Activation of Nrf2 Pathways Correlates with Resistance of NSCLC Cell Lines to CBP501 In Vitro

Naoki Mine1, Sayaka Yamamoto1, Donald W. Kufe2, Daniel D. Von Hoff3, and Takumi Kawabe1

Abstract

CBP501 is an anticancer drug candidate that was investigated in two randomized phase II clinical trials for patients with nonsquamous non–small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MPM). CBP501 has been shown to have two mechanisms of action, namely calmodulin modulation and G2 checkpoint abrogation. Here, we searched for a biomarker to predict sensitivity to CBP501. Twenty-eight NSCLC cell lines were classified into two subgroups, CBP501-sensitive and -insensitive, by quantitatively analyzing the cis-diamminedichloro-platinum (II) (CDDP)–enhancing activity of CBP501 through treatments with short-term (1 hour) coexposure to CDDP and CBP501 or to either alone. Microarray analysis was performed on these cell lines to identify gene expression patterns that correlated with CBP501 sensitivity. We found that multiple nuclear factor erythroid-2–related factor 2 (Nrf2) target genes showed high expression in CBP501-insensitive cell lines. Western blot and immunocytochemical analysis for Nrf2 in NSCLC cell lines also indicated higher protein level in CBP501-insensitive cell lines. Moreover, CBP501 sensitivity is modulated by silencing or sulforaphane-induced overexpression of Nrf2. These results indicate that Nrf2 transcription factor is a potential candidate as a biomarker for resistance to CBP501. This study might help to identify those subpopulations of patients who would respond well to the CBP501 and CDDP combination treatment of NSCLC.

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Introduction

Estimating quantifiable markers that predict sensitivity to a particular drug is an aim of personalized medicine. During the last decade, several anticancer drugs were developed on the basis of known oncogenic mutations, and the presence of the mutation by itself has been found to be a useful biomarker for these drugs. Examples include some tyrosine kinase inhibitors, including gefitinib and erlotinib, that target epidermal growth factor receptor (EGFR) in NSCLC and for which efficacy depends on the presence of particular EGFR mutations (1, 2); crizotinib, which is highly effective when NSCLC cells harbor the EML4–ALK fusion gene (3); and the BRAF inhibitor vemurafenib, which is highly effective when melanoma cells carry the V600E mutation of BRAF (4).

CBP501 is a peptide anticancer drug candidate for which two phase II clinical studies in malignant pleural mesothelioma (MPM) and non–small cell lung cancer (NSCLC) have recently been completed. In phase I trials, the combination of cis-diamminedichloro-platinum (II) (CDDP) plus CBP501 showed hint of clinical activity in patients with platinum-resistant ovarian carcinoma and MPM (5). The primary endpoint for efficacy was achieved in the phase II study on MPM (6). G2 checkpoint abrogation had been proposed as a mechanism of action (MOA) based on the observation that CBP501 (i) inhibits multiple kinases that phosphorylate CDC25C at Ser216, (ii) binds directly to 14-3-3 protein, which forms suppressive complexes with phospho-CDC25C, (iii) attenuates phosphorylation of CDC25C at Ser216, and (iv) reduces the accumulation of cancer cells at G2–M upon lengthy combined exposure to CDDP or bleomycin (7, 8).

We recently demonstrated an additional MOA for CBP501. This entails direct interaction of CBP501 with calmodulin, which leads to increased uptake of CDDP into cancer cells upon much shorter exposure to and at lower doses of CBP501 than the conditions required for G2 checkpoint abrogation (9). CDDP uptake is known to depend on multiple transporters and channels (10). Because it is still not yet clear which of the many transporters and channels are most affected by the calmodulin–CBP501 interaction, it is challenging to rationally predict biomarkers that might be useful for this mode of action of CBP501.

Nuclear factor erythroid-2–related factor 2 (Nrf2) is a transcription factor that regulates the expression of many antioxidant genes, including those related to glutathione (GSH) synthesis (11). In tumorigenesis, the expression of...
particular oncogenic alleles of Kras, BRAF, and Myc increases the levels of intracellular antioxidants by inducing Nrf2 expression (12). Nrf2 also regulates the expression of genes related to drug detoxification and transport (13).

