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Gene Expression Profiling of 2-(4-Aminophenyl)benzothiazole-resistant MCF-7 Cells Using cDNA Microarrays

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Abstract. *Background: CJM126, 2-(4-aminophenyl) benzothiazole, is a potent inhibitor of human-derived breast carcinoma cell lines. Previous studies have shown that CJM126 elicits concentration-dependent, biphasic growth inhibitory effects against a panel of estrogen receptor-positive and receptor-negative human mammary carcinoma cell lines by a mechanism which has not been fully elucidated. Materials and Methods: In an effort to understand the mechanism(s) of resistance to CJM126, the present study used cDNA microarrays (Clontech Laboratories, Inc.) representing 1,176 human cancer-related genes to analyze expression profile changes of two CJM126-resistant cell lines, MCF-7^{10nM126} and MCF-7$^{10\mu M126}$, previously created by exposing MCF-7 cells to 10 nM and 10 μM CJM126, respectively. Results: Expression changes in the CJM126-resistant MCF-7 cell lines were observed in genes involved in a variety of cell signaling pathways. Gene expression changes common to MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells, as compared to sensitive MCF-7^{wt} cells, were the shut-down of transcription factor Oct-2 and the up-regulation of the negative apoptosis regulator MCL-1, the G1-to-S-phase regulator ubiquitin carrier protein and the GTP-binding protein GST1-HS. These findings indicate the association of a CJM126-resistance phenotype with profound gene transcription dysregulation, decreased apoptotic activity and increased proliferation. Specific changes unique to each of the CJM126-resistant cell lines were also observed. Genes involved in the DNA mismatch-repair pathway, such as MSH2, DNA repair protein RAD51 and damage-specific DNA binding protein were down-regulated in MCF-710nM126, while genes involved in the nucleotide-excision repair pathway, such as ERCC1, RFC and PCNA were overexpressed in MCF-7$^{10\mu M126}$. Conclusion: The differential changes in the DNA-repair pathways between MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cell lines indicate that different processes may have been employed to circumvent the growth inhibition produced by exposure to CJM126. This would also suggest that CJM126 may have concentration-dependent mechanisms of growth inhibition.*

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A significant clinical problem in the treatment of many cancers is the development of acquired drug resistance. Gaining an understanding of a drug-resistant phenotype and the mechanisms that mediate this process would aid in the development of effective drugs and treatment regimens to circumvent chemoresistance, thereby enhancing therapeutic effectiveness. Several mechanisms of drug resistance in tumors are well recognized and include over-expression of the multidrug resistance gene MDR1 (1), multidrug resistance-associated protein (2,3) and DNA repair proteins (4). In addition, changes in a diverse group of gene products that include cell cycle, transcription and cell death regulators, growth factor receptors, tumor suppressors and oncogenes also affect cellular sensitivity to chemotherapeutic agents (5). Since resistance to different therapeutic drugs may involve different molecular pathways, the use of cDNA microarrays is the only method currently available that allows for the examination of the expression of many genes simultaneously. The data obtained can then be used to generate an expression profile for a series of genes and their contribution to a drug resistant phenotype.

2-(4-Aminophenyl)benzothiazole (CJM126) is a potent growth inhibitor of human- derived breast carcinoma cell lines (6-8). Previous studies with CJM126 revealed a biphasic, concentration-dependent growth inhibitory effect against a panel of estrogen receptor (ER)-positive and ER-negative human mammary carcinoma cell lines (9). *In vivo* studies also showed strong inhibitory effects of CJM126 on the growth of breast, ovarian and colon cancer xenografts (6,10). In addition

Table I. *Gene expression profile changes in MCF-7^{10nM126} cells as compared to MCF-7^{wt}.*

