Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway

Deborah Chirnomas,[1,2] Toshiyasu Taniguchi,[1]
Michelle de la Vega,[1] Ami P. Vaidya,[3]
Maria Vasserman,[1] Anne-Renee Hartman,[1]
Richard Kennedy,[1] Rosemary Foster,[4]
Jennifer Mahoney,[4] Michael V. Seiden,[4]
and Alan D. D'Andrea[1]

[1]Department of Radiation Oncology, Dana-Farber Cancer
Institute; [2]Department of Pediatric Hematology/Oncology,
Dana-Farber Cancer Institute/Boston Children's Hospital,
Harvard Medical School; [3]Division of Gynecologic Oncology,
Massachusetts General Hospital Cancer Center; and [4]Division of
Hematology Oncology, Massachusetts General Hospital,
Boston, Massachusetts

Abstract

**Cisplatin resistance occurs, at least in part, through the
function of the Fanconi anemia (FA)/BRCA pathway, a
DNA-damage response pathway required for repair of
cisplatin cross-links. In the current study, we designed a
cell-based screening strategy to identify small-molecule
inhibitors of the FA/BRCA pathway with the hypothesis
that such molecules could restore sensitivity to platinum
agents. We identified four inhibitors, including three
protein kinase inhibitors (wortmannin, H-9, and alsterpaul-
lone) and one natural compound (curcumin) that inhibit the
FA/BRCA pathway. We show that curcumin, a compound
that is generally regarded as safe, inhibits the monoubiqui-
tination of the FANCD2 protein as predicted by the screen
and consequently sensitizes ovarian and breast tumor cell
lines to cisplatin through apoptotic cell death. We believe
that this study shows an efficient, high-throughput method
for identifying new compounds that may sensitize cancer
cells to DNA-damaging chemotherapy. [Mol Cancer Ther
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Note: The current address for T. Taniguchi is Division of Human Biology,
Fred Hutchinson Cancer Research Center, Seattle, WA 98109-1024.

Requests for reprints: Alan D. D'Andrea, Dana-Farber Cancer Institute,
Department of Radiation Oncology, Harvard Medical School, 44 Binney
Street, Boston, MA 02115. Phone: 617-632-2112; Fax: 617-632-5757.
E-mail: alan_dandrea@dfci.harvard.edu

Introduction

Ovarian cancer is the most prevalent lethal gynecologic
malignancy in the United States and Europe, and patients
often present with advanced disease (1, 2). Although
chemotherapy with cisplatin and paclitaxel are effective
in providing initial cytoreduction of the tumors, the
majority of ovarian cancer patients experience tumor
recurrence and the eventual development of cisplatin-
resistant tumors (3, 4). Breast cancer also responds to
cisplatin treatment as a primary therapy, but patients who
have previously received DNA-damaging treatments show
high levels of cisplatin resistance (5–8). Strategies designed
to enhance cisplatin sensitivity may be of clinical benefit in
both ovarian and breast cancer patients (9).

The mechanism of cisplatin resistance in ovarian cancers
has been widely studied. Cisplatin resistance may result
from increased cell surface expression of the P-glycoprotein
multidrug resistance transporter (10), increased DNA
repair by nonhomologous end joining, decreased expres-
sion of the mismatch repair proteins MSH2 and MLH1 (11),
or from increased expansion of the variant polymerase, pol
β (12, 13). Recent studies indicate that acquired cisplatin
resistance in ovarian tumors may in part be explained by
reactivation of the FA/BRCA pathway (14). Furthermore,
breast tumors that have been treated with a combination of
cyclophosphamide and doxorubicin show up-regulation of
Fanconi anemia G protein, a component of the Fanconi
pathway (15).

