATP5G3*, *CD59*, *EIF4A1*, *FLJ35093*, *HMGB1*, *JUN*, *PCCB*, *RPL13*, *SHMT2*
Thiotepa	6396	*FLJ35093*, *PRDX5*
Triethylenemelamine	9706	*FLJ35093*, *PRDX5*
Uracil mustard	34462	*FLJ35093*, *FTH1*, *PRDX5*
Yoshi 864	102627	*FLJ35093*
T1		
Camptothecin	94600	*FLJ35093*
7-Chloro-camptothecin	249910	*FLJ35093*
9-Methoxy-camptothecin	176323	*DAB2IP*
9-Amino-20-(*RS*)-camptothecin	629971	*FLJ35093*
9-Amino-20-(*S*)-camptothecin	603071	*FLJ35093*
10-Hydroxy-camptothecin	107124	*VBP1*
11-Formyl-camptothecin	606172	*EIF4A1*, *FLJ35093*, *HIRIP3*, *LTBR*
11-Hydroxymethyl-camptothecin	606173	*EIF4A1*, *HIRIP3*, *LTBR*, *VBP1*
Camptothecin, 20-ethylglycinate ester	606497	*FLJ35093*
Irinotecan	616348	*FLJ35093*
7-Ethyl-10-hydroxy-camptothecin	673596	*VBP1*
Topotecan	609699	*FLJ35093*
Rebeccamycin	359079	*EIF4A1*, *HIRIP3*, *VBP1*
6,*N*-Diethylaminoethyl-rebeccamycin	640199	*CD59*,*VBP1*
BMY 27557	655649	
T2		
Amonafide	308847	*FLJ35093*, *PRPS1*, *RPL13*, *SHMT2*
Amsacrine	249992	*FLJ35093*, *PRPS1*, *TOP2A*
Anthrapyrazole derivative	355644	*PRPS1*, *TOP2A*
Bisantrene	337766	*SEC14L1*
Daunorubicin	82151	*NPC2*, *VBP1*
Daunorubicinol	180510	*FTH1*, *PRPS1*, *LTBR*,
Deoxydoxorubicin	267469	*RPL13*, *SEC14L1*
Doxorubicin	123127	*VBP1*
Ellipticine	71795	*C20orf45*, *CD59*, *NEDD5*, *PRKAR1A*
Etoposide	141540	*EIF4A1*, *PRPS1*
Idarubicin	256439	*CD59*, *FLJ35093*, *HIRIP3*, *TOP2A*
Iododoxorubicin	378901	
Menogaril	269148	*FLJ35093*, *TOP2A*
9-Methoxy-ellipticine	69187	*C20orf45*, *CD59*, *DNCL1*, *HIRIP3*, *HSPC152*, *NEDD5*, *NPC2*, *PRKAR1A*, *PSAP*, *RPL13*
Mitoxantrone	301739	*PRPS1*, *TOP2A*, *VBP1*

TABLE 1—(*Continued*)

Mechanism of Action & Drug	NSC No.	Correlated Genes
*N*2-Methyl-9-hydroxy-ellipticine	264137	*LTBR*, *TOP2A*
Piroxantrone	349174	*PRPS1*, *TOP2A*, *VBP1*
Teniposide	122819	*FLJ35093*, *TOP2A*
Zorubicin	164011	
Aclarubicin	208734	*C20orf45*, *SHMT2*
Db		
Cyano-doxorubicin	357704	*NDUFB3*
Hycanthone	142982	
Morpholino-doxorubicin	354646	*NDUFB3*
N,*N*-Dibenzyl-daunorubicin	268242	*EIF4A1*, *HIRIP3*, *RPL13*, *SHMT2*
Pyrazoloacridine	366140	*HIRIP3*
Di		
5,6-Dihydro-5-azacytidine	264880	*SEC14L1*
α-2′-Deoxythioguanosine	71851	*ATP5B*, *CPE*, *FLJ35093*, *HIRIP3*, *NEDD5*, *PC4*
Azacytidine	102816	*NEDD5*, *SHMT2*
Thioguanine	752	*CPE*, *FLJ35093*, *NEDD5*, *PC4*
Dr		
Guanazole	1895	*FLJ35093*
Hydroxyurea	32065	*FLJ35093*, *RPL13*
Pyrazoloimidazole	51143	*RPL13*
Ds		
Aphidicolin-glycinate	303812	*FLJ35093*
Cyclocytidine	145668	*EIF4A1*, *FLJ35093*, *RPL13*
Cytarabine	63878	*EIF4A1*, *FLJ35093*, *RPL13*
Floxuridine	27640	*NPC2*
Fluorouracil	19893	*ATP5G3*, *C20orf45*, *CD59*, *FLJ35093*
Ftorafur	148958	*SEPHS2*
Gemcitabine	613327	*FLJ35093*, *RPL13*
Thiopurine	755	*NEDD5*, *PC4*
Rs		
Acivicin	163501	*ATP5B*, *FLJ35093*, *NPC2*, *JUN*
Lawsone	126771	*ATP5B*, *ATP5G3*, *HIRIP3*, *PSMB1*, *SHMT2*
Brequinar	368390	*ATP5G3*, *CD59*, *HIRIP3*, *NPC2*, *SHMT2*
L-Alanosine	153353	*EIF4A1*
N-Phosphonoacetyl-L-aspartate	224131	*ATP5G3*, *EIF4A1*
Pyrazofurine	143095	*CD59*, *FLJ35093*, *FTH1*, *HIRIP3*, *HSPC152*, *NPC2*, *PC4*, *PRKAR1A*, *PSAP*, *PSMB1*, *RPL13*
Df		
Aminopterin	132483	*ATP5B*, *EIF4A1*, *NPC2*, *RPL13*
Aminopterin derivative	134033	*EIF4A1*
Aminopterin derivative	184692	*ATP5B*, *EIF4A1*
Antifolate	623017	*CD59*, *FLJ35093*, *JUN*, *NPC2*, *RPL13*
Baker's antifolate	139105	*ATP5B*, *ATP5G3*, *EIF4A1*, *JUN*, *NPC2*
Methotrexate	740	*EIF4A1*, *FLJ35093*, *HIRIP3*, *NPC2*
Methotrexate derivative	174121	*C20orf45*, *FLJ35093*, *HIRIP3*, *NPC2*, *PCCB*, *PSAP*, *PSMB1*, *RHOT2*, *RPL13*
Trimetrexate	352122	*FLJ35093*, *NPC2*
Raltitrexed	639186	
Pemetrexed	698037	*CD59*, *EIF4A1*, *NPC2*
TU		
Colchicine	757	
Colchicine derivative	33410	
Dolastatine 10	376128	*C20orf45*
Halichondrin B	609395	*NEDD5*
Maytensine	153858	
Trityl-cysteine	83265	*NPC2*, *SEC14L1*
Vinblastine	49842	*SEC14L1*
Vincristine	67574	
Paclitaxel	125973	*C14orf87*, *NPC2*, *SEC14L1*
Taxol analog	600222	*ATP5G3*, *C14orf87*, *DNCL1*, *JUN*, *NPC2*, *RAB6A*, *SEC14L1*, *SERP1*
Docetaxel	628503	*C14orf87*, *NPC2*, *RAB6A*
P90		
Geldanamycin	330500	*SLMO2*
Unknown		
Thiosemicarbazone	107392	*DAB2IP*
Inosine-glycodialdehyde	118994	*CD59*, *HIRIP3*, *JUN*, *SEPHS2*, *SERP1*
Pi		
L-Asparaginase	109229	
mTi		
Rapamycin (sirolimus)	226080	*DPP4*, *JUN*
Rapamycin analog	606698	
Rapamycin analog	606699	
Temsirolimus	683864	