Up-regulated genes and genes turned on*	ratio±SD
Cell cycle regulating kinases and regulators	
CDK4	4.15±0.51
Serine/threonine-protein kinase PLK1	4.12±0.42
GTP-binding protein GST1-HS	4.00±0.40
M-phase inducer phosphatase 2	3.41±0.25
AIM; ARK2	2.71±0.25
Transcription factors	
Retinoic acid receptor alpha 1	2.30±0.31
RelA proto-oncogene; NFKB3	2.15±0.15
Metabolic pathways	
uroporphyringen III synthase	3.75±0.22
ribonucleotide reductase	3.31±0.4
thymidylate synthase	3.13±0.26
adenylosuccinate lyase	2.68±0.15
IMP dehydrogenase 1	2.58±0.30
Ornithine decarboxylase	2.49±0.28
Fatty acid synthase	2.39±0.25
Ribonucleotide reductase	2.34±0.26
Thioredoxin reductase	2.21±0.25
Cyclins	
G2/mitotic-specific cyclin B1 (CCNB1)	3.49±0.25
Signal transduction	
dishevelled homolog1	3.41±0.18
T3 receptor-associating cofactor1	3.01±0.55
Zyxin+zyxin-2	2.32±0.22
JUP; desmoplakin III	2.07±0.19
Growth factors and chemokine receptors	
*tumor necrosis factor C receptor	19265±6082
fibroblast growth factor receptor	2.32±0.25
Oncogenes	
*TIMP3; MIG5	41895±13562
active breakpoint cluster region-related protein	3.18±0.28
γ-interferon-inducible protein; IP-30	3.17±0.31
neurogenic locus notch protein (N)	2.35±0.23
bcl-1 oncogene; cyclin PRAD1	2.11±0.18
N-ras; transforming p21 protein	2.18±0.19
MCL-1	2.03±0.17
Kinase substrates and inhibitors	
protein kinase C inhibitor 1 (PKCI1)	2.97±0.24
stratifin; epithelial cell marker protein	2.81±0.29
Calmodulin 1; delta phosphorylase kinase	2.66±0.22
Protein kinase DYRK4	2.46±0.18
ERK activator kinase 2	2.25±0.19
Tyrosine phosphatases	
Dual-specificity protein phosphatase 8	2.74±0.23
Phosphotyrosyl phosphatase activator	2.50±0.20
Protein phosphatase 2C gamma	2.17±0.17
DNA replication factors	
MCM4 DNA replication licensing factor	2.62±0.21
Replication factor C (RFC38)	2.01±0.22
Protein turnover	
Ubiquitin protein ligase	2.50±0.70
Cathepsin D precursor	2.30±0.19
Stress response proteins	
*BCKDH E1-alpha	30294±9397
*paxillin	30949±8682
Other	
*endothelin 3	26402±8083

Down-regulated genes and genes shut-down**	ratio±SD
Transcription factors and related oncogenes	
**octamer-binding transcription factor 2 (Oct-2)	-37024±12300
CHD3	0.13±0.011
STAT5A+STAT5B	0.34±0.024
Jun-B	0.23±0.017
c-rel proto-oncogene protein	0.42±0.037
Tumor suppressors	
Von Hippel-Lindau tumor suppressor protein	0.21±0.018
Cell cycle regulating kinases	
CDC-like kinase 3 (CLK3)	0.22±0.017
DNA repair proteins	
RecA-like protein; DNA repair protein RAD51 Homolog	0.24±0.023
damage-specific DNA binding protein p48 subunit (DDBBp48)	0.31±0.029
DNA mismatch repair protein MSH2	0.35±0.026
Trafficking proteins	
interferon-induced protein p78 (IFI-78K)	0.27±0.023
Cyclins	
G2/mitotic-specific cyclin A	0.39±0.032
G proteins	
Guanine nucleotide-binding protein gamma-10 unit	0.41±0.033
Tyrosine phosphatases	
protein phosphatase WIP1	0.43±0.036
Apoptosis associated proteins	
**inhibitor of apoptosis protein 3 (API3)	25748±8882
ionizing radiation resistance-conferring protein + death-associated protein 3 (DAP3)	0.43±0.037

Table I. *Continued.*

Up-regulated genes and genes turned on*	ratio±SD
*KIAA0022	18216±5967
*KIAA0324	24469±7301
CD59 glycoprotein precursor	2.89±0.21
HSPA5; BIP	2.25±0.17
RPD3 protein	2.14±0.13
glycyl tRNA synthetase	5.65±0.41
menin	3.70±0.23
60S ribosomal protein L5	3.66±0.31
CSBP; transformation-upregulated nuclear protein	3.24±0.33
mRNA cap-binding protein	2.09±0.22
IgG receptor FC large subunit p51 precursor	2.17±0.23
Tyrosine-protein kinase receptor tyro3 precursor	2.17±0.16
insulin-induced protein 1	3.86±0.29
early growth response alpha	2.26±0.21
interferon-induced protein (IFI-56K)	2.24±0.18
Sentrin; GAP modifying protein 1	2.20±0.19
Ninjurin-1	2.20±0.25
Melanoma antigen p15	2.21±0.17
Transmembrane protein 21 precursor	2.38±0.24
Basigin precursor; CD147	2.35±0.18
Cytokeratin 2E	2.23±0.17

Values are shown as the mean ratio of the normalized intensity of MCF-$7^{10nM126}$ to that of MCF-7^{wt} ± SD. Where the genes are "turned on" (*) or "shut down" (**), the values shown are the mean normalized intensity ± SD. The average background intensity for MCF-7^{wt} and MCF-$7^{10nM126}$ are 8704±3202 and 12048±2986, respectively.