The FA/BRCA pathway regulates the cellular response to
cisplatin and other DNA cross-linking agents (16). The
pathway is governed by the coordinate activity of several
FA proteins. In response to cross-linkers, a protein complex
composed of at least eight FA proteins (A, B, C, E, F, G,
L, M) monoubiquitinates the FANCD2 protein (17–19).
Monoubiquitinated FANCD2 is subsequently targeted to
chromatin where it interacts with the FANCD1/BRCA2
protein (20). This interaction seems to be required for
normal homologous recombination repair and ultimately
for cross-link repair. The molecular relationship between
FANCD2-Ub and BRCA2 in homologous recombination
repair is not known, although the proteins may cooperate
in the timed release of RAD51 at sites of DNA repair
(21, 22). Another FA protein, FANCJ, may also function
downstream of FANCD2 monoubiquitination or indepen-
dently of the FA/BRCA pathway (23–26). A protein kinase,
ataxia telangiectasia related (ATR), has been shown to be
required for efficient activation of the FA/BRCA pathway
(27). Disruption of the FA/BRCA pathway, by germ line
mutations in *FA* genes or the *ATR* gene, results in cross-
linker hypersensitivity.

The FA/BRCA pathway is disrupted in a variety of
primary sporadic cancers, including ovarian (14), head and
neck squamous cell carcinoma (28), lung (28), cervical

cancers (29), and pancreatic cancers (30–33). In the case of ovarian cancer, disruption of the pathway results, at least in part, from methylation of the *FANCF* promoter and silencing of the *FANCF* gene, leading to decreased FANCF protein expression and a corresponding loss of the FA protein complex (A/B/C/E/F/G/L/M complex; ref. 14). Whereas *FANCF* promoter methylation accounts for the chromosome instability and initial cisplatin hypersensitivity of a subset of ovarian cancers, *FANCF* promoter demethylation accounts for restoration of the FA/BRCA pathway and a potential mechanism of acquired cisplatin resistance. In principle, inhibitors of the FA/BRCA pathway would be predicted to sensitize tumors with an intact FA/BRCA pathway to cisplatin (34, 35) and may have a role in future combination chemotherapy strategies for primary ovarian cancer.

In the current study, we designed a high-throughput, cell-based screening test to identify inhibitors of the FA/BRCA pathway. Through this screen, we identified three synthetic kinase inhibitors and the natural product, curcumin, as potent inhibitors of the pathway. We show that curcumin, a compound that is generally regarded as safe in humans and has a wide range of anti-inflammatory activities inhibits the FA/BRCA pathway and sensitizes cells to cisplatin to induce increased cell death through apoptosis.

Materials and Methods

Cell Lines and Cell Culture

HeLa cells, PD20 (FA-D2) fibroblasts, and GM6914 (FA-A) fibroblasts were grown as previously described (36). The FANCF-deficient ovarian tumor line (2008) and FANCF cDNA corrected 2008F cells were previously described (14). Breast cancer cell line MCF7 was purchased from the American Type Culture Collection (Manassas, VA). 2008 cells were provided by S. Chaney (University of North Carolina, Chapel Hill, NC).

Plasmids and Retroviral Infection

The retroviral expression vectors, pMMP-puro (37), pMMP-HA-FANCD2 (38), and pMMP-FANCD2, were described previously (39, 40). pMMP-enhanced green fluorescent protein (EGFP)-FANCD2 was constructed by adding EGFP cDNA sequence [from pEGFP-N1 (Clontech, Mountain View, CA)] to the NH_2 terminus of the FANCD2 cDNA sequence. The cDNA insert was verified by direct DNA sequencing. Production of pMMP retroviral supernatants and infection of fibroblasts (PD20 fibroblasts) were done as previously described (41). After 48 hours, cells were trypsinized and selected in medium containing puromycin (1 μg/mL). Dead cells were removed and surviving cells were grown under continuous selection in puromycin. Subcloning of PD20 fibroblasts infected with pMMP-EGFP-FANCD2 was done by limited dilution, and a clone that showed clear EGFP-FANCD2 foci formation in response to ionizing radiation treatment (15 Gy, 10 hours) was selected (PD20F-EGFP-FANCD2 clone 7) for the drug screening study.