In this report, we focus on the effect of CBP501 on promoting CDDP uptake rather than G2 checkpoint abrogation by examining cells only upon short-term exposure to CBP501 and CDDP. Twenty-eight NSCLC cell lines were quantitatively defined as sensitive or insensitive to CBP501 by analyzing their response to a short (1 hour) coexposure to CDDP and CBP501 as compared with either drug alone. Comprehensive gene expression analysis (microarray) was performed on these cell lines to identify the sensitivity markers for this MOA of CBP501. Multiple genes regulated by the Nrf2 transcription factor were identified as having high expression in CBP501-insensitive cell lines. In addition, knockdown of Nrf2 by using Nrf2 shRNA revealed that Nrf2 expression is required for resistance to CBP501.

Materials and Methods

Cell culture and reagents

Human NSCLC cell lines were cultured in a variety of media, each supplemented with 10% fetal bovine serum (Invitrogen) at 37°C with 5% CO2/air. The media used was RPMI-1640 (Sigma-Aldrich) for NCI-H1755, NCI-H2030, NCI-H1437, NCI-H2122, NCI-H2172, NCI-H2228, NCI-H1563, NCI-H1944, NCI-H1993, NCI-H1734, NCI-H1568, NCI-H2444, NCI-H2291, NCI-H2347, NCI-H1838, NCI-H1299, HCC827, NCI-H1975, NCI-H1155, NCI-H522, and NCI-H1703; RPMI-1640 supplemented with 2 mmol/L l-glutamine (Invitrogen) for NCI-H727; RPMI-1640 supplemented with 4.5 g/L d-glucose (Sigma-Aldrich), 10 mmol/L HEPES (Sigma-Aldrich), and 1 mmol/L sodium pyruvate (Sigma-Aldrich) for NCI-H358, NCI-H520, NCI-H647, and NCI-H838; Ham’s F12K (Sigma-Aldrich) for A549. CDDP was purchased from Sigma-Aldrich. Sulforaphane was purchased from Sigma-Aldrich. All NSCLC cell lines were purchased from ATCC and short tandem repeat (STR) analysis had been performed by ATCC to authenticate the identity of each cell line. All cell lines were used within 3 months after thawing.

Cell-cycle analysis

Cells were plated in 24-well plates and incubated for 24 hours. The cells were treated with or without CDDP plus or minus CBP501 at the indicated concentration for the indicated times. The cells were harvested and stained with Krishan’s solution (0.1% sodium citrate, 50 µg/mL propidium iodide, 20µg/mL RNase A, and 0.5% NP-40). Stained cells were analyzed by FACScalibur (Becton Dickinson) using CELLQuest software (Becton Dickinson).

Antibodies

Antibodies were purchased from the indicated sources: anti-NR0B1, anti-Grx, anti-Ptx, anti-γGCSc, anti-γGCSm, and anti-Gpx1 (Santa Cruz Biotechnology); anti-MLC, anti-G6PD, anti-ABCC1, anti-KEAP1, anti-BACH1, anti-Trx1, anti-SOD1, anti-HO1, anti-NQO1, anti-IQGAP1, and anti-ATM (Cell Signaling Technology); anti-GSR, anti-AKR1C1, anti-AKR1C3, anti-AKR1B10, and anti-Nrf2 (Epitomics).

Microarray and gene expression analysis

NSCLC cell lines were grown in a monolayer before RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Agilent Expression Array analysis of the isolated total RNA samples was performed at TAKARABIO Inc. Each analysis of total RNA of a cell line was performed in triplicate. Preliminary statistical treatment (one-way ANOVA and Benjamini–Hochberg method: false discovery rate ≤ 0.05) of the gene expression data was performed by TAKARABIO Inc. The accession number for microarray data for the Gene Expression Omnibus (GEO) repository is GPL10332.