TABLE 1—(*Continued*)

Mechanism of Action & Drug	NSC No.	Correlated Genes
TKI		
Erlotinib	718781	*AIDA*, *FEN1*, *HSD17B4*
Dasatinib	732517	*AIDA*, *ATP5B*, *ATP5G3*, *ATP6V1F*, *EIF4A1*, *HMGB1*, *JUN*, *PCCB*, *SPC12*
Imatinib	716051	*ATP5G3*, *FEN1*
Lapatinib	727989	*DPP4*, *ERBB2*, *HIC1*, *PRDX5*, *RHOA*, *VBP1*
Nilotinib	747599	*ATP5G3*, *ATP6V1F*, *DAD1*, *JUN*, *PCCB*, *PSMB1*, *SEPHS2*
Gefitinib	715055	*FEN1*, *LTBR*, *PRDX5*, *VBP1*
Sunitinib	736511	*ATP5B*, *ATP5G3*, *HMGB1*, *PGK1*, *SLMO2*, *TMPRSS2*
Sorafenib	724772	*HSD17B4*

NSC, National Safety Council; AA A2/A6, agents alkylating at position N2 or O6 of guanines; AA A7, agents alkylating at position N7 of guanines; T1, topoisomerase I inhibitors; T2, topoisomerase II inhibitors; Db, DNA-binding agents; Di, DNA-incorporating agents; Dr, ribonucleotide reductase inhibitors; Ds, DNA synthesis inhibitors; Rs, RNA synthesis inhibitors; Df, antifolates; TU, tubulin inhibitors; P90, heat shock protein 90 inhibitors; Pi, protein synthesis inhibitors; mTi, mammalian target of rapamycin inhibitors; TKI, tyrosine kinase inhibitors; PCNU, *N*-[2-chloroethyl]-*N*′-[2,6-dioxo-3-piperidinyl]-*N*-nitrosourea; Yoshi 864, 1-propanol-3,3′-iminodidimethanesulfonatehydrochloride; BMY 27557, 1,11-dichloro-6-[2-(diethylamino)ethyl]-12,13-dihydro-12-(4-*O*-methyl-β-D-glucopyranosyl)-5*H*-indolo(2,3-a)pyrrolo(3,4-c)carbazole-5,7(6*H*)-dione.