to its superior antitumor activity, the ease of synthesis and stability of CJM126 and related analogs (11) have led to the development of a clinical candidate. However, the mechanisms of action of CJM126 and its molecular targets remain largely unknown (7). Possible modes of action for the growth inhibitory effects of CJM126 have been evaluated. The results of these studies have ruled out an association of tyrosine kinase, cdc2 kinase, cdc25 phosphatase, EGF receptor, ER, aromatase, lyase, protein kinase C, topoisomerase II and telomerase in the mechanism(s) of action of this agent (M. Stevens; unpublished observations). It has been suggested that selective metabolism may be important for the antitumor activity of CJM126 and its 3'-analogs, which undergo acetylation and hydroxylation, respectively, in sensitive cell lines *in vitro* (12).

The COMputerized Pattern Recognition (COMPARE) algorithm revealed that CJM126 did not COMPARE with any category of standard anti-tumor agents evaluated in the National Cancer Institute *in vitro* cell panel. Molecular target COMPARE analyses failed to identify any biological target when compared with known clinically active classes of chemotherapeutic agents (7,9).

In order to explore the mediators of CJM126 growth inhibition, as well as those processes involved in the development of resistance, two CJM126 resistant cell lines, MCF-$7^{10nM126}$ and MCF-$7^{10\mu M126}$, were previously developed from MCF-7^{wt} cells following long-term exposure to 10 nM and 10 μM CJM126 (7). Previous work has demonstrated that, within the concentration range of 1 pM to 100 μM, CJM126 showed progressively increased cytotoxicity to MCF-7^{wt} cells from 1 pM to 10 nM. Cytotoxicity essentially reached a plateau from 10 nM to 300 nM, followed by a progressive decrease to a nadir at 10μM. Cytotoxicity then progressively increased from 10 μM to 100 μM (9). This unique biphasic effect of CJM126 has made it an interesting agent to study mechanisms of breast cancer chemoresistance and provided the basis of the gene expression studies reported here. The concentrations used to develop the two CJM126-resistant cell lines correspond to the initial cell growth inhibitory phase (10 nM) and the subsequent partial cell revival phase (10 μM).

Using cDNA microarrays that contain 1,176 known human cancer-related genes, we examined the differential gene expression patterns associated with the CJM126-resistant phenotype and the mechanisms involved in the

Table II. *Gene expression profile changes in MCF-7$^{10\mu M126}$ cells as compared to MCF-7^{wt} cells.*

Up-regulated genes and genes turned on*	ratio±SD
Cell cycle regulating kinases and regulators	
GTP-binding protein GST1-HS	2.75±0.26
CDC37 homolog	2.43±0.23
Cell division protein kinase 5 (CDK5)	2.27±0.21
Cell cycle protein p38-2G4 homolog	2.21±0.16
Transcription factors	
Jun activation domain binding protein	2.47±0.23
Transcription factor erf-1	2.23±0.18
Metabolic pathways	
thymidylate synthase TYMS	2.77±0.22
adenine phosphoribosyltransferase (APRT)	2.75±0.23
UMK	2.63±0.19
Branched-chain amino acid aminotransferase mitochondrial precursor	2.60±0.23
ribonucleotide reductase	2.35±0.21
adenylosuccinate lyase	3.08±0.27
Ornithine decarboxylase	2.38±0.19
Purine nucleoside phosphorylase (PNP)	2.36±0.20
Methylenetetrahydrofolate dehydrogenase-Formyltetrahydrofolate synthetase	2.27±0.18
DNA repair proteins	
DNA excision repair protein ERCC1	2.62±0.24
G/T mismatch-specific thymine DNA glycosylase	2.40±0.22
RAD51C truncated protein	2.32±0.17
BCL family	
MCL-1	2.24±0.18
G proteins	
Guanine nucleotide-binding protein gamma-10 unit	2.75±0.24
transforming protein rhoB; ARHB	2.47±0.23
Apotosis-associated kinase/protein	
Fas-activated serine/threnoine (FAST) kinase	2.74±0.26
Sentrin; ubiquitin-like protein SMT3C	2.38±0.18
PDCD2	2.38±0.17
Signal transduction	
dishevelled homolog1	2.56±0.23
guanylate kinase (GMP kinase)	2.55±0.19
Kinase substrates and inhibitors	
*STAT-induced STAT inhibitor 3	29268±10015
protein kinase C inhibitor 1 (PKCI1)	2.15±0.18
stratifin; epithelial cell marker protein 1	2.12±0.2
DNA replication factors	
MCdM2 DNA replication licensing factor	2.52±0.21
Activator 1 40kDa subunit; RFC2	2.47±0.20
Replication factor C (RFC38)	2.37±0.20
Activator 1 37-kDa subunit	2.14±0.17
PCNA	2.09±0.16
Protein turnover	
Ubiquitin protein ligase	2.50±0.23
Other	
*EB1 protein	27946±9305
KIAA0078	3.57±0.33
ribosomal protein S21	2.38±0.23
HSC70-interacting protein	3.30±0.29
CD59 glycoprotein precursor	2.45±0.23
Calmodulin 1; delta phosphorylase kinase	3.33±0.32
CSBP; transformation-upregulated nuclear protein	2.71±0.29
Arginine/serine-rich splicing factor7	2.64±0.27
HNRNPK; CSBP	2.09±0.22
Protein phosphatase 2C gamma	3.01±0.29

