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## CBP501-Calmodulin Binding Contributes to Sensitizing Tumor Cells to Cisplatin and Bleomycin

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### Abstract

CBP501 is an anticancer drug currently in randomized phase II clinical trials for patients with non-small cell lung cancer and malignant pleural mesothelioma. CBP501 was originally described as a unique G<sub>2</sub> checkpoint-directed agent that binds to 14-3-3, inhibiting the actions of Chk1, Chk2, mitogen-activated protein kinase-activated protein kinase 2, and C-Tak1. However, unlike a G<sub>2</sub> checkpoint inhibitor, CBP501 clearly enhances the accumulation of tumor cells at G<sub>2</sub>-M phase that is induced by cisplatin or bleomycin at low doses and short exposure. By contrast, CBP501 does not similarly affect the accumulation of tumor cells at G<sub>2</sub>-M that is induced by radiation, doxorubicin, or 5-fluorouracil treatment. Our recent findings point to an additional mechanism of action for CBP501. The enhanced accumulation of tumor cells at G<sub>2</sub>-M upon combined treatment with cisplatin and CBP501 results from an increase in intracellular platinum concentrations, which leads to increased binding of platinum to DNA. The observed CBP501-enhanced platinum accumulation is negated in the presence of excess Ca<sup>2+</sup>. Some calmodulin inhibitors behave similarly to, although less potently than, CBP501. Furthermore, analysis by surface plasmon resonance reveals a direct, high-affinity molecular interaction between CBP501 and CaM ( $K_d = 4.62 \times 10^{-8}$  mol/L) that is reversed by Ca<sup>2+</sup>, whereas the  $K_d$  for the complex between CBP501 and 14-3-3 is approximately 10-fold weaker and is Ca<sup>2+</sup> independent. We conclude that CaM inhibition contributes to CBP501's activity in sensitizing cancer cells to cisplatin or bleomycin. This article presents an additional mechanism of action which might explain the clinical activity of the CBP501-cisplatin combination. *Mol Cancer Ther*; 10(10); 1929-38. ©2011 AACR.

### Introduction

Calmodulin (CaM) is a ubiquitous, highly conserved, calcium-binding protein that contains 4 EF-hand Ca<sup>2+</sup> binding sites and is responsible for much of the modulation of intracellular processes by calcium ions (1, 2). CaM regulates intracellular cyclic AMP (cAMP) concentrations in a very complex manner, modulating the activity of both cAMP-producing adenylate cyclases and cAMP-hydrolyzing phosphodiesterase (3). CaM also regulates numerous kinases, phosphatases, and other enzymes with opposing physiologic functions (4, 5).

In the field of cancer therapeutics, numerous studies report that inhibition of CaM increases the effectiveness of cytotoxic agents as anticancer drugs (6, 7). On the basis of these reports, the possibility of combination therapy with CaM inhibitors and DNA-damaging agents was raised for the treatment of cancer. In practice, earlier human clinical trials with such combination therapy [e.g., trifluoperazine and bleomycin for patients with glioblastoma] were carried out in 1980s (8); however, there have been no drugs that have been approved or progressed beyond phase II clinical evaluation with CaM inhibition as a mechanism of action. Cisplatin is one such widely used anticancer agent that had been reported to have its cytotoxicity enhanced by the naphthanesulfonamide class of CaM inhibitors (9-11).

CBP501 is an anticancer drug candidate currently in 2 randomized phase II clinical trials (for non-small cell lung cancer and for malignant pleural mesothelioma). It clearly enhances the clinical activity of cisplatin (12). In phase I trials, the combination of cisplatin plus CBP501 showed clinical activity in patients with ovarian carcinoma and malignant pleural mesothelioma. G<sub>2</sub> checkpoint abrogation had been proposed as the mechanism of action based on the observation that CBP501 (i) inhibits multiple kinases that can phosphorylate CDC25C at Ser216, (ii) binds 14-3-3 that forms suppressive complexes with phospho-CDC25C, (iii) attenuates

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phosphorylation of CDC25C at Ser216, and (iv) reduces accumulation of cancer cells at G<sub>2</sub>-M upon lengthy combined exposure with cisplatin or bleomycin (ref. 13; unpublished data).

Here, we reveal an additional role for CBP501 as an inhibitor of CaM and present evidence that this role is important for CBP501's mechanism of action in combination therapy. Several CaM inhibitors are shown to behave similarly to, although much less potently than, CBP501 *in vitro*. Moreover, a direct, high-affinity molecular interaction between CBP501 and CaM is showed by surface plasmon resonance (SPR). We conclude that CBP501 possesses a unique CaM-inhibiting activity that leads to the sensitization of cancer cells to cisplatin or bleomycin, especially at short exposures, and that this contributes to the activity profile of CBP501 as a codrug, as has been observed in preclinical studies. This article presents an additional mechanism of action for CBP501 and the prospects afforded by CBP501 as an entry point for the development of a new class of anticancer drugs.

## Materials and Methods

### Cell culture and reagent

Cells were cultured in a variety of media, each supplemented with 10% FBS (Invitrogen) at 37°C with 5% CO<sub>2</sub>/air. The media used was RPMI1640 (Sigma-Aldrich) for HCT15, a human colon cancer cell line; RPMI1640 supplemented with 2 mmol/L L-glutamine (Invitrogen) for COR-L23, a large lung cell carcinoma cell line; RPMI1640 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-H226, a human mesothelioma/non-small cell lung cancer cell line; Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) with 2.5% horse serum (Invitrogen) for MIA-PaCa2, a human pancreas carcinoma cell line; McCoy's 5A medium for the 2 human colon cancer cell lines, HCT116 and HT29; and McCoy's 5A medium supplemented with hydrocortisone, VEGF, ascorbic acid, gentamicin, amphotericin B, hFGF-B, R3-IGF-1, heparin, and hEGF (Sanko Junyaku) for human umbilical vein endothelial cell (HUVEC). CBP501 was manufactured for CanBas by Lonza. bleomycin and cisplatin were purchased from Wako and Sigma-Aldrich, respectively. Calmidazolium chloride (CMZ), W-7, W-12, and trifluoperazine were purchased from Sigma-Aldrich. The chelator, 1,2-bis (o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), was purchased from DOJIN.