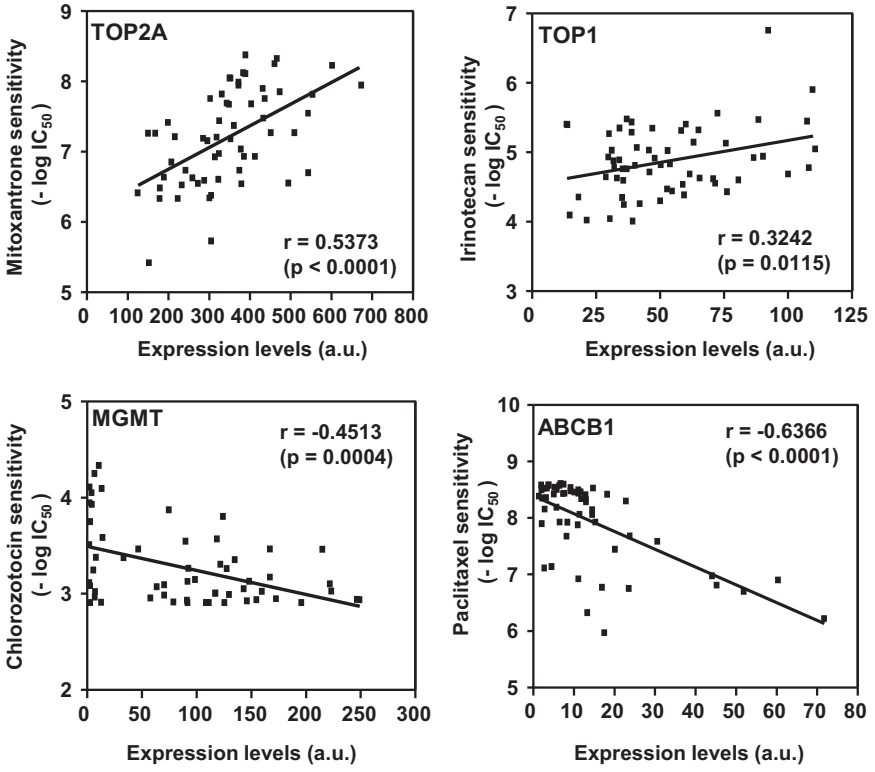

Fig. 2. Correlations between cell sensitivities to mitoxantrone, irinotecan, chlorozotocin, and paclitaxel and levels of expression of the corresponding target genes or genes involved in the mechanisms of drug action, i.e., *TOP2A*, *TOP1*, *MGMT*, and *ABCB1*, respectively, as identified with our NCI-60 database–based in silico approach. The *r* values indicate Pearson coefficients, and the corresponding *p* values are indicated in parentheses. a.u., arbitrary units.

rays (Supplemental Fig. 1C). The genes *HSD17B3*, *HACE1*, *FTH1*, *MYBPC1*, and *CD63* were eliminated from the analyses because of the absence of correlations between the different expression data sets available. Such discrepancies are likely attributable to improper selection of the probes used with the arrays to quantify the expression of these genes.

Next, studies of correlations between gene expression levels and cell sensitivities to a representative panel of 152 core drugs were performed through the calculation of Pearson coefficients. In the multiple-test situation, p values of $<3 \times 10^{-4}$, corresponding to Pearson coefficients below -0.45 or above $+0.45$, were considered significant at the 0.05 level. With such a threshold, however, only a limited number of correlations could be identified. For this reason, Pearson coefficients below -0.2 or above $+0.2$ were taken into account ($p < 0.05$) for a first screening, to optimize the number of potential correlations that could be validated with a functional in vitro approach. A detailed list of the 382 correlations that were found is shown in Table 1. As a control, we determined whether our approach could indicate correlations between gene expression levels and cell sensitivities to drugs for which target genes or genes involved in the mechanism of action had already been identified (Fig. 2). We verified that increased levels of *TOP1* (encoding topoisomerase I) and *TOP2A* (encoding topoisomerase IIα) gene expression were correlated with sensitivity to irinotecan (topoisomerase 1 inhibitor) and mitoxantrone (topoisomerase 2 inhibitor), respectively, which confirmed previous studies that showed that higher levels of these enzymes were associated with better responses to their inhibitors (Burgess et al., 2008). We also found that increased levels of expression of *MGMT*, which encodes an enzyme that is essential for the repair of O^6-methylguanine adducts (Kaina et al., 2010), were correlated with greater resistance to the alkylating agent chlorozotocin. Similarly, increased expression of the *ABCB1* (*MDR1*) gene was significantly correlated with greater resistance to paclitaxel, which confirmed the many studies that demonstrated the role of this pump in the active transport of

tubulin poisons, leading to multidrug resistance (Hall et al., 2009).

To test the functional relevance of our in silico approach, we decided to focus on the correlations that were obtained for platinum derivatives (Table 1). We identified correlations that were specific for either "classic" platinum compounds (cisplatin and carboplatin) or diaminocyclohexyl platins (oxaliplatin and tetraplatin). Levels of expression of *PRDX5*, *RAB6A*, *AZGP*, and *TMPRSS2*, the last of which is rearranged in >50% of advanced prostate cancers (Clark and Cooper, 2009), were correlated only with sensitivities to cisplatin and carboplatin (Table 1). Conversely, levels of expression of *ATP5G3*, *CD59*, *EIF4AI*, *RHOT2*, *SHMT2*, *RPL13*, *PCCB*, *JUN*, *DPM1*, *CDKN2C*, and *FLJ35093* were correlated significantly with oxaliplatin cytotoxicity (Fig. 3) and in most cases with tetraplatin cytotoxicity (Table 1) but not with cisplatin cytotoxicity (Fig. 4). *HMGB1* was the only gene for which expression levels were correlated with sensitivities to all platinum derivatives (Supplemental Fig. 2).