Down-regulated genes and genes shut-down**	ratio±SD
Transcription factors	
**octamer-binding transcription factor 2 (Oct-2)	-37024±10341
CHD3	0.44±0.04
EGF response factor 1	0.39±0.04
Stress response proteins	
Glutathione-S-transferase (GST) homolog	0.39±0.04
Oncogenes	
Fau	0.31±0.02
c-myc binding protein MM-1	0.36±0.03
ras homolog gene family member A transforming protein rhoA H12	0.41±0.03
Cytoskeleton and motility proteins	
Cytokeratin 8	0.39±0.03
Cytokeratin 19	0.42±0.04
Extracellular communication proteins	
Macrophage migration inhibitor factor	0.39±0.03
PDGF associated protein	0.43±0.04
Receptor-associated proteins	
Growth factor receptor-bound protein 2	0.44±0.04
CDK inhibitor	
CDK inhibitor 1; WAF1	0.45±0.04
Cytokines	
**Macrophage inhibitory cytokine 1 (MIC1)	-61744±20981
Cell adhesion proteins	
**alpha E-catenin	-39625±12208

Table II. *Continued.*

Up-regulated genes and genes turned on*	ratio±SD
Suppressor for yeast mutant	2.68±0.20
insulin-induced protein 1	2.38±0.24
antigen KI-67 (MK167)	2.42±1.99
High mobility group protein HMG2	2.78±0.29
Chromatin assembly factor-I P150 subunit	2.41±0.22
Chromatin assembly factor 1 p48 subunit	2.41±0.21
Cytohesin-1; Sec7p-like protein	2.24±0.17
Ninjurin-1	2.34±0.20
LAMR1	2.36±0.18
BCL7B protein	2.80±0.30

Values are shown as the mean ratio of the normalized intensity of MCF-$7^{10\mu M126}$ to that of MCF-7^{wt} ± SD. Where the genes are "turned on" (*) or "shut down" (**), the values shown are the mean normalized intensity ± SD. The average background intensity for MCF-7^{wt} and MCF-$7^{10\mu M126}$ are 8704±3202 and 13056±3426, respectively.

resistance to these two CJM126 concentrations. In addition, we compared the expression profiles of MCF-$7^{10nM126}$ and MCF-$7^{10\mu M126}$ with that of an Adriamycin-resistant MCF-7 subline, MCF-7/ADR. The objective of this study was to elucidate the biochemical mechanisms of CJM126-induced chemoresistance.

Materials and Methods

cDNA microarray membranes. The cDNA microarray membrane, Atlas Human Cancer 1.2, obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA), is an 8 x 12 cm nylon membrane containing 1,176 cDNAs of known cancer-related human genes, composing cell cycle regulating kinases and their regulators, DNA replication factors, transcription factors, growth factors and receptors, chemokines and receptors, apoptosis-associated proteins, tumor suppressors, oncogenes, DNA repair proteins, proteins involved in various cell signaling pathways, cell skeleton and trafficking proteins, *etc*. (www.clontech.com/atlas/gene). It also contains 9 housekeeping genes as positive control and normalization spots, and 3 phage genes as negative controls.

Cell culture and RNA preparation. MCF-7^{wt} and CJM126-resistant sublines MCF-$7^{10nM126}$ and MCF-$7^{10\mu M126}$ were cultivated under conditions as previously described (7). Adriamycin-resistant MCF-7/ADR cells were cultured in DMEM supplemented with 10% fetal bovine serum, 110 mg/L sodium pyruvate, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD, USA), maintained at 37˚C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested at about 80% confluence. RNA was harvested from the cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions, followed by an additional phenol-chloroform extraction.