### Cell-cycle analysis

Cells were plated in 24-well plates and incubated for 24 hours. The cells were treated with or without DNA-damaging agents and with CBP501 absent or 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, 20 µg/mL RNase A, and 0.5% NP-40). Stained cells were analyzed

using a FACSCalibur (Becton Dickinson) instrument and CELLQuest Software (Becton Dickinson).

### Colony formation analysis

Approximately 300 cells were seeded into 6-well plates. After cells attached onto the plate, cells were treated with compounds for 1 hour, replaced into fresh media, and cultured for 7 to 8 days. The colonies were fixed and stained with crystal violet (Sigma-Aldrich) and counted.

### Cell viability assay

MIA-PaCa2, NCI-H226, or HCT15 cell lines that had been cultured to log phase were harvested and plated in a 96-well microplate at a density of 4,000 per well. Cells were incubated with drugs for 1 hour and then replaced into fresh media. After incubation for 72 hours, cell viability assays were carried out using a Cell Counting Kit-8 (Dojindo). Briefly, WST-8 reagent solution was added to each well, after which the microplate was incubated for 2 hours at 37°C. The absorbance at 450 nm was then measured using a microplate reader (Tecan Group Ltd.).

### Antibody

Commercially available anti-MLC, anti-phospho-Ser19 MLC, anti-phospho-Thr286 CaM-dependent protein kinase II (anti-phospho-Thr286 CaMKII; Cell Signaling), and anti-CaMKII (Epitomics) were used.

### Intracellular platinum concentration and DNA-platinum adducts

NCI-H226 cells were treated with CBP501 (0.2 and 2.0 µmol/L) and cisplatin (1, 3, and 9 µg/mL) for 3 hours and either harvested or switched to fresh medium and incubated for an additional 45 hours (until 48 hours after the initial treatment with CBP501). HUVEC, HT29, and MIA-PaCa2 cells that had been treated with 3 µg/mL cisplatin plus or minus 10 µmol/L CBP501 were harvested at 3 hours. As an alternative set of conditions, MIA-PaCa2 cells were treated with 10 µg/mL cisplatin plus or minus 1 µmol/L CBP501 and were harvested at 1 hour. COR-L23 cells were treated with 2.5 µg/mL cisplatin plus or minus 1 µmol/L CBP501 and were harvested at 1 hour. To analyze for platinum concentration (Toray Research Center, Inc.), cells were first dissolved by heating them in nitric acid and hydrogen peroxide. The samples were then diluted with aqua regia (4% v/v), and the platinum concentration was measured by inductively coupled plasma mass spectroscopy analysis. To detect the concentration of DNA-platinum adducts, genomic DNA was isolated from the cell lines by methods previously described (14).

### Western blot analysis

Cells (50% confluence) were treated with or without CBP501 or CMZ 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, 50 mmol/L NaF, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{mol/L}$  microcystin, proteinase inhibitors cocktail (Roche)]. The lysates were clarified by centrifugation (15,000 rpm, 20 minutes, 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  $\mu\text{g}$ ) were analyzed by 10% to 12% SDS-PAGE. Protein from each gel was transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked at room temperature for 1 hour in TBS and Tween 20 (TBST; 10 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, and 0.05% Tween-20) containing 2% ECL Advance Blocking Reagent (GE Healthcare) and incubated with primary antibody overnight at 4°C. After washing, the membrane was incubated further with anti-peroxidase-conjugated secondary antibody (Cell Signaling) for 1 hour at room temperature. And after washing, the signals detected using the enhanced chemiluminescence detection system (ECL Advance Western Blotting Detection Kit, GE Healthcare). Detected bands were quantified using a Lumino-mater LAS-4000 instrument (Fujifilm).

### SPR analysis

Binding of CBP501 to CaM (Enzo Biochem Inc.) or to 14-3-3 (Enzo Biochem) was analyzed by SPR using a BIAcoreT 100 (GE Healthcare) instrument. CBP501 dose-response experiments were carried out at 25°C using 10 mmol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, 0.005% surfactant P20, pH 7.4, as running buffer at a flow rate of 20  $\mu\text{L/min}$ .  $\text{CaCl}_2$  dose-response experiments were carried out at 25°C using 10 mmol/L HEPES, 0.15 mol/L NaCl, 0.005% surfactant P20, pH 7.4, as running buffer at a flow rate of 20  $\mu\text{L/min}$ . CaM and 14-3-3 were coupled to CM-5 sensor chips utilizing an amine-coupling kit (GE Healthcare). The binding reactions were carried out by injecting various concentrations of CBP501 over the immobilized CaM or 14-3-3 on the chip surface. Background responses from a reference cell were subtracted from the experimental responses. All of the sensorgrams were analyzed using BIAcoreT 100 Software, version 2.0 (GE Healthcare).

## Results

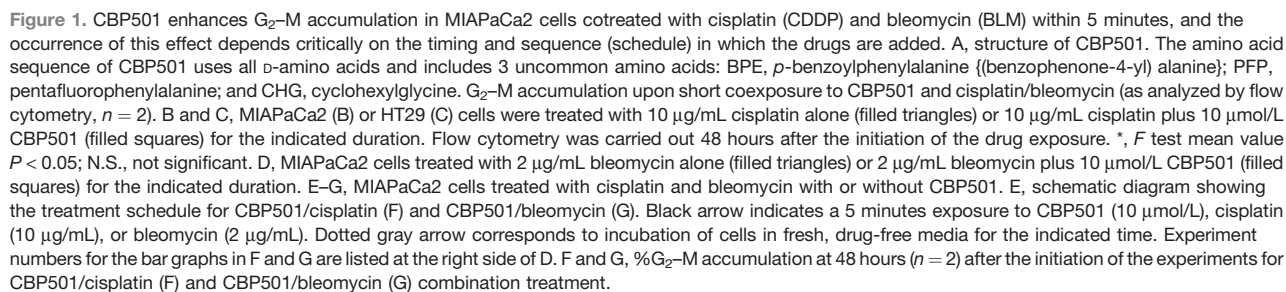
### CBP501 enhances cisplatin and bleomycin cytotoxicity after short exposure