Lentivirus infection

Nrf2 shRNA lentivirus particles and control shRNA lentivirus particles were purchased from Santa Cruz Biotechnology. Lentivirus infection was performed according to the manufacturer’s instructions. Cells (50% confluence) were treated with 5 µg/mL Polybrene (Santa Cruz Biotechnology) before virus infection. One day after addition of virus, the cells were transferred to fresh medium and cells were selected by treatment with Puromycin (Santa Cruz Biotechnology).

Immunofluorescence

Cells growing on coverslips were fixed with 4% paraformaldehyde (15 minutes), washed with phosphate-buffered saline (PBS; 5 minutes, three times) and then blocked in 1× PBS with 1% Block Ace (DS Pharma). They were then incubated with antibodies using standard protocols. Primary antibodies included anti-Nrf2 (1:100), anti-AKR1C1 (1:100), anti-AKR1C3 (1:100), and anti-AKR1B10 (1:100). Secondary antibodies were labeled with Alexa Fluor 488 (Invitrogen) and Hoechst 33342 (DOJINDO) was used to stain DNA. Confocal microscopy was performed using FV10i confocal microscope (OLYMPUS).

Western blot analysis

Cells (50% confluence) were treated with or without sulforaphane at indicated concentrations for the indicated times. The cells were harvested and lysed (30 minutes on ice) in lysis buffer [50 mmol/L Tris–HCl (pH 8.0), 5 mmol/L EDTA (pH 8.0), 100 mmol/L NaCl, 0.5% NP-40, 2 mmol/L dithiothreitol (DTT), 50 mmol/L NaF, 1 mmol/L Na3VO4, 1 µmol/L microcystin, and proteinase inhibitors cocktail (Roche)]. The lysates were clarified by centrifugation (20,600 × g; 4°C), and the supernatants were assayed for protein content using the detergent-compatible protein assay kit (Bio-Rad) according to the manufacturer’s instructions. The whole-cell lysates (60 µg) were run on 10% to 12% SDS-PAGE. Protein
from each gel was transferred onto a polyvinylidene difluoride, (PVDF), membrane (Bio-Rad). The membrane was blocked at room temperature for 1 hour in TBST [10 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, and 0.05% Tween-20] containing 1% Block Ace (DS Pharma) and incubated with primary antibody overnight at 4°C. The membrane was incubated further with anti-peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 hour at room temperature after washing, and the signals were detected using the enhanced chemiluminescence detection system (ECL Advance Western Blotting Detection Kit; GE Healthcare). Detected bands were quantified using Lumino-meter LAS-4000 instrument (Fujifilm).

Figure 1. CBP501 sensitivity is characterized in both H1703 and H1437 NSCLC cell lines by 1-hour cotreatment with CDDP. A–D, H1703. E–H, H1437. A and E, cell-cycle distribution change on sub-G1 population by flow cytometry analysis (n = 2). B and F, G1-phase (n = 2). C and G, S-phase (n = 2). D and H, G2–M phase (n = 2). Chi-square tests were performed to compare the change in the cell-cycle distribution between CBP501 minus and plus at each CDDP dose point. *, statistical significance (P < 0.001); N.S., not significant (P > 0.05); error bars, standard deviation from duplicate flow cytometry analyses.
**Mutation search for NSCLC cell lines**

Search for Keap1 mutations in NSCLC cell lines was performed using the online database, Catalogue of somatic mutations in cancer (COSMIC; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

### Results

**NSCLC cell lines and the sensitivity to CBP501**

CBP501 sensitizes tumor cells to CDDP, as shown by various methods, including colony formation assays, *in vitro* viability assays, and flow cytometry analysis, which measures systematic changes in cell-cycle distribution. The results between the different methods correlate well (8, 9, and data not shown) and differential sensitivity to CBP501 varies consistently for different cell lines (9).

Here, the change in the cell-cycle distribution, as indicated by flow cytometry analysis, was used to estimate the sensitivity of NSCLC cell lines to CBP501-enhanced cytotoxicity to CDDP. Such changes were monitored in cells treated with CDDP in the presence or absence of CBP501. The utility of this analysis for assessing the effectiveness of anticancer therapy is based on the absence of functioning G$_{1}$-S checkpoint regulation in the cell cycle of many types of cancer cells (14). Lack of a functional G$_{1}$-S checkpoint leads to an accumulation of cancer cells in G$_{2}$-M phase upon their exposure to DNA-damaging anticancer agents (14, 15).