Labeling, hybridization and scanning of microarray. The labeling and hybridization procedures were conducted as specified by the manufacturer. Ten μg of total RNA was used to synthesize α-^{32}P-labeled cDNA probes (www.clonetech.com/atlas/atlaspure/index.html). RNA was single-pass transcribed by reverse transcriptase in the presence of α-^{32}P-dATP and specific primers for the 1,176 genes represented on the Atlas Human Cancer 1.2 Array. Probes were purified by column chromatography as supplied and instructed by the manufacturer. The array membranes were prehybridized at 68˚C for 30 min in an Express Hybrid solution (Clontech Laboratories, Inc.) containing 100mg/ml freshly boiled sheared salmon sperm DNA, after which cDNA probes were hybridized onto the membranes at 68˚C overnight. The following morning, the membranes were washed four times in low stringency wash buffer (2X SSC/1% SDS) and once in high stringency wash buffer (0.1X SSC/0.5%SDS) at 68˚C for 30 min each. Washed membranes were than exposed to a phosphorimager screen for 18 h. The phosphorimager screen was then scanned on a Fuji Bas 1800 phosphorimager at 50 mM resolution. The experiments were conducted twice on different array membranes and the results were averaged to create a composite array when analyzing. Scanned image files were analyzed with the Atlas Image1.0 software (Clontech Laboratories, Inc.). Signals were normalized to 40S ribosomal protein S9 and/or cytoplasmic beta-actin. The Normalization Coefficient (NC) for a single gene Z was calculated as follows: $NC = (\text{intensity} - \text{background})_{\text{geneZ, array1}} / (\text{intensity} - \text{background})_{\text{geneZ, array2}}$. If multiple genes were selected, the normalization coefficient was calculated by averaging the above ratio for all the genes in the set. On each array, a signal was regarded as positive if its intensity was at least twice its background. When comparing gene expression on different arrays, a gene was regarded as overexpressed if the signal intensity ratio to its corresponding spot was equal to or greater than 2.0. Likewise a gene was regarded as down-regulated if the signal intensity ratio to its corresponding spot was equal to or less than 0.5. A gene was considered "turned on" when it expressed on one array but not on the corresponding spot on the control array. Likewise, a gene was

Table III. *Common gene expression changes in MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells.*

Overexpressed genes and genes turned on*	Down-regulated genes and genes shut-down**
Cell cycle regulating kinases and regulators	Transcription factors
GTP-binding protein GST1-HS	**octamer-binding transcription factor 2 (Oct-2)
Metabolic pathways	CHD3
ribonucleotide reductase	
adenylosuccinate lyase	
ornithine decarboxylase	
Signal transduction	
DVL1	
Oncogenes	
MCL-1	
Kinases and inhibitors	
PKCI1	
stratifin	
calmodulin 1	
Protein turnover	
ubiquitin carrier protein, CDC34	
Other	
CD59 glycoprotein precursor	
insulin-induced protein 1	
sentrin	
ninjurin-1	
CDC21	

regarded as "shut down" when there was no expression on one array but was expressed on the corresponding spot on the control array.

Results

In MCF-7^{10nM126} and MCF-7$^{10\mu M126}$, expression profile changes occurred in a diverse group of genes as shown in Table I and Table II, respectively. Compared with MCF-7^{wt} cells, 6.9% in MCF-7^{10nM126} and 6% in MCF-7$^{10\mu M126}$ of the analyzed genes showed expression changes. Altered expression of genes common to the two resistant cell lines, involving about 1.5% of the genes analyzed, was also observed, as shown in Table III. Typical scanned phosphorimages of MCF-7^{wt}, MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ are shown in Figure 1.

Common down-regulated genes in MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells. The most striking observation was the shut-down of the POU family transcription factor octamer-binding transcription factor-2 (Oct-2), as shown in Table III. Another transcription factor, CHD3, was also down-regulated in both CJM126-resistant cell lines.

Common overexpressed genes in MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells. Overexpressed genes common to the two CJM126-resistant cell lines are shown in Table III. The majority of these genes are involved in the regulation of cell proliferation and apoptosis. Specific examples include the negative apoptosis regulator MCL-1 and genes involved in G1-to-S-phase transition, such as GTP-binding protein GST1-HS and ubiquitin carrier protein.

Differential gene expression changes in MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells. As shown in Tables I and II, both MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells showed expression changes in multiple cell signaling pathways. The most distinct difference in the expression profiles between the two cell lines concern different DNA repair pathways. In MCF-7^{10nM126} cells, genes involved in the DNA mismatch-repair pathway were down-regulated. These down-regulated genes included MSH2, DNA repair protein RAD 51 and damage-specific DNA binding protein. In MCF-7$^{10\mu M126}$ cells, genes involved in both the nucleotide-excision repair and the mismatch-repair pathways were overexpressed. These genes included ERCC1, RFC, PCNA, G/T mismatch specific DNA glycosylase and RAD51C truncated protein.