We used the specific signature obtained for oxaliplatin to validate our results at the functional level. For this purpose, we determined whether transient silencing of each of these genes could alter cell sensitivity to oxaliplatin but not cisplatin (which was used as a negative control), as predicted by the in silico approach. Because of the lack of appropriate high-grade prostate cancer cell models, gene silencing was performed with the moderately aggressive DU145 cell line that belongs to the panel, the more-aggressive, hormone-sensitive, LNCaP and hormone-insensitive, bone-metastatic, LNCaP-derivative, C4-2B cell lines, and normal BPH cells. We first checked the efficiency of gene repression with quantitative RT-PCR assays and showed that only nine genes

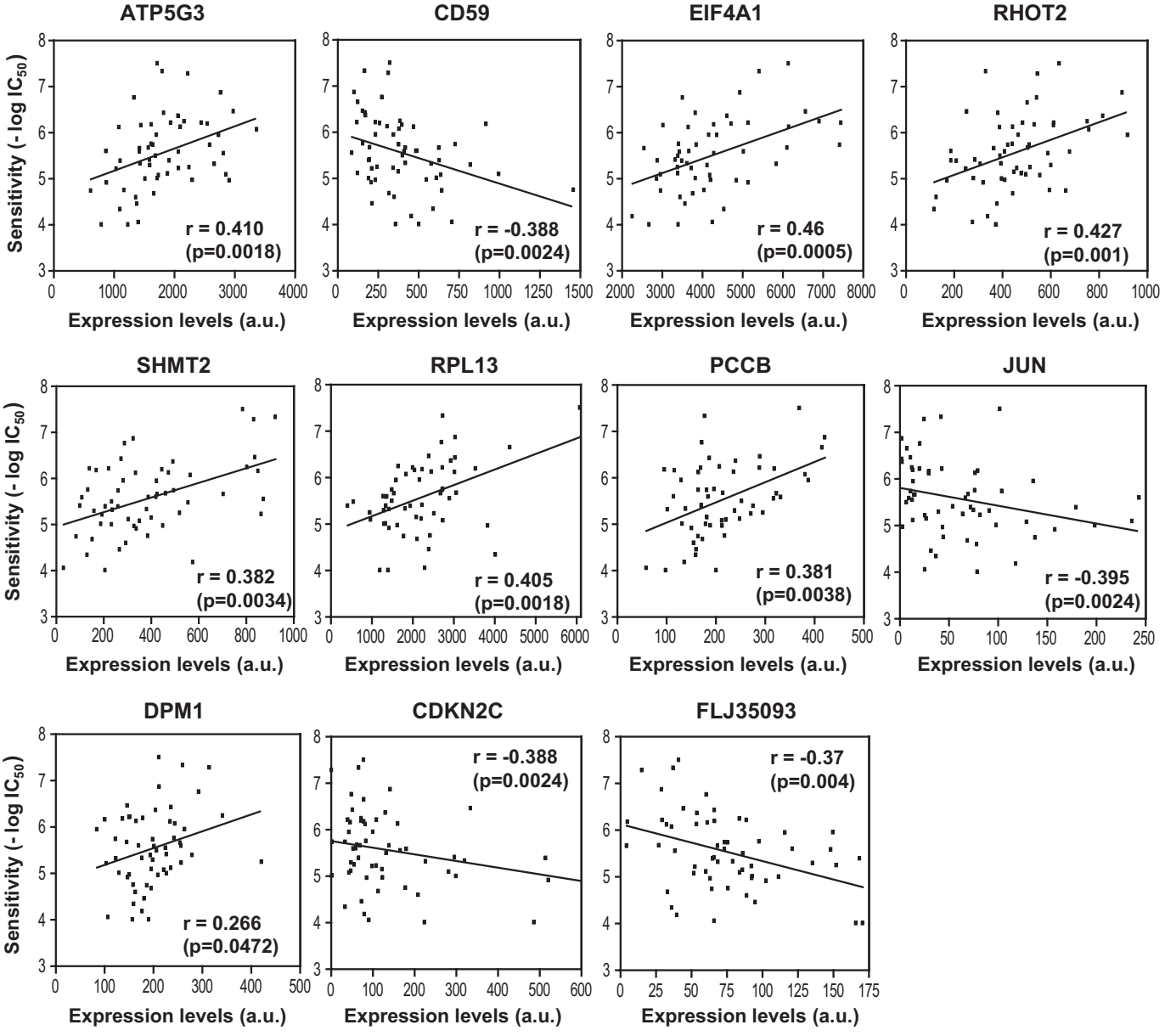

Fig. 3. Correlations between gene expression levels and cell sensitivities to oxaliplatin, as identified with the in silico approach. In each graph, the gene expression levels in the 60 cell lines are plotted as a function of the corresponding $-\log IC_{50}$ values for oxaliplatin. The *r* values indicate Pearson coefficients, and the corresponding *p* values are indicated in parentheses. a.u., arbitrary units.