To assess the CDDP-enhancing activity of CBP501, one can examine either of two dose–response curves in which the X-axis indicates the dose of CDDP and Y-axis indicates either the number of cells in sub-G$_{1}$ or in G$_{2}$-M. The transition and peak in either dose–response curve will shift to the left in the presence of CBP501 for CBP501-sensitive cells, indicating the enhanced ability of CDDP to change the cell-cycle distribution. By such an analysis of NCI-H1703 cells, the presence of CBP501 resulted in an approximate 8-fold increase in the effectiveness of CDDP to alter the cell-cycle distribution (Fig. 1A–D). On the other hand, the NCI-H1437 cell line did not exhibit any detectable shift in the dose–response curve of CDDP caused by CBP501 (Fig. 1E–H). Using this criterion based on CBP501’s ability to shift the CDDP-induced sub-G$_{1}$ or G$_{2}$-M dose–response curve, 12 NSCLC cell lines were classified as being either CBP501-sensitive or CBP501-insensitive. Cell lines that shifted the curve 2-fold or more were classified as CBP501-sensitive cells and those that shifted the curve less than 2-fold were classified as CBP501-insensitive (Table 1).

**Comprehensive gene expression analysis suggests that some Nrf2 target genes might have upregulated expression in CBP501-insensitive cell lines**

Comprehensive gene expression (*Microarray*) analysis was performed on the 12 NSCLC cell lines listed in Table 1. Upon setting the threshold signal value for the expression level to be 5,000, 40 genes showed more than 70% of correlation with CBP501 sensitivity (Fig. 2A). These identified genes were subdivided into two categories: genes highly expressed in sensitive cell lines or genes highly expressed in insensitive cell lines. Examination of the average expression level values for groupings of genes individually classified as sensitive or insensitive indicated that some groupings of insensitive genes consistently exhibited discernable differences in expression level when CBP501-sensitive and -insensitive cell lines were compared (Supplementary Fig. S1). Western blot analysis was also performed on the 12 NSCLC cell lines listed in Table 1 for more than 40 proteins that act in a variety of biologic processes, including stress response, drug resistance, metabolism, differentiation, and antioxidant response (data not shown). The expression level of Nrf2 protein, but not mRNA, was found to correlate well with CBP501 sensitivity in the same set of NSCLC cell lines (Fig. 2B and C and data not shown). On the basis of these analyses, we then focused on the mRNA expression levels of glutathione reductase (GSR), glucose-6-phosphate dehydrogenase (G6PD), ATP-binding cassette sub-family C member 2 (ABC2), Aldo-keto reductase family 1 C1 (AKR1C1), and AKR1C3 from Fig. 2A because the expression of these genes is known to be regulated by a common transcription factor Nrf2 (16–20). We first confirmed that the levels of protein expression correlated with mRNA expression by Western blot analysis (Fig. 2D).

Next, we used immunocytochemistry to examine intracellular localization of Nrf2 in several NSCLC cell lines. These tests revealed both a high degree of nuclear localization for Nrf2 as well as a relatively higher level of whole-cell expression for Nrf2 in CBP501-insensitive cell lines compared with CBP501-sensitive cell lines (Fig. 3). Taken together, the results presented in Figs. 2 and 3 suggest that high levels of expression of Nrf2 protein in CBP501-insensitive cell lines result in high levels of target gene expression.