The changes in the cell-cycle distribution caused by varying the time of exposure of cancer cells to binary treatment with CBP501 and cisplatin (or bleomycin) were examined by flow cytometry. The use of this analysis for assessing the effectiveness of anticancer therapy is based on the absence of a functioning  $G_1$ -S checkpoint in the cell cycle of many types of cancer cells (16). 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 (15, 16). For MIA PaCa2, a

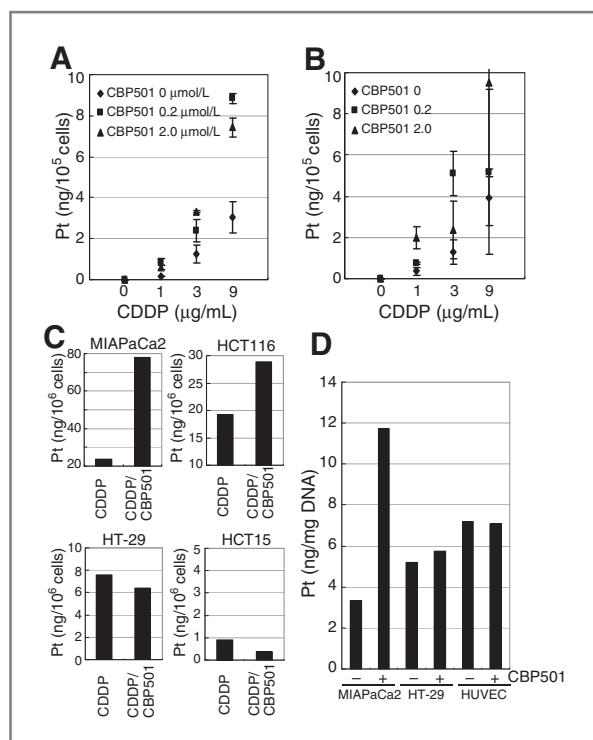
CBP501-sensitive cell line (sensitivity as defined in Supplementary Figs. S1 and S2; ref. 13), just a 5-minute combined exposure to CBP501 and cisplatin or to CBP501 and bleomycin leads to a significant increase in the  $G_2$ -M phase population relative to treatment with cisplatin or bleomycin alone (Fig. 1B and D). CBP501 exposure alone does not alter the cell-cycle distribution (ref. 13 and data not shown). In contrast, accumulation at  $G_2$ -M phase decreases upon longer combined exposure (Fig. 1B and D) due to an increase of the sub- $G_1$  population. This increase at sub- $G_1$  is indicative of the onset of cell death (data not shown). On the contrary, HT29, a CBP501-insensitive cell line, does not show a similarly increased accumulation upon combined treatment with CBP501 and cisplatin (Fig. 1C) or bleomycin (data not shown). Following these initial observations, we examined the effects of changing exposure times and relative introduction times (scheduling) for each individual component in the binary treatment (Fig. 1E). Accumulation at  $G_2$ -M phase was clearly apparent when the DNA-damaging agent was added concomitantly with CBP501 or if it was added after CBP501. However,  $G_2$ -M accumulation was undetectable if the DNA-damaging agent was added first, and CBP501 was added after changing the cells to drug-free growth medium (Fig. 1F and G). The increased accumulation at  $G_2$ -M, the rapid observed response, and the observed schedule dependency suggested that the augmentation of cytotoxicity of cisplatin/bleomycin by CBP501 may not solely be due to  $G_2$ -M checkpoint abrogation.

### CBP501 enhances platinum accumulation and platinum-DNA adduct formation in CBP501 sensitive cells

One possible way that CBP501 might induce cisplatin to become more cytotoxic would be if CBP501 affects the intracellular accumulation of cisplatin. To examine this possibility, we measured the intracellular concentration of platinum in a variety of cell lines after cotreatment with cisplatin and CBP501 at various concentrations. The results show that some level of dose-dependent platinum accumulation occurs in CBP501-sensitive NCI-H226 cells treated with cisplatin in either the presence or absence of CBP501 (Fig. 2A and B). However, cotreatment with CBP501 leads to a relative increase in the concentration of platinum in the cell samples, either upon harvesting after 3 hours (Fig. 2A) or upon changing to fresh growth medium at 3 hours and incubating for an additional 45 hours (Fig. 2B). The similarity in platinum retention at either 3 or 45 hours implies that the accumulation of platinum stems largely from an increase in the rate of influx of platinum rather than the rate of efflux in this cell line. Similar platinum accumulation was also confirmed for other cell lines. Upon 3-hour treatment, CBP501-sensitive cell lines MIA PaCa2 and HCT116 showed increases in platinum concentration when CBP501 was present, but CBP501-insensitive cell lines HT29 and HCT15 showed no change (Fig. 2C).



To examine the molecular mechanism for this increase in platinum accumulation caused by CBP501, we first scanned a variety of physiologically compatible,



**Figure 2.** Coadministration of CBP501 to cisplatin (CDDP) enhances platinum accumulation and platinum-DNA adduct formation in CBP501-sensitive cancer cells. A and B, graphs show platinum concentration in NCI-H226 cells. A, cells were treated with CBP501 and cisplatin for 3 hours ( $n = 4$ ) and harvested for platinum analysis. B, cells were treated with CBP501 and cisplatin for 3 hours, then they were transferred to fresh medium and cultured for 45 hours ( $n = 4$ ) before harvesting for the analysis. C, platinum concentration in CBP501-sensitive and -insensitive cell lines. Platinum concentrations were determined after 3-hour treatment with cisplatin alone or cisplatin (10  $\mu\text{g/mL}$ ) and CBP501 (10  $\mu\text{mol/L}$ ) in combination. MIAPaCa2 and HCT116 cells are sensitive to CBP501. HT-29 and HCT15 cells are insensitive to CBP501 ( $n = 1$ ). D, DNA-bound platinum. Platinum concentration in genomic DNA isolated from CBP501-sensitive (MIAPaCa2) and -insensitive cancer (HT-29) cells and noncancer (HUVEC) cells treated with cisplatin (10  $\mu\text{g/mL}$ ) and CBP501 (10  $\mu\text{mol/L}$ ) for 3 hours ( $n = 1$ ). Pt, platinum.