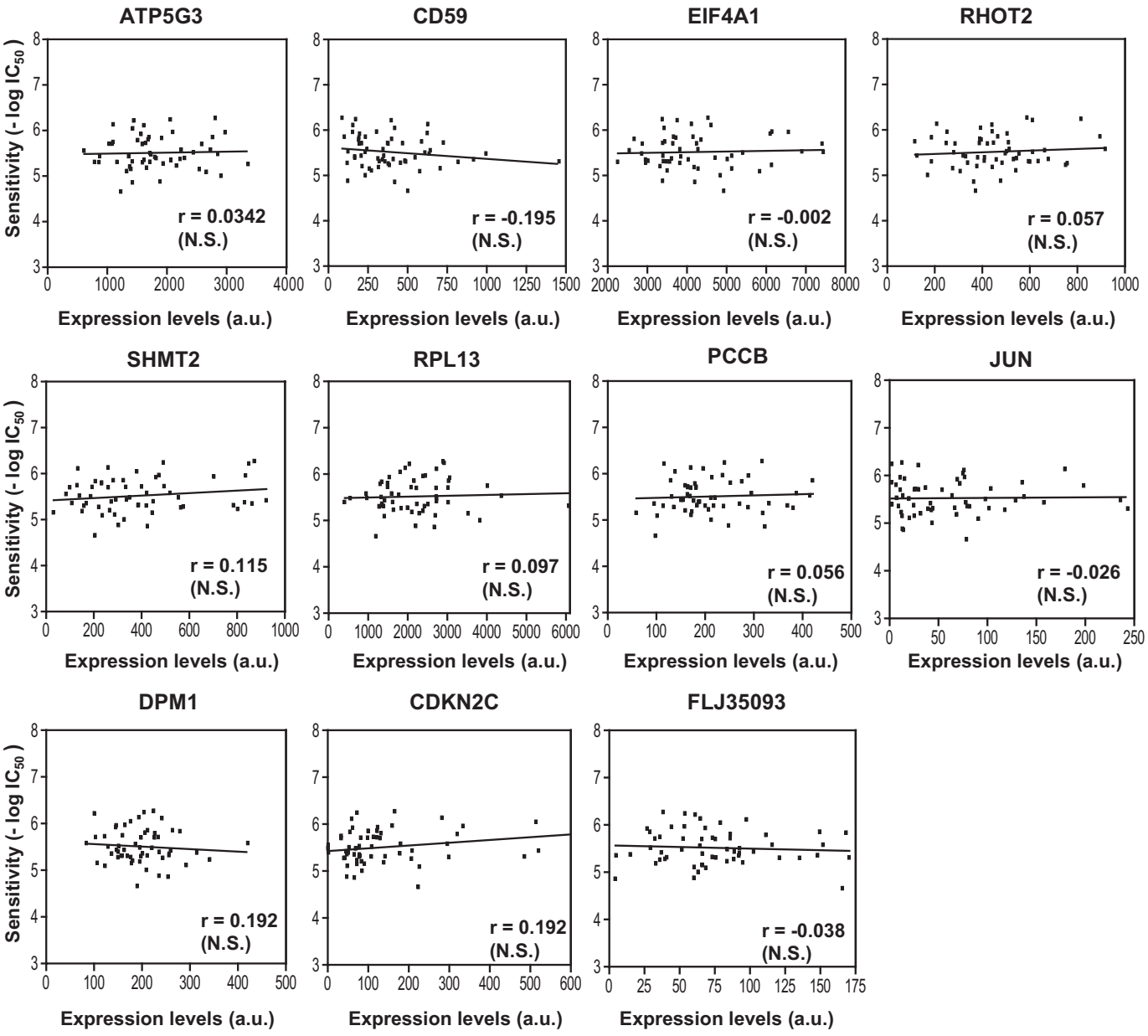

Fig. 4. Correlations between gene expression levels and cell sensitivities to cisplatin, as identified with the in silico approach. In each graph, the gene expression levels in the 60 cell lines are plotted as a function of the corresponding $-\log IC_{50}$ values for cisplatin. The *r* values indicate Pearson coefficients, and the corresponding *p* values are indicated in parentheses. N.S., not significant; a.u., arbitrary units.

could be repressed efficiently (by ≥50%, compared with non-targeting siRNA) (Fig. 5A). Such decreases in mRNA levels were generally associated with effects on cell growth that were consistent with additional assessments of the effects of gene silencing on drug-induced cell growth inhibition (Fig. 5B). Under these conditions, the effects of gene silencing on cisplatin or oxaliplatin sensitivity were evaluated in DU145, LNCaP, and C4-2B cancer cells and in normal BPH cells (Fig. 6). We found that transient repression of *PCCB*, *SHMT2*, *DPM1*, *RHOT2*, *CD59*, *EIF4AI*, and *JUN* mRNA levels altered the growth inhibition of DU145, LNCaP, and/or C4-2B cells treated with oxaliplatin, with >2-fold changes in IC_{50} values, but had no significant effect on cell sensitivity to cisplatin (Fig. 6). It is interesting to note that overall the in silico predictions were confirmed at the functional level in three prostate cancer cell lines; down-regulation of *PCCB*, *SHMT2*, *DPM1*, and *RHOT2* induced resistance to oxaliplatin in DU145, LNCaP, and/or C4-2B cells, whereas repression of *CD59* and *JUN* sensitized cells to the drug. Although it conferred resistance to the drug for C4-2B cells, repression of *EIF4A1* conferred greater sensitivity to oxaliplatin for DU145 and LNCaP cells, in contrast to the in silico prediction (Figs. 3 and 4). Repression of *RPL13* also induced opposite effects depending on the cell line, and repression of *CDKN2C* affected the sensitivity of LNCaP cells to cisplatin (Fig. 6). We also tested the effects of gene silencing in normal BPH cells and in the MDA-MB-468 breast carcinoma cell line. We found that gene silencing had no significant effect on the sensitivity of BPH cells to oxaliplatin (Fig. 6). When gene silencing was performed with MDA-MB-468 cells, no change in sensitivity to oxaliplatin was observed with *PCCB*, *SHMT2*, *DPM1*, or *RHOT2* repression, similar to findings for BPH cells. Increased