### Table 1. Classification of CBP501 sensitivity in NSCLC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Folds increase of CDDP effect by CBP501</th>
<th>Definition of CBP501 sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H2030</td>
<td>1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H1437</td>
<td>1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H2122</td>
<td>1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H2172</td>
<td>1.5</td>
<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H2228</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H1944</td>
<td>1.5</td>
<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H1568</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H2444</td>
<td>2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H2291</td>
<td>2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H2347</td>
<td>4</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H1838</td>
<td>4</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H1703</td>
<td>8</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>
CBP501 sensitivity is affected by the availability of Nrf2

Western blot analyses were performed to examine the expression levels of additional known gene targets for Nrf2 (16–20). NAD(P)H dehydrogenase, quinine 1 (NQO1), AKR1B10, γ-glutamyl cysteine synthetase modifier subunit (γGCSm), and glutathione peroxidase 1 (GPX1) each showed higher levels of expression in CBP501-insensitive cell lines (Fig. 4A). These results support the possibility that the expression of Nrf2 target genes is enhanced in CBP501-insensitive cell lines under normal culture conditions.

To demonstrate a direct causal relationship between CBP501 sensitivity and Nrf2 expression, we established a stable Nrf2 knockdown cell line of H1703, which is sensitive to CBP501 (Fig. 4B). The triple exposure of this cell line to CBP501, CDDP, and sulforaphane, a known Nrf2 activator (17), was studied. As shown in Fig. 4B, sulforaphane caused Nrf2 protein levels to increase in a dose-dependent manner in the cells transfected with a control shRNA. Sulforaphane did not cause a similar increase in the levels of Nrf2 protein in cells transfected with Nrf2 shRNA. Addition of sulforaphane attenuated CBP501’s effect in cells transfected with control shRNA (Fig. 4C and

Figure 2. Microarray gene expression analysis indicates an increase in Nrf2–dependent gene expression in CBP501–insensitive cell lines. A, heatmap display of the expression of isolated genes as determined by microarray analysis. Left, the CBP501–insensitive cell lines. Right, the CBP501–sensitive cell lines. Green, a low level of expression; red, a high level of expression; black, intermediate levels of expression (a value ~−5,000). Experiments were performed in triplicate. B, Western blot analysis for Nrf2 signaling components in NSCLCs. C, quantitative analysis of Nrf2 protein levels in NSCLCs (n = 3). D, Western blot analysis for a series of genes initially identified by microarray analysis.
However, the effect of sulforaphane was abrogated in the Nrf2 knockdown strain (Fig. 4B–D).

Next, we investigated the effect of Nrf2 knockdown in the CBP501-insensitive cell line H1437. Nrf2 knockdown was found to cause reduced levels of expression for AKR1C3, G6PD, and GSR (Fig. 5A). For this knockdown cell line, CBP501 was found to increase the effects of CDDP on changes in the cell-cycle distribution of sub-G1 and G2–M, reversing the initial CBP501 insensitivity. On the other hand, a cell line transfected with control shRNA showed no difference in CDDP activity with or without CBP501 (Fig. 5B and C). These results suggest that high Nrf2 expression levels might induce resistance to CBP501.

The high expression of Nrf2 protein is a candidate marker to predict resistance to CBP501; Nrf2 targets, at either the protein or mRNA level, are additional candidate markers

Although the expression of Nrf2 or its several downstream transcription targets may possibly predict resistance to CBP501, the general reliability of these predictive markers for CBP501 sensitivity remains to be established. Toward this end, we analyzed the combination effect of CBP501 and CDDP in 16 additional NSCLC cell lines, classifying each as CBP501-sensitive or -insensitive by the prior criteria (Table 2). These cell lines were then subjected to the same Microarray analysis as for the initial 12 cell lines. Gene expression heatmaps for the expanded set of 28 cell lines were again analyzed to identify prediction marker candidates for CBP501 sensitivity having more than 70% accuracy. Seven such genes, including three Nrf2 target genes GSR (85.7% prediction rate), AKR1C3 (75%), and G6PD (85.7%), were identified (Fig. 6A).