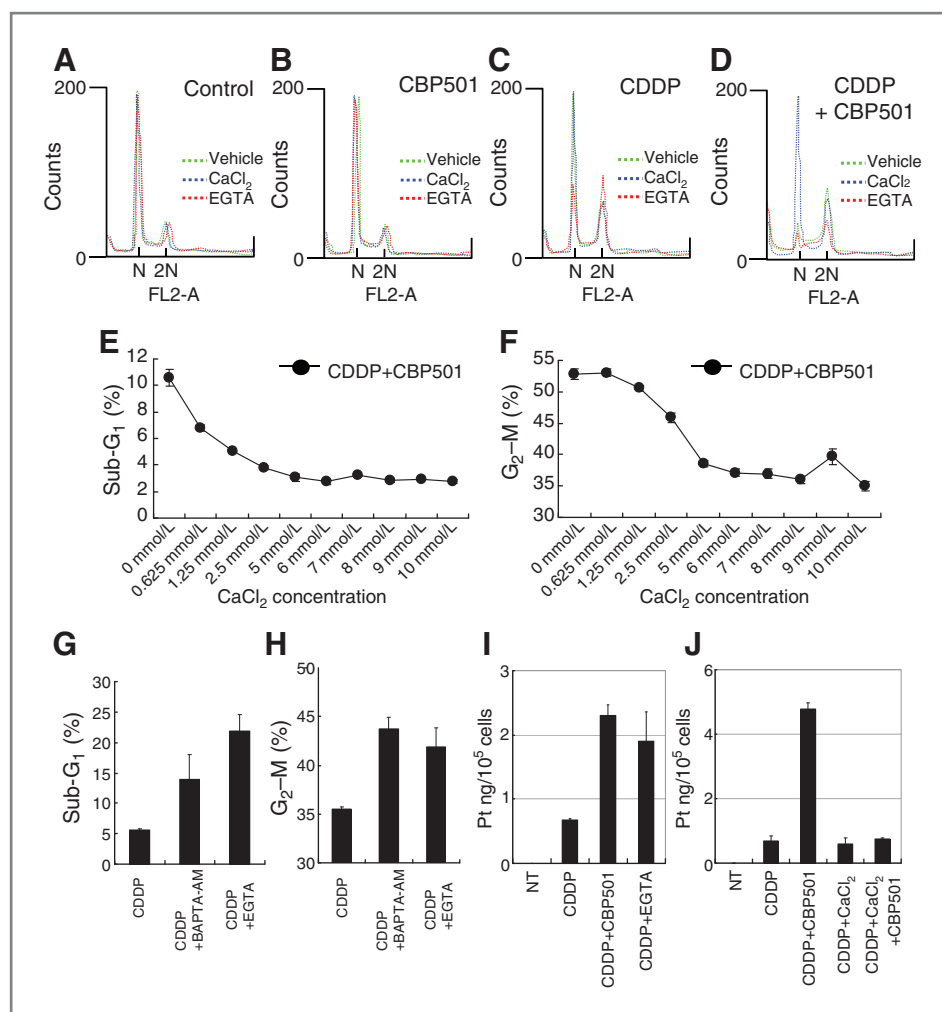
commercially available reagents to examine how they perturbed the effect seen upon combined treatment with cisplatin/CBP501. Of more than 60 such reagents tested, the most pronounced effect was found when  $\text{CaCl}_2$  (10 mmol/L) was present in the medium. In the presence of  $\text{CaCl}_2$ , the CBP501-induced increase in cisplatin-mediated  $\text{G}_2\text{-M}$  phase accumulation (codrug effect) was completely abrogated in the CBP501-sensitive cancer cell lines, and there was no effect on cell-cycle distribution in the absence of cisplatin (Fig. 3A–F). Furthermore, upon combined treatment of cells with cisplatin and the  $\text{Ca}^{2+}$  chelating agent EGTA (3 mmol/L, 1 hour), the percentage of cells accumulating at  $\text{G}_2\text{-M}$  phase increased significantly relative to that found for cisplatin alone (Fig. 3C) and increased cell death was observed for the cisplatin/CBP501/EGTA triple combination (Fig. 3D), which might be rationalized as a further enhancement of CBP501's effect on cisplatin. Thus, the overall effect on accumula-

tion at  $\text{G}_2\text{-M}$  phase that is caused by a high concentration of EGTA is similar to that found with low doses of CBP501. Next, we examined the effects of BAPTA-AM, a cell-permeable reagent that chelates intracellular  $\text{Ca}^{2+}$ . Combined treatment with BAPTA-AM and cisplatin also resulted in a significantly increased population of cells accumulated at  $\text{G}_2\text{-M}$  phase (Fig. 3H). Besides causing accumulation at  $\text{G}_2\text{-M}$  phase, cotreatment with EGTA/cisplatin also led to an increase of intracellular platinum (Fig. 2I). The nearly 3-fold increase in intracellular platinum concentration upon cotreatment with EGTA and cisplatin (relative to treatment with cisplatin alone) is analogous to the effect caused when CBP501 is the codrug. Furthermore, the presence of added  $\text{CaCl}_2$  abolishes CBP501-induced  $\text{G}_2\text{-M}$  accumulation as well as sub- $\text{G}_1$  accumulation and platinum uptake (Fig. 3D–F and J). These results implicate intracellular calcium signaling as a basis for CBP501's codrug effect in combination with cisplatin.

### CaM inhibitors show similar activity to CBP501

Because CaM is a major cellular mediator of calcium signaling (17), we wondered whether CaM might be involved in the CBP501 codrug effect. To investigate this possibility, the effect of CMZ, a CaM antagonist, was examined in COR-L23, a CBP501-sensitive cell line. CMZ treatment alone did not affect the distribution of cells throughout the cell cycle (data not shown). However, combined treatment with CMZ/cisplatin increased the  $\text{G}_2\text{-M}$  phase population over that obtained with cisplatin treatment alone (Fig. 4B). This CMZ-cisplatin synergism was canceled by adding calcium salts into the growth medium, as had been observed with CBP501 (data not shown for this cell). To test whether CMZ has same site of action as CBP501, we first examined the potential synergy of the codrug effects for CBP501 and CMZ. The codrug effect of CBP501 reaches a plateau in activity at 0.5  $\mu\text{mol/L}$  (Fig. 4C). The CMZ response increases with concentrations between 2 and 10  $\mu\text{mol/L}$  (Fig. 4D). When the concentration of CMZ was varied (2–10  $\mu\text{mol/L}$ ) in the presence of cisplatin, CMZ exhibited a codrug effect, increasing the population of  $\text{G}_2$  phase arrested cells in a dose-dependent manner (Fig. 4E). When CBP501 was present at a concentration above saturation (1  $\mu\text{mol/L}$ ), further significant enhancement of  $\text{G}_2$  phase cell-cycle arrest by CMZ was not observed (Fig. 4B). Next, we examined 2 additional CaM inhibitors, W-7 and W-12. Treatment with W-7 or W-12 alone did not affect the cell cycle. The combined treatment with either W-7/cisplatin or W-12/cisplatin showed slight, but significant increases in  $\text{G}_2\text{-M}$  phase population over cisplatin alone (Fig. 3F–H). When we examined melittin, a peptidic inhibitor of CaM from bee venom (18), the combined treatment with melittin/cisplatin showed relative increases in the  $\text{G}_2\text{-M}$  population (Fig. 4I).