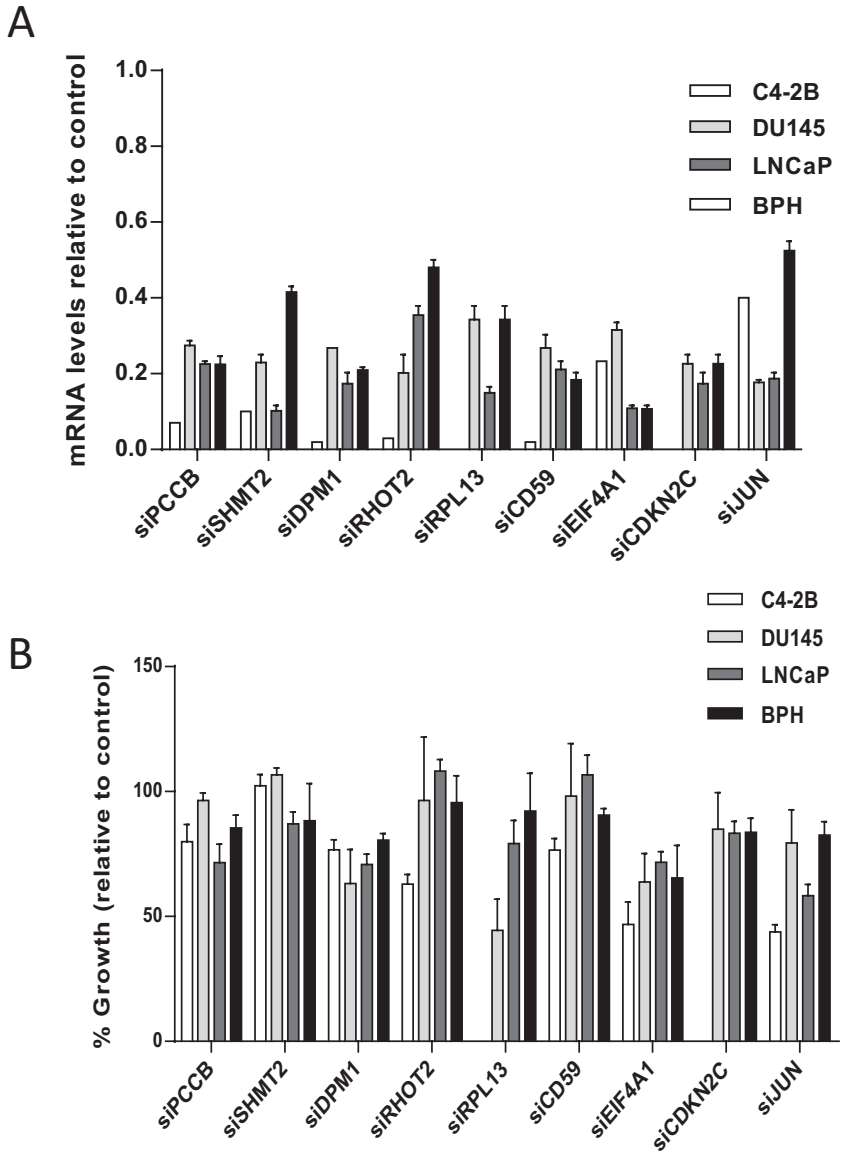

Fig. 5. A, siRNA effects on gene expression, as validated with quantitative RT-PCR assays (described under *Materials and Methods*). mRNA levels were quantified 48 h after transfection with siRNA. For each cell line, results are expressed as ratios of mRNA levels measured in cells transfected with siRNA targeting a specific gene to levels in cells transfected with nontargeting siRNA. Results are mean ± S.D. of three independent experiments. B, effects of the gene silencing performed in A on the growth of DU145, LNCaP, C4-2B, and BPH cells, as measured with MTT assays.

resistance to the drug was noted with *EIF4A1* and *JUN* repression (Supplemental Fig. 3A), which was associated with marked (<50%) effects of gene silencing on cell growth (Supplemental Fig. 3C) and/or lower efficiency of gene silencing (in the case of *JUN*) (Supplemental Fig. 3D). Together, these results suggested that a specific prostate tumor background is necessary for observation of the selective effects of gene modulation on responses to oxaliplatin.

Discussion

The goal of our study was to identify alternatives to taxanes for the treatment of high-grade prostate cancers, by using a rational approach that takes into account the genetic background of these highly aggressive tumors. Several studies reported transcriptomic signatures that allowed discrimination of prostate cancer tissues from their normal counterparts (Dhanasekaran et al., 2001; Luo et al., 2001, 2002; Welsh et al., 2001; Best et al., 2005), but only three studies took the tumor grades into account in their analyses (Singh et al., 2002; Lapointe et al., 2004; True et al., 2006). The first two studies provided gene signatures involving 29 and 41 genes, respectively, which could be used as classifiers on the basis of tumor Gleason scores (corresponding to the sum of the two most-represented grades) (Singh et al., 2002; Lapointe et al., 2004). In those studies, expression profiles were obtained with mRNA extracted from nonmicrodissected tissues, which might yield a bias attributable to potential contaminations with adjacent normal tissue, inflammatory cells, or endothelial cells. For example, it is known that the expression of certain genes (such as *SPARC*) is highly perturbed in stromal cells in various cancers (Cunha and Matrisian, 2002). Moreover, these gene classifiers could not discriminate between mixed grades of 3 + 4 and 4 + 3, which both correspond to a Gleason score of 7 but clinically are associated with low-grade and high-grade tumors, respectively. A third study identified an 86-gene expression signature on the basis of Gleason grades, by using microdissected tumors as the starting material (True et al., 2006). Although only 7700 genes were present in the microarrays used in that study, the signature could predict the Gleason grades of