### Table 2. Classification of CBP501 sensitivity in additional NSCLC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Folds increase of CDDP effect by CBP501</th>
<th>Definition of CBP501 sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H1755</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H358</td>
<td>1</td>
<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H727</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H1563</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H647</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H1155</td>
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<td>Insensitive</td>
</tr>
<tr>
<td>NCI-H520</td>
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<td>Sensitive</td>
</tr>
<tr>
<td>A549</td>
<td>2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H1993</td>
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<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H1734</td>
<td>2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H522</td>
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</tr>
<tr>
<td>NCI-H838</td>
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<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H1975</td>
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<td>Sensitive</td>
</tr>
<tr>
<td>HCC827</td>
<td>4</td>
<td>Sensitive</td>
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<td>NCI-H23</td>
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<td>Sensitive</td>
</tr>
<tr>
<td>NCI-H1299</td>
<td>8</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

Figure 3. A high degree of Nrf2 nuclear localization correlates with high levels of Nrf2 expression. A, immunocytochemistry for Nrf2 in NSCLCs. Left, Nrf2 in green; middle, Hoechst dye in red; right, the merged images; scale bar, 100 μm. B and C, quantification of Nrf2 intensities from the five different images from A (n = 5). B, Nrf2 intensities in whole cells. C, Nrf2 intensities in nuclei. Error bars, standard deviations of Nrf2 intensity as determined from the five different images.
Expression levels of the corresponding proteins in these cell lines were also confirmed by Western blotting (Fig. 6B). Notably, the high expression levels of AKR1C3 and GSR remained comparably correlated with resistance to CBP501 and Nrf2 expression (Fig. 6B and C). To investigate the practical utility of this observation, we examined several NSCLC cell lines to determine whether detection of protein expression of AKR1C3 by immunocytochemical methods might serve as a possible predictive marker for CBP501 sensitivity. Such methodology promises to be generally applicable for marker detection, because it is sufficiently sensitive to detect expressed protein levels in the small tissue samples that are typically available from NSCLC tumor biopsies (21). From these tests, it was concluded that higher levels of AKR1C3 could indeed be detected in CBP501-insensitive cell lines compared with CBP501-sensitive cell lines (Fig. 6D and E) similar to the results with Nrf2 (Figs. 3 and 6B). These results indicate that immunohistochemical detection of Nrf2 and a series of Nrf2 gene target molecules can be highly reliable markers for predicting resistance to CBP501, at least in vitro.

Discussion

As shown in our previous reports, CBP501 has at least two MOA as an anticancer drug candidate (8, 9). One of these, G2 checkpoint abrogation, occurs for treatments of longer duration and at higher doses (8). A second anticancer activity of CBP501, the enhancement of CDDP uptake through the binding to calmodulin, occurs for treatments of shorter duration and at lower doses (9). In this report, we focus on the latter effect and characterized the CBP501 sensitivity of many different NSCLC cell lines by treating cells for shorter duration with lower doses of CBP501. The detailed molecular mechanism of this secondary anticancer activity of CBP501 remains to be elucidated because the CBP501–calmodulin interaction seems to affect several of the numerous channels and transporters implicated in CDDP uptake (10 and data not shown).
shown). These ambiguities about CDDP uptake complicated the identification of single CDDP transport pathway most uniquely affected by CBP501. Therefore, we took the approach of trying to predict CBP501 sensitivity by identifying major differences in gene expression profiles between CBP501-sensitive and -insensitive cell lines, as determined by microarray analysis. This comprehensive analysis of gene expression initially led to several candidates for predictive markers of CBP501 sensitivity. Increased expression of genes from a common transcriptional pathway regulated by Nrf2 was identified as a possible indicator of insensitivity to CBP501. Nrf2 is known to be a key transcription factor for genes related to cytoprotective function (22–26). Under homeostatic conditions, Nrf2 is maintained at very low intracellular concentration by the proteasomal degradation system through its association with Kelch-like ECH-associated protein 1 (Keap1) and the Cul3 E3 ligase (27–30). We found here that Nrf2 protein expression levels were higher in CBP501-insensitive cell lines than in CBP501-sensitive cell lines. However, Keap1 expression levels did not correlate as well with sensitivity to CBP501 (Fig. 2B). This result might indicate that the ability of Nrf2 protein to degrade under homeostatic conditions varies among different cell lines. Several reports indicated a relationship between Keap1 mutation and poor prognosis or chemoresistance in NSCLC cell lines and tumors (31–33). Keap1 mutations are present in a series of NSCLC cell lines that were used in this study (Supplementary Table S1). Keap1 mutations had a tendency to be present in CBP501-insensitive cell lines. This suggests that Keap1 mutation status might be a useful indicator of
resistance to CBP501. However, the role of each mutation type needs to be further verified.