To compare the codrug effects of CMZ and CBP501, various combination treatments were examined in



**Figure 3.** Calcium plays a role in CBP501 action. A–D, histograms from flow cytometry analysis. MIA PaCa2 cells were treated for 1 hour with or without cisplatin (CDDP; 10  $\mu$ g/mL) and/or CBP501 (1  $\mu$ mol/L) in the presence or absence of CaCl<sub>2</sub> (10 mmol/L) or EGTA (3 mmol/L). N in x-axis corresponds to a FL2-A value of 200 units. E and F, sub-G<sub>1</sub> and G<sub>2</sub>-M population in percentages after 1-hour treatment with cisplatin (10  $\mu$ g/mL), CBP501 (1  $\mu$ mol/L) with indicated concentration of CaCl<sub>2</sub>, and flow cytometry analysis at 48 hours ( $n = 2$ ). G and H, sub-G<sub>1</sub> and G<sub>2</sub>-M population in percentages after 1-hour treatment with BAPTA-AM or EGTA in MIA PaCa2. I and J, platinum concentration in MIA PaCa2. MIA PaCa2 cells were treated for 1 hour with cisplatin (10  $\mu$ g/mL) and/or CBP501 (1  $\mu$ mol/L) in the presence or absence of CaCl<sub>2</sub> (10 mmol/L) or EGTA (3 mmol/L;  $n = 2$ ). Pt, platinum; NT, nontreatment.

CBP501-sensitive and CBP501-insensitive cell lines. In CBP501-sensitive cell lines, such as HCT116 and MIA-PaCa2, cotreatment with CBP501 enhanced the accumulation of cells at G<sub>2</sub>-M in response to cisplatin and bleomycin, but not to doxorubicin (DOX; Fig. 5A and C). CMZ cotreatment showed effects similar to those of CBP501 in these cell lines (Fig. 5B and D). In contrast, in the CBP501-insensitive HT-29 cell line, CMZ exhibited no codrug effect (Fig. 5E and F). These results suggest that CBP501 and CMZ share a common target, the Ca<sup>2+</sup>/CaM signaling pathway.