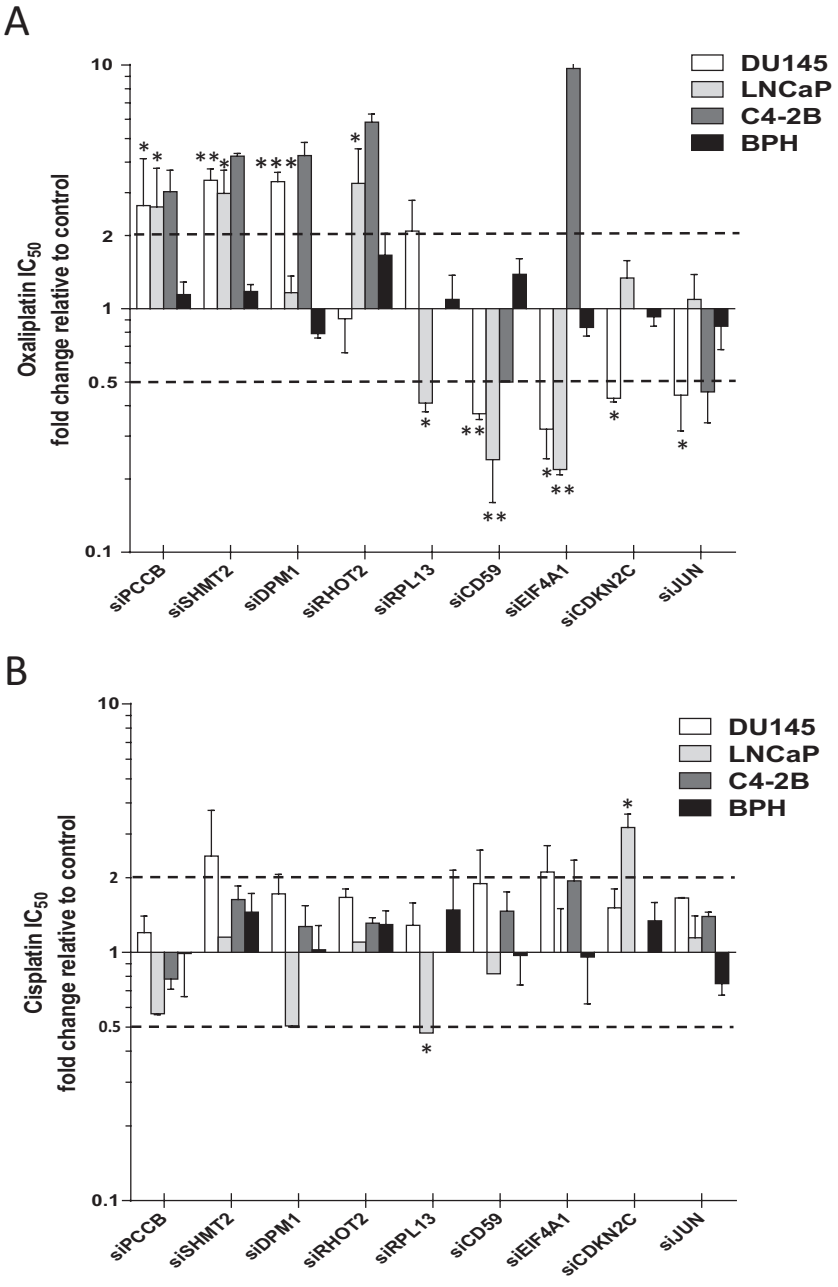

Fig. 6. Functional validation of the correlations for oxaliplatin obtained with the in silico approach. The effects of gene silencing on DU145, LNCaP, C4-2B, and BPH cell sensitivity to oxaliplatin (A) or cisplatin (B) were measured with MTT assays, according to the protocol described in *Materials and Methods*. The results are expressed as the ratios of IC_{50} values for cells transfected with siRNA and cells transfected with nontargeting (control) siRNA and are the mean ± S.D. of at least two independent experiments performed in triplicate. Ratios of >2 or <0.5 indicated that repression of the target gene conferred 2-fold resistance or sensitization, respectively, of the cells to the drug. Statistical differences were evaluated by using unpaired Student's *t* tests. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

tumors with 76% accuracy in an independent validation set of 32 primary prostate carcinomas (True et al., 2006).

By using this signature, we identified 382 correlations between gene expression levels for 40 genes and in vitro sensitivity to at least one of the 152 anticancer drugs. Gene expression levels were generally found to be correlated with the majority of drugs in a given class, which suggests correlations with specific mechanisms of action. It is interesting to note that 40% of the identified genes are involved in cellular metabolic pathways, according to the Human Genome Organization classification, which probably reflects the metabolic changes associated with prostate cancer progression and the acquisition of metastatic potential (Sreekumar et al., 2009). Our results also revealed the drug-specific nature of these correlations within a class of compounds, with the identification of gene signatures specific to classic platinum derivatives and to diaminocyclohexyl platins. These results are in accordance with a previous in silico study that used the same NCI database (Vekris et al., 2004). These data strongly suggested that drug-induced cytotoxicity triggered distinct molecular pathways when cells were treated with cisplatin or carboplatin, as opposed to oxaliplatin or tetraplatin.

By using the correlations obtained specifically for oxaliplatin, we demonstrated that transient repression of *PCCB*, *SHMT2*, *DPM1*, *RHOT2*, *CD59*, *EIF4AI*, and *JUN* mRNA levels significantly altered the growth inhibition of DU145, LNCaP, and/or C4-2B cells treated with oxaliplatin but had no major effect on cell sensitivity to cisplatin (Fig. 6). Overall, the in silico predictions were confirmed at the functional level in three prostate cancer cell lines; down-regulation of *PCCB*, *SHMT2*, *DPM1*, and *RHOT2* induced resistance to oxaliplatin in DU145, LNCaP, and/or C4-2B cells, whereas repression of *CD59* and *JUN* sensitized cells to the drug. The weight of each gene in the prediction of oxaliplatin responses

remains unknown. As an example, we tested the effects of the concomitant repression of *PCCB* and *SHMT2* in DU145 and LNCaP cells on oxaliplatin and cisplatin sensitivity (Supplemental Fig. 4). We found that simultaneous repression of *SHMT2* and *PCCB* conferred resistance to oxaliplatin, with no significant change in cisplatin sensitivity. However, this resistance was of the same order of magnitude as the resistance observed when the two genes were repressed independently (Fig. 6). This absence of a cooperative effect suggests that *SHMT2* or *PCCB* alone would not be of better predictive value, a result that cannot be extrapolated to other genes in the signature.