The two CJM126-resistant cell lines also showed differences in regulatory genes involved in a diverse range of cell signaling pathways. Genes involved in cell cycle-regulation were differentially dysregulated in the two cell lines. In the MCF-7^{10nM126}, CDK4, PLK1, CCNB1, M-phase inducer phospatase 2 and ARK2 were overexpressed, while CLK3 and G2/mitotic-specific cyclin A were down-regulated. In the MCF-7$^{10\mu M126}$, CDK5, CDC37 and cell cycle protein p38-2G4 were overexpressed.

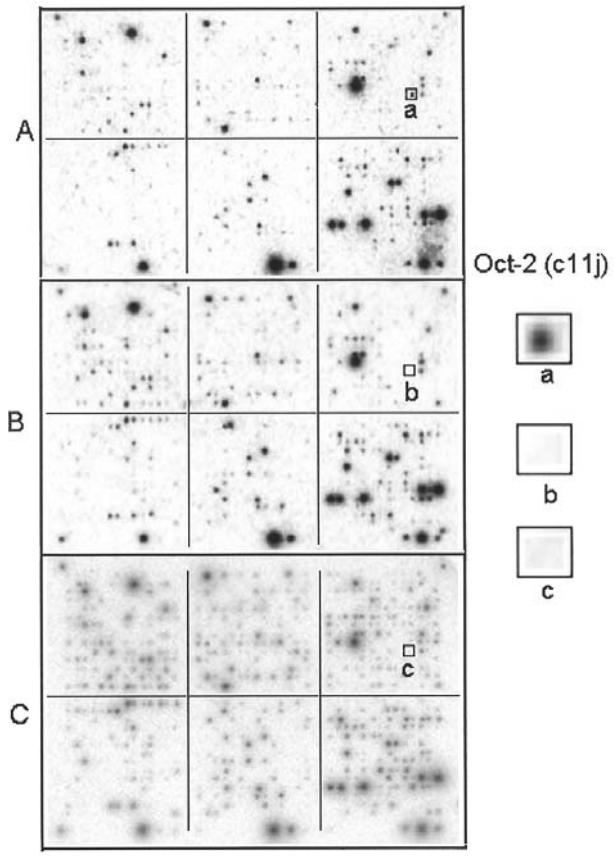

Figure 1. *Phosphorimager scans of cDNA expression arrays of MCF-7^{wt} (A), MCF-7^{10nM126} (B) and MCF-7$^{10\mu M126}$ (C), showing the shut-down of the Oct-2 gene in the two CJM126-resistant cell lines. (a), (b) and (c) show enlargements of the Oct-2 signal for MCF-7^{wt}, MCF-7^{10nM126} and MCF-7$^{10\mu M126}$, respectively.*

Genes involved in a variety of metabolic pathways were also differentially overexpressed in the two cell lines. In the MCF-7^{10nM126}, uroporphyringen III synthase, IMP dehydrogenase1, fatty acid sythase, ribonucleotide reductase and thioredoxin reductase were overexpressed. In the MCF-7$^{10\mu M126}$, APRT, UMK, PNP, branched-chain amino acid aminotransferase mitochondrial precursor and methylenetetrahydrofolate dehydrogenase-formyltetrahydrofolate synthetase were overexpressed.

Several transcription factors and related oncogenes were dysregulated in the two CJM126-resistant cell lines. In the MCF-7^{10nM126}, NF-kappa B3 and retinoic acid receptor-alpha1 were overexpressed, while STAT5A+STAT5B, Jun-B and c-rel were down-regulated. In the MCF-7$^{10\mu M126}$, Jun activation domain binding protein and transcription factor erf-1 were overexpressed, whereas EFG response factor 1 was down-regulated.

Apoptosis-associated genes were also observed to be differentially dysregulated in the two cell lines, indicating profound changes in apoptosis-regulating pathways in the CJM126-resistant cell lines. In the MCF-7^{10nM126}, the inhibitor of apoptosis protein 3 was shut down, along with the down-regulation of the death-associated protein DAP3. In the MCF-7$^{10\mu M126}$, STAT-induced STAT inhibitor 3 was induced, along with the overexpression of Fas-activated serine/threonine (FAST) kinase, sentrin and PDCD2.

The two proliferation indicators, Cathepsin D and Ki-67, were also overexpressed in MCF-7^{10nM126} and MCF-7$^{10\mu M126}$ cells, respectively.

Comparison of the two CJM126-resistant cell lines with MCF-7/ADR. When we compared the expression profile changes of the two CJM126-resistant cell lines with the Adriamycin-resistant MCF-7/ADR cell line, we found that although many changes occurred in the MCF-7/ADR cells when compared with MCF-7^{wt}, only a few expression changes were common to MCF-7/ADR and the CJM126-resistant cells. All three resistant cell lines showed overexpression of CD59 glycoprotein precursor, the membrane inhibitor of reactive lysis, ornithine decarboxylase and stratifin (data not shown).