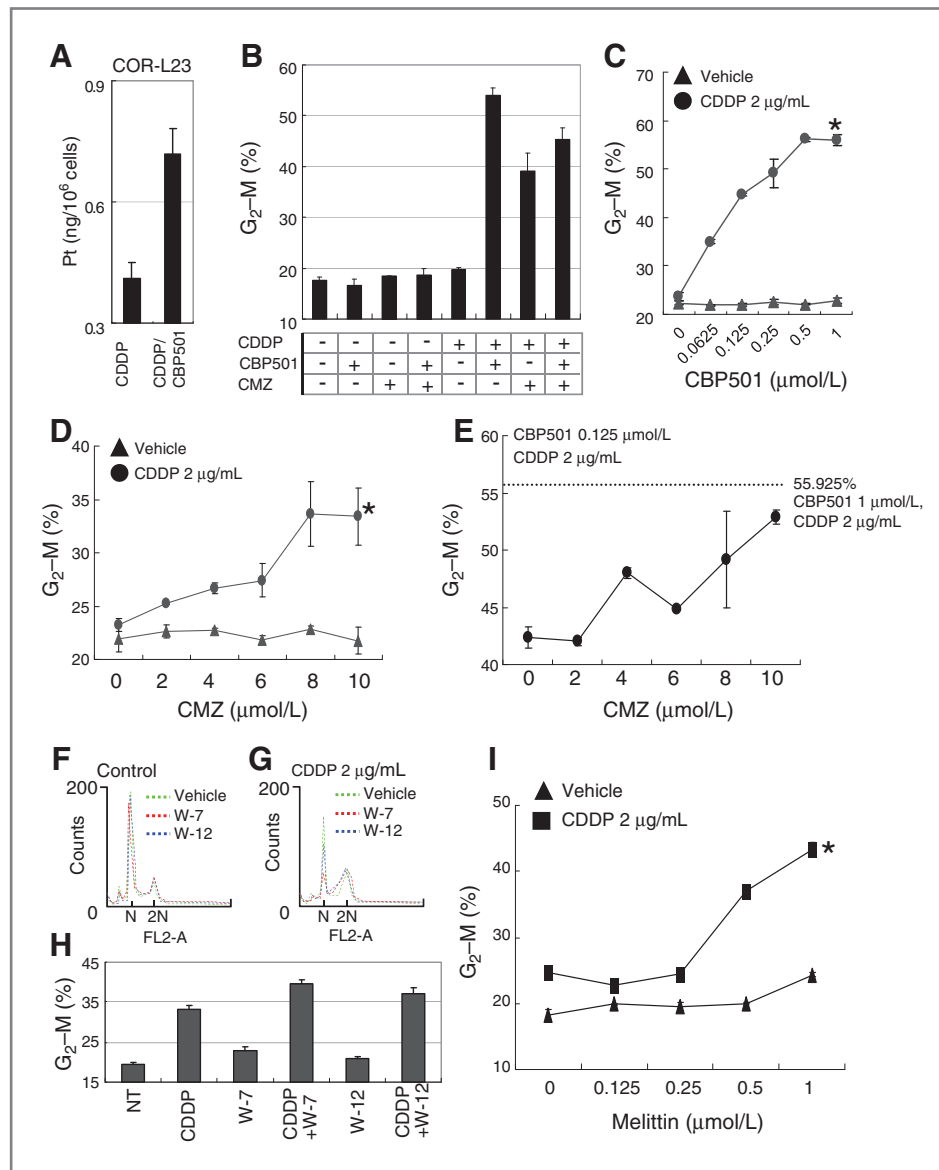
#### CaM is a target of CBP501

Because the codrug effect of CBP501 is similar to that of CaM inhibitors, we conducted immunoblot analysis of several downstream targets of CaM. MLC is known to be phosphorylated at Ser19 by the Ca<sup>2+</sup>/CaM-activated kinase, MLC kinase (MLCK; ref. 19). The level of MLC-p-Ser19 was found to be decreased upon 15 minutes treatment with either CBP501 or CMZ (Fig. 6A). Next, the presence of phosphorylated CaMKII was detected after exposure to CBP501 and CMZ. The level of

p-Thr286 did not decrease upon 15 minutes exposure to either CBP501 or CMZ (Fig. 6A). However, upon 18-hour exposure to CBP501, the level of p-CaMKII decreased significantly (Fig. 6B and Supplementary Fig. S3C). These results indicate that CBP501 suppresses a common upstream regulator of the CaMKII and MLCK pathways, which led us to the examination of CaM. To investigate this possibility, we analyzed the direct interaction between CBP501 and CaM by SPR spectroscopy.

First, we confirmed that CBP501 binds to 14-3-3 $\zeta$ , as had been found earlier (unpublished data). The binding response between CBP501 and 14-3-3 $\zeta$  increased in a dose-dependent manner (Fig. 6C). The binding response between CBP501 and CaM also increased in a dose-dependent manner (Fig. 6D), and we found that the affinity of CBP501 to CaM is nearly 10-fold higher than that of CBP501 and 14-3-3 $\zeta$ . Because the codrug effect of CBP501 is negated by the presence of calcium ions, we also examined the effects of Ca<sup>2+</sup> on both equilibria. We found that the binding of CBP501 to 14-3-3 $\zeta$  is not affected by the presence of Ca<sup>2+</sup> (Fig. 6E); however, the binding of

**Figure 4.** CaM inhibitors mimic CBP501's codrug effect. **A**, graph shows platinum concentration in large lung cell carcinoma cell line COR-L23. COR-L23 cells were treated for 1 hour with cisplatin (2.5  $\mu\text{g/mL}$ ) with or without CBP501 (1  $\mu\text{mol/L}$ ;  $n = 2$ ). Pt, platinum. **B**, graph shows percentage of cells in  $G_2$ -M. COR-L23 cells were treated for 1 hour with cisplatin (2  $\mu\text{g/mL}$ ), CBP501 (1  $\mu\text{mol/L}$ ), and CMZ 10  $\mu\text{mol/L}$ ;  $n = 2$ . **C**, dose-dependent effect of CBP501 combined with cisplatin (2  $\mu\text{g/mL}$ ) for COR-L23 cells ( $n = 2$ ). \*,  $F$  test mean value  $P < 0.01$ . **D**, dose-dependent effect of CMZ combined with cisplatin (2  $\mu\text{g/mL}$ ) for COR-L23 cells ( $n = 2$ ). \*,  $F$  test mean value  $P < 0.01$ . **E**, dose-dependent effect of CMZ combined with cisplatin (2  $\mu\text{g/mL}$ ) with CBP501 present at subsaturation level (0.125  $\mu\text{mol/L}$ ) for COR-L23 cells ( $n = 2$ ). **F** and **G**, histograms from flow cytometric analysis. COR-L23 cells were treated for 1 hour with W-7 (100  $\mu\text{mol/L}$ ) or W-12 (600  $\mu\text{mol/L}$ ). Then cisplatin (2  $\mu\text{g/mL}$ ) was added and cultured for 1 hour.  $N$  means FL2-A value 200 units. **H**, bar graph shows the percentage of cells in  $G_2$ -M phase based on **E** and **F**. NT, nontreatment. **I**, dose-dependent effect of melittin combined with cisplatin (2  $\mu\text{g/mL}$ ) for COR-L23 cells ( $n = 2$ ). \*,  $F$  test mean value  $P < 0.01$ . CDDP, cisplatin.



CBP501 to CaM decreased significantly in the presence of  $\text{Ca}^{2+}$  (Fig. 6F). The higher affinity of CBP501 to CaM and the decrease in affinity in the presence of calcium ions imply that the formation of a CBP501–CaM complex is at least one aspect of the basis for the CBP501 codrug effect.

## Discussion

The finding that CBP501 in combination with cisplatin has documented clinical antitumor activity emphasized the importance of defining the mechanism of action of CBP501. Previously, we reported that CBP501 acted as a  $G_2$  checkpoint abrogator based on observations that treatment of cancer cells with CBP501 attenuated phosphorylation of CDC25C-Ser216 and reduced accumulation of

cells at  $G_2$ -M phase when combined with cisplatin and bleomycin at long-exposure times (13). Here, we report 3 key findings that suggest an additional mechanism of action to account for the codrug activity of CBP501 when combined at lower doses and shorter exposures compared with our previous work (13). First, CBP501 exhibits significant codrug activity with cisplatin or bleomycin within 5 minutes of treatment at a dose around 100 nmol/L, which is much shorter than the time (6 hours) and lower than the dose (2  $\mu\text{mol/L}$ ) required to confer dephosphorylation of Ser216 on CDC25C (13). Second, CBP501-sensitive cells accumulate to a greater extent at  $G_2$ -M phase when combined with suboptimal doses of cisplatin or bleomycin, which implies activation rather than abrogation of the  $G_2$  checkpoint. Third, the codrug