As expected for such approaches, discrepancies with the in silico predictions could be observed. For example, repression of *EIF4A1*, *RPL13*, and *CDKN2C* had different effects depending on the cell line, and those genes could not be used as markers of oxaliplatin responses. *EIF4A1* repression conferred resistance to oxaliplatin in C4-2B cells but had opposite effects, compared with the in silico results, in both DU145 and LNCaP cancer cell lines. These discrepancies are probably inherent in the cell models used, because the models cannot fully mimic high-grade prostate cancers and are known for their genetic background differences. LNCaP cells are sensitive to androgens, express PSA, and express wild-type p53, whereas bone-metastatic, LNCaP-derivative, C4-2B cells are insensitive to androgens. DU145 cells are resistant to androgen stimulation, do not express PSA, and exhibit p53 mutations (Carroll et al., 1993). It is possible that such alterations would differentially affect cell responses to oxaliplatin. The fact that gene silencing in normal BPH cells or in MDA-MB-468 breast carcinoma cells did not yield the same pattern of responses to oxaliplatin confirms the idea that a specific prostate tumor background is necessary for observation of the selective effects of gene modulation on responses to this platinum derivative.

Together, our results represent a proof of concept demonstrating the relevance of our approach for the identification of alternative treatments for high-grade prostate cancers. By using this strategy, we identified and validated at the functional level an expression signature that could predict the selective sensitivities of prostate cancer cell lines to oxaliplatin, which suggests that this platinum derivative might represent an alternative treatment for high-grade prostate cancers. Although response rates were disappointing, clinical trials with platinum derivatives alone or in association with 5-fluorouracil or capecitabine demonstrated some interesting biological responses (Droz et al., 2003; Gasent Blesa et al., 2011). It would be of interest to consider additional clinical trials of oxaliplatin with stratification of cases on the Gleason grading scale and with use of the oxaliplatin gene signature to select patients who might benefit most from this therapy. Oxaliplatin is routinely used, in association with 5-fluorouracil, for the treatment of colorectal cancers. Two recent studies reported gene expression signatures for clinical responses to oxaliplatin among patients with colorectal cancer. The first study included 40 patients with colorectal cancer who were treated with oxaliplatin, and it identified 27 genes that were differentially expressed among responders and nonresponders (Watanabe et al., 2011). The second study was performed with 14 patient-derived colorectal cancer explants, and it identified 120 probe sets (corresponding to approximately 90 genes) that could predict sensitivity to oxaliplatin (Kim et al., 2012). None of our genes was present in those signatures, although some are probably involved in the same biological pathways, such as *RNPS1*, *RPS25*, *RPL7*, and *RPL18* (involved in ribosomal protein synthesis), *RPL13*, the *RAS* homolog gene family member *RHOC*, and *RHOT2* (Kim et al., 2012). By using unpublished expression data sets for two independent cohorts of patients with colorectal cancer who were treated with oxaliplatin, we also failed to demonstrate that our gene signature could predict clinical responses to oxaliplatin (data not shown). These differences could be explained on the basis of various factors, such as the small numbers of patients included in these studies, differences in the endpoints used for oxaliplatin responses in the clinical setting, the doses of oxaliplatin administered, and the fact that cotreatment with 5-fluorouracil and/or adjuvant therapy might affect tumor responses. The differences also reflect the complexity of the pathways that are specifically regulated by these genes in each tumor type and the difficulty of determining the functional basis for the association of these signatures with drug sensitivities.

During the course of this work, two studies identified new gene expression signatures, involving 31 and 157 genes, the expression patterns of which were claimed to be specific for high-grade prostate cancers (Cuzick et al., 2011; Penney et al., 2011). Among the 31 genes primarily involved in cell cycle progression, five were present on the microarray chip used by True et al. (2006), and none was present in the 86-gene signature. Of the 157 genes, 41 were present on the microarray chip used by True et al. (2006) and four genes, *AZGP1*, *DPP4*, *MYBPC1*, and *SHMT2* (the last of which we validated in our study) were common to the 86-gene signature (True et al., 2006). Inclusion of these genes might be useful to increase the predictive value of gene signatures for specific agents.

In conclusion, we showed that our in silico approach could provide access to potential drugs for which cell sensitivity could be predicted on the basis of the levels of expression of specific genes that characterize high-grade prostate tumors. To our knowledge, none of the genes in our signature was known to be involved directly or indirectly in the mechanism of oxaliplatin sensitivity, which supports our interest in exploring the NCI-60 panel databases to identify new predictive markers of anticancer drug responsiveness and to provide new insights regarding the roles of these genes in the mechanisms of action of these drugs (Shoemaker, 2006).

Authorship Contributions

Participated in research design: Houédé, Richaud, Robert, and Pourquier.

Conducted experiments: Puyo.

Contributed new reagents or analytic tools: Kauffmann and Robert.

Performed data analysis: Puyo, Kauffmann, Richaud, Robert, and Pourquier.

Wrote or contributed to the writing of the manuscript: Puyo, Houédé, Kauffmann, Robert, and Pourquier.

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