In this report, the differential expression of several Nrf2 target genes at the RNA or protein level was identified as a means to identify candidates for markers to predict CBP501 insensitivity. Of these initial candidates, G6PD, GSR, and AKR1C3 showed significant differences in both mRNA and protein expression levels between CBP501-sensitive and -insensitive cell lines. Regulation of the expression of these identified genes was recognized as...
being under the common control of Nrf2-binding elements called AREs (antioxidant response elements; ref. 23). Several known transcription factors can antagonize Nrf2 by competing for interaction at AREs. These include the small v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (MAF) proteins, BACH1, c-FOS, and FRA1 (23, 34, 35). These transcriptional regulators might be involved in controlling the differential expression of different Nrf2 target genes. In fact, BACH1 protein is one for which expression in NSCLC cell lines seems to increase in CBP501-sensitive cell lines (Fig. 2B).

Nrf2 participates in a diverse spectrum of different biologic phenomena, including metabolism, the xenobiotic response, and the antioxidant response, by inducing the expression of numerous genes (22–26). For instance, inducing the expression of G6PD and GSR elevates GSH levels and this leads to the antioxidant response (36, 37). AKR1C3 can mediate pathways leading to the synthesis of testosterone and to prostaglandin formation (38, 39).

Although Nrf2 knockdown experiments indicate that Nrf2 might modulate the CBP501 sensitivity, there is still no definitive answer about which Nrf2 target gene or phenomena directly affects CBP501 sensitivity. One or several Nrf2 target genes might be involved in determining cell sensitivity to CBP501’s effects on CDDP uptake. Such direct effects might be difficult to detect by conventional microarray analysis, which can sometimes be hindered by technical limitations such as variable sensitivity in the detection of individual probes. Although our previous reports indicated that the CBP501–CDDP combination effect on short-term expression correlated well with CDDP uptake in at least seven cell lines (9), platinum accumulation in NSCLC cell lines was not demonstrated. Therefore, there was a possibility that CBP501 affected not only enhancement of CDDP uptake but also inhibition of the Nrf2-dependent cytoprotective effect against CDDP. For example, reduced GSH, which can be upregulated by Nrf2, binds to CDDP and can decrease toxicity (40). However, measured levels of intracellular reduced GSH in NSCLC cell lines do not correlate with CBP501 sensitivity (data not shown). Clearly, further investigation is needed to establish a concrete mechanism by which Nrf2 regulates CBP501 insensitivity.

For the actual selection of patients who might be benefited by CBP501 sensitivity, though, immunohistochemistry (IHC) promises to be a first-line method of choice because it may be possible to obtain a clear indication of sensitivity from the small tumor samples that are available through biopsy. Smaller samples can be analyzed by IHC than by Western blotting (21). Actually, our investigations into immunocytochemical measurements for Nrf2, AKR1C1, AKR1B10, and AKR1C3 showed that they reliably reproduced the results obtained in Western blotting experiments (Figs. 3 and 6C and D; Supplementary Figs. S2 and S3). Further validation of the use of IHC to detect these proteins in tumor biopsy samples and further validation of the correlation with CBP501 sensitivity might ultimately provide a useful tool to predict a patient’s increased responsiveness to combined CDDP + CBP501 therapy.

Disclosure of Potential Conflicts of Interest

D.W. Kufe and D.D. Von Hoff are consultant/advisory board members for CanBas Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: N. Mine
Development of methodology: N. Mine, S. Yamamoto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Mine, S. Yamamoto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Mine
Writing, review, and/or revision of the manuscript: D.W. Kufe, D.D. Von Hoff
Study supervision: T. Kavabec

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References


Molecular Cancer Therapeutics

Activation of Nrf2 Pathways Correlates with Resistance of NSCLC Cell Lines to CBP501 *In Vitro*


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