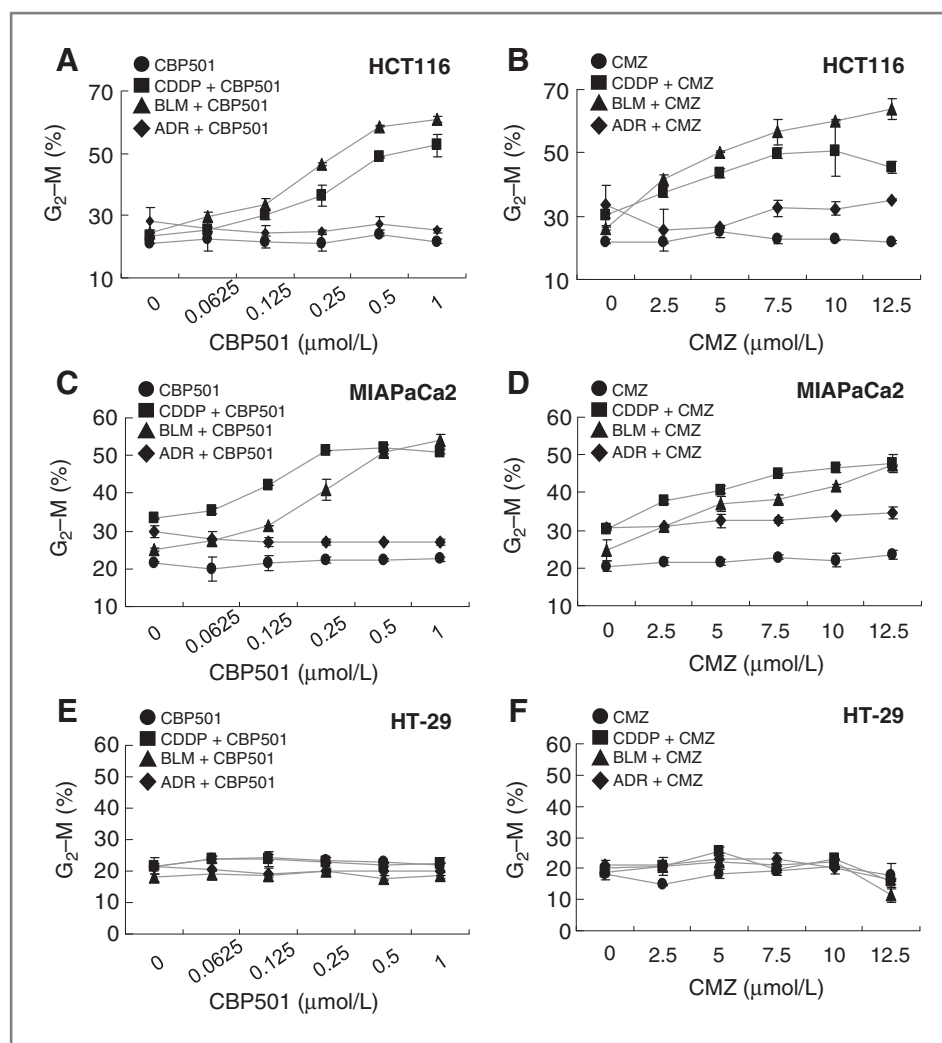


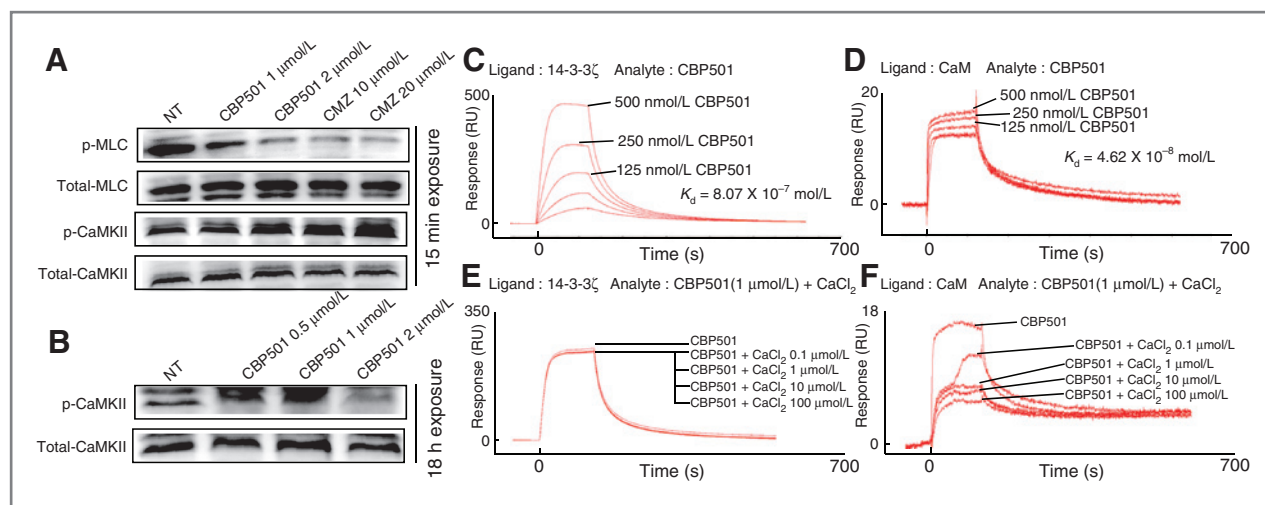
Figure 5. CMZ shows similar activity to CBP501 when accumulation of cells at G<sub>2</sub>-M is monitored in response to the combination with cisplatin (CDDP), bleomycin (BLM), or DOX. Each graph shows the percentage of cells in G<sub>2</sub>-M on y-axis with the dose of CBP501 or CMZ in x-axis. After 1 (CBP501) or 1.5 hours (CMZ) of drug treatment, cells were cultured in fresh media for 48 hours before flow cytometric analysis ( $n = 2$ ). A, C, and E, 1-hour treatment with CBP501 in combination with or without cisplatin (8  $\mu$ g/mL), bleomycin (2  $\mu$ g/mL), or DOX (0.4  $\mu$ g/mL) in HCT116 (A), MIAPaCa2 (C), and HT29 (E) cells. B, D, and F, 30 minutes pretreatment with CMZ and an additional 1 hour culture with cisplatin (8  $\mu$ g/mL), bleomycin (2  $\mu$ g/mL), or DOX (0.4  $\mu$ g/mL) in HCT116 (B), MIAPaCa2 (D), and HT29 (F) cells.

effect of CBP501 with cisplatin correlates with increased platinum concentration and platinum-DNA adduct formation in cells, which is considered unrelated to G<sub>2</sub> checkpoint abrogation.