Discussion

The role of a number of gene products has been recognized in the induction of tumor chemoresistance (5). These include drug transporters, DNA repair enzymes, cell cycle, transcription and apoptosis regulators, growth factor receptors and oncogenes. CJM126 is a cytotoxic drug which has been shown to have a concentration-dependent, biphasic growth inhibitory effect against human mammary carcinoma cells *in vitro* (6) and potent inhibitory activity on breast, ovarian and colon cancer xenografts (6,10). In an effort to understand the mechanism(s) of the CJM126 action, two resistant cell lines, MCF-7^{10nM126} and MCF-7$^{10\mu M126}$, were previously established (7). The current gene expression analyses of these two CMJ126-resistant cell lines revealed common alterations in genes involved in diverse signaling pathways and certain transcription factors, yet divergent changes in the expression of DNA repair genes.

It was observed that the POU family transcription factor Oct-2 was shut down in both MCF-7^{10nM126} and MCF-7$^{10\mu M126}$. Oct-2 is a single gene that produces a primary RNA transcript. This transcript then undergoes alternative splicing to yield a variety of different mRNAs encoding Oct-2 isoforms that either activate or repress gene expression (13). Though Oct-2 has been reported to be expressed only in B lymphocytes, monocytes and neuronal cells (14), we did demonstrate its expression in the MCF-7^{wt} cell line (as shown in Figure 1). As transcription factors

dictate gene expression patterns as well as regulating the cell cycle and proliferation, the complete depletion of Oct-2 transcripts may play a key role in producing resistance by profoundly affecting down-stream target gene expression.

Another transcription factor, CHD3, which is a chromatin-remodeling factor involved in repression of transcription (15), was also found to be down-regulated in both CJM126-resistant cell lines. In the MCF-$7^{10nM126}$ cells, the down-regulation of CHD3 was 8.6-fold, which was among the most prominently changed genes (data not shown). Other transcription factors such as NF-kappa B, which has been reported to regulate genes important for tumor invasion, metastasis and chemoresistance (16), Jun-B and c-rel, were also dysregulated in the CJM126-resistant MCF-7 cells.

We also observed overexpression of MCL-1 with no significant changes in bcl-2 and bcl-xL transcripts in the CJM126-resistant MCF-7 sublines. The bcl family oncogene MCL-1 has been implicated as a negative regulator of apoptosis. Cells overexpressing MCL-1 were found to be resistant to a variety of chemotherapeutic agents (17) and, in some cases, resistance was not affected by the level of other apoptosis inhibitors such as bcl-2 and bcl-xL (18). The overexpression of MCL-1 suggests an important role of the apoptosis inhibitor in the development of CJM126-resistance, independent of bcl-2/bcl-xL expression. In the MCF-$7^{10\mu M126}$, the down-regulation of p21, a downstream target transcript of the tumor suppressor p53, together with the induction of STAT-induced-STAT inhibitor 3, which suppresses the apoptotic effect of JAK-STAT pathway (19,20), further indicate a decreased apoptotic activity in this resistant cell line.

The present results indicate an association between enhanced cell proliferation and the CJM126-resistant phenotype. The biphasic dose response elicited by CJM126 on MCF-7^{wt} cells showed maximum growth arrest following exposure of cells to 100 nM and 300 nM. At concentrations between 3 μM and 30 μM, healthy proliferating cells were observed amongst dying cells. Proliferation associated with 3' methyl or halogen analog treatment was abolished following extended exposure (>3 days) (9). The MCF-$7^{10nM126}$ cell line was established following long term exposure to 10 nM CJM126, a concentration within the growth inhibitory phase of the dose response curve, and MCF-$7^{10\mu M126}$ to 10 μM, a concentration within the proliferative phase of the curve (7). The overexpression of the proliferation marker Ki-67 (21,22) in the MCF-$7^{10\mu M126}$ cells indicates that the MCF-$7^{10\mu M126}$ cells may have developed from the proliferating cells under 10μM CJM126, retaining and inducing stronger proliferative capacity. Overexpression of cathepsin D precursor was also observed in the MCF-$7^{10nM126}$ cells. Cathepsin D is a proliferation indicator previously found over-expressed in many tumor types, including those of the breast and colon (23,24). It was also shown to be associated with increased proliferation and decreased sensitivity to chemotherapeutic agents in ovarian cancer (25).

We observed significant changes in DNA repair pathways in the CJM126-resitant cells. Studies have suggested that, for those therapeutic drugs that induce DNA crosslinks or double strand-breaks, enhanced DNA damage repair, such as double-strand break repair, could be a primary cause for development of drug resistance (18,26-28). Protein expression studies have suggested an association of enhanced excision repair pathways with the resistance to platinum drugs (29,30), chloroethylnitrosoureas, nitrogen mustards and other alkylating agents (4,31). Defective mismatch repair has been suggested in cell lines that are resistant to methylating agents and cisplatin (32-34).

In the nucleotide-excision repair pathway, which removes non-bulky or bulky lesions from DNA in the form of 27-29mers by incising the damaged strand on both sides of the lesion, essential genes have been identified, including excision repair cross-complementing group ERCC2, ERCC3, ERCC4 and ERCC1 (35). Following excision, the gap is filled by the DNA replication proteins RPA, RFC (replication factor C), PCNA and DNA polymerase δ and ε, and then the patch is ligated (36,37). The expression profile of MCF-$7^{10\mu M126}$ showed overexpression of genes involved in this pathway, such as ERCC1, RFC and PCNA. On the other hand, some components of the DNA mismatch-repair pathway, such as MSH2, DNA repair protein RAD51 and damage-specific DNA binding protein, which were also previously reported to be down-regulated in breast cancer patients (38), were down-regulated in MCF-$7^{10nM126}$ cells. This suggests that the activity of the multi-enzyme excision-repair pathway may be enhanced in MCF-$7^{10\mu M126}$ cells such that an augmentation of this pathway, among other things, may have aided in the development of the resistant phenotype. In contrast, a defective mismatch repair pathway may have evolved in the MCF-$7^{10nM126}$ cells and played a role in the resistant phenotype of these cells.

The differential DNA-repair regulation between the two CJM126-resistant cell lines may be explained by an examination of the nature of CJM126. The unique biphasic dose-response of CJM126 may suggest a multi-target mechanism. The cytoplasmic CJM126 localization in MCF-$7^{10nM126}$ and the nuclear CJM126 localization in MCF-$7^{10\mu M126}$ cells (7) further suggests that CJM126, at these concentrations, targets two different sets of biomolecules. Accordingly, it would be expected that cells could develop different mechanisms to overcome drug toxicity and thereby become resistant. It is possible that, with an increase in CJM126 concentration, MCF-7 cells were induced to enhance a nucleotide-excision repair pathway in order to correct the CJM126 toxicity with drug resistance as a result. This process may also account for the second cell reviving phase of the biphasic curve.

In MCF-$7^{10nM126}$ cells, CJM126 is predominately localized in the cytoplasm, producing negligible DNA strand breaks (7). Down-regulated DNA mismatch repair may allow the cells to undergo more un-repaired genetic changes, facilitating the selection for more aggressive phenotypes, which in the present case is drug resistance. CJM126 also accesses the nuclei in these cells thereby causing damage to DNA. This damage is much less than that seen in the MCF-7^{wt} cell line (7). Enhanced nucleotide-excision repair could correct CJM126-induced toxicity leading to the development of a resistant phenotype.

Acquired resistance to CJM126 appeared unrelated to the multidrug-resistant phenotype since sensitivity to adriamycin and tamoxifen was retained by both MCF-$7^{10nM126}$ and MCF-$7^{10\mu M126}$ cell lines (7). Comparison of the expression profiles of the two CJM126-resistant cell lines and MCF-7/ADR cells revealed few gene expression changes that were common among the three cell lines. Genes found overexpressed in all three cell lines included CD59 glycoprotein precursor, a membrane attack complex inhibitor that helps cells escape cytolysis from complement-mediated damage and keep cell integrity (39); stratifin, a protein induced by DNA damaging agents (40), and ornithine decarboxylase, a growth-associated gene. Therefore, these changes only reflect the common growth capacity and enhanced resistance to cytolysis that are common to the drug-resistant phenotype.

Taken together, the findings of the present study suggest that the CJM126-resistant phenotype is associated with profound dysregulation of transcription factors, most prominently the shut-down of Oct-2. The depletion of Oct-2 triggers diverse downstream expression changes in cell signaling pathways. Overexpression of the apoptosis inhibitor MCL-1 could contribute to the resistance phenotype by overcoming the apoptotic stress exerted on cells. Changes in DNA repair pathways, namely, down-regulated DNA mismatch-repair in MCF-$7^{10nM126}$ and enhanced nucleotide-excision repair pathway in the MCF-$7^{10\mu M126}$, may also contribute to the CJM126-resistant phenotype at the relevant CJM126 concentrations.

These findings provide significant information regarding the biochemical mechanisms that regulate the development of resistance to CJM126. In addition, the findings support the utility of cDNA microarrays as a useful method for the examination of the cellular mechanisms of drug action.

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