This rapid action of CBP501 is suppressed by high concentrations of calcium and is mimicked by high concentrations of 2 calcium chelating reagents, EGTA and BAPTA-AM (chelates intracellular calcium), and by some CaM inhibitors, such as CMZ, W-7, W-12, and melittin. These observations led us to examine and ultimately to show (i) a high affinity binding interaction between CBP501 and CaM and (ii) CaM inhibitory activity by CBP501 as indicated by the suppression of MLC and CaMKII phosphorylation. CaMKII is a particularly well-studied target of Ca<sup>2+</sup>/CaM (20, 21). In its quiescent state, CaMKII exhibits a closed conformation that undergoes autophosphorylation, but only slowly, leaving Thr286 predominantly unphosphorylated. Upon binding to Ca<sup>2+</sup>/CaM, CaMKII switches from the closed conformer to an open one, allowing subsequent autophosphor-

ylation at Thr286. In cells, this phosphorylated form, CaMKII-p-Thr286, acts as a protein kinase for downstream signaling (20–22).

An important finding presented here is that several CaM antagonists show similar activity to CBP501 when combined with cisplatin or bleomycin, although they require higher doses and have a narrow window in terms of the optimal duration of treatment for activity. Of the low-MW inhibitors tested, CMZ binds most tightly to CaM with an affinity 500 times higher than that of trifluoperazine (23); however, even CMZ shows weaker codrug activity than CBP501. As found for CBP501, CMZ enhanced the cytotoxicity of cisplatin or bleomycin (EC<sub>50</sub> for CMZ around 5  $\mu$ mol/L), but not that of DOX. Other CaM inhibitors W-7 and W-12 (24) show similar but weaker activity compared with CBP501. The affinity of CBP501 to CaM shown by SPR analysis is 10-fold higher than that of CBP501 to 14-3-3 $\zeta$ . In contrast, KN93, an inhibitor of CaMKII, and ML-7, an inhibitor of MLCK, had no synergistic effect with cisplatin (data not shown),



**Figure 6.** CaM is a direct target of CBP501. A and B, Western blot analysis with MLC, p-MLC, CaMKII, and p-CaMKII antibodies. A, COR-L23 cells were treated with CBP501 or CMZ for 15 minutes ( $n = 3$ ). B, COR-L23 cells were treated with CBP501 for 18 hours ( $n = 3$ ). C–F, SPR analysis. C, interaction between 14-3-3 $\zeta$  and CBP501. D, interaction between CaM and CBP501. E, the interaction between 14-3-3 $\zeta$  and CBP501 with increasing doses of CaCl<sub>2</sub>. F, the interaction between CaM and CBP501 with increasing doses of CaCl<sub>2</sub>.

which indicates that these kinases are not likely the downstream target of CBP501 codrug effects.

Similar increases in the effectiveness of cytotoxic chemotherapeutic agents upon inhibition of CaM had been reported as early as the 1980s (6, 7). However, although several such combinations were investigated in earlier human clinical trials [e.g., the combination of trifluoperazine and bleomycin as a treatment for patients with glioblastoma (ref. 8)], there have been no subsequent reports of advanced clinical trials since then.

Although we have no clear answer for how CaM inhibition leads to increased platinum levels in cells, a multidrug resistant efflux transporter, multidrug resistance 1, was suggested to be regulated by CaM (25, 26). In addition, SK4 (small conductance calcium-activated potassium channel 4), a calcium-dependent potassium ion channel, is activated by CaM and has been shown to participate in platinum uptake (27). Unlike the earlier reported CaM inhibitors (28, 29), CBP501 strongly enhances the cytotoxicity of cisplatin or bleomycin, but not that of many other cytotoxics. The variations in pharmacologic effects exhibited by different CaM inhibitors might result from distinct interactions of different CaM–inhibitor complexes with each CaM target. CaM is composed of 2 globular domains, one at each terminus of the peptide chain (1, 2). CaM may assume a vast range of conformations in which the relative position of its 2 globular domains varies. These conformations are classified as open or closed; however, within each class there is much variation. The observed conformation of CaM depends not only on the number of bound Ca<sup>2+</sup> ions but also on CaM's interactions with different target proteins (e.g., CaMKII, MLCK, anthrax adenyl cyclase, etc.) and with other ligands (17). Given that the observed range

of CaM conformations appears to differ markedly when different ligands are bound, and given that the conformation of CaM required for binding to each of its target proteins seems to be unique (17), it is reasonable to suppose that different CaM ligands may differentially affect the interaction of CaM with each of its targets. Although this concept remains to be shown directly, it may account in part for the differences in the pharmacologic effects displayed by different CaM inhibitors in our system. Alternatively, it is also possible that the previously tested CaM inhibitors bind to other unknown targets besides CaM, which may have adversely affected their utility as anticancer chemotherapeutics in the earlier clinical trials.

An occasionally observed adverse activity of CBP501 that has been found to occur in human clinical studies has been the histamine release syndrome (12), the effects of which seem to be ameliorated, to some extent, by responsive treatment with antihistamines. Several reports had indicated that Ca<sup>2+</sup>/CaM acts to modulate histamine release from mast cells (30). It would be interesting, then, to see whether the efficacy of CBP501 correlates with the occurrence of histamine release syndrome in patients in these ongoing clinical studies.

These findings, taken together, indicate that there is an additional mechanism of action for CBP501 as a unique CaM inhibitor. However, G<sub>2</sub> checkpoint abrogation still remains a possible factor in the action of CBP501, especially as a longer term effect, because the reduced phosphorylation at CDC25C-Ser216 is observed 6 hours after treatment with CBP501 at 2  $\mu\text{mol/L}$  when the clinical C<sub>max</sub> of CBP501 is 2 to 4  $\mu\text{mol/L}$  (12). Such short- and long-term effects might work together to contribute to CBP501's strong codrug

effect in combination with cisplatin or bleomycin and to CBP501's apparently wide therapeutic window of activity.

### Disclosure of Potential Conflicts of Interest

N. Mine, S. Yamamoto, Naoya Saito, C. Suda, and M. Ishigaki are employees of CanBas Co., Ltd. CanBas Co., Ltd. owns the patent; T. Kawabe and M. Ishigaki are the inventors. D.W. Kufe and D.D. Von Hoff are consultants for CanBas Co., Ltd.

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