Cytotoxicity Assay

Human cells (HeLa cells, MCF7, PD20 fibroblasts, or 2008/2008F cells, where indicated; ref. 14) were seeded onto 12-well plates at 9×10^4 per well in DMEM-15% FCS (5 mL). After cells attached for 16 to 24 hours, the medium was replaced with DMEM-15% FCS containing cisplatin or mitomycin C (Sigma, St. Louis, MO) at various concentrations, with or without a kinase inhibitor or curcumin (Sigma) at either variable or the same concentrations. The cells were incubated at 37°C. After incubation for 5 to 6 days, monolayers were washed twice with PBS and then fixed for 5 to 10 minutes at room temperature in 10% (v/v) methanol and 10% (v/v) acetic acid. Adherent colonies were stained for 2 to 10 minutes at room temperature with 1% (w/v) crystal violet (Sigma) in methanol (0.5 mL per well). Plates were rinsed in distilled water, and the adsorbed dye was resolubilized with methanol containing 0.1% (w/v) SDS (0.5 mL per well) by gentle agitation for 1 to 4 hours at room temperature. Dye solution (150 μL) was transferred to 96-well plates and diluted (1:3) in methanol. Crystal violet concentrations were measured photometrically (595 nm) in a model 3550 microplate reader (Bio-Rad, Hercules, CA). For quantitation, readings of absorbance at 595 nm were normalized to those obtained from untreated cells (concentration of cisplatin = 0 nmol/L), assumed to yield 100% cell survival.

Immunofluorescence Microscopy

Cells were seeded onto four-well chamber slides (Becton Dickinson, San Jose, CA) and cultivated for 16 to 24 hours. Slides were rinsed with PBS, and adherent cells were fixed for 20 minutes in paraformaldehyde [4% (w/v) in PBS] and permeabilized with Triton X-100 [0.3% (v/v) in PBS] for 10 minutes at room temperature. Staining with primary (affinity-purified anti-FANCD2:E35 1:200; ref. 39) and secondary (fluorescein-conjugated goat anti-rabbit) antibodies was for 2 hours at room temperature, followed by counterstaining for 5 minutes at room temperature with 4′,6-diamidino-2-phenylindole (10 μg/mL in PBS; Sigma). Slides were mounted in Vectashield (Vector Laboratories) and analyzed by fluorescence microscopy.

High-Throughput Screen for Small-Molecule Inhibitors of the FA/BRCA Pathway

Initially, we transduced PD20 (FA-D2) fibroblasts with the pMMP-EGFP-FANCD2 retroviral supernatant and 20 individual puromycin-resistant colonies were selected. One clone (clone 7) had a low level of expression of EGFP-FANCD2 in the nucleus, judged by fluorescence microscopy, but formed bright EGFP foci in response to ionizing radiation. Clone 7 was used in subsequent experiments.

Cell-based screening was done at the Institute for Chemistry and Cell Biology.[5] For the high-throughput screen, clone 7 cells were plated in 384-well plates (4,000 per well). One hour after plating, a chemical compound from a commercial library (42) was added to each well, at a

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[5] http://iccb.med.harvard.edu.

single concentration of ~20 to 40 μmol/L. Library compounds were added to duplicate plates. After a 12-hour incubation, the plates were irradiated (15 Gy) and, following an additional 12-hour incubation, cells were fixed for EGFP microscopy. Photomicrographs were obtained for each well and wells with significant (50%) reduction in EGFP foci were identified by visual inspection. Also, visualization of EGFP-FANCD2 foci by direct microscopy was done.

The chemical libraries, ICCB bioactives (489 compounds) and Commercial Diversity Set 1 (5,056 compounds), have been previously described (42, 43). Twenty-seven positive compounds were identified from the library in the initial screening and four compounds were confirmed as true positives. Further positive compounds are still being evaluated. For subsequent studies, these four compounds were purchased directly from Sigma (curcumin) or Calbiochem (San Diego, CA; H-9, alsterpaullone, and wortmannin).

Immunoblotting

Whole cell lysates were electrophoresed by SDS-PAGE [3–8% Tris-acetate gradient gel for FANCD2 analysis and 4–12% Bis-Tris for poly(ADP)ribose polymerase analysis]. Proteins were transferred to nitrocellulose, blocked, and incubated with primary antibodies as described (27). Antibodies included anti-FANCD2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000; ref. 40) and anti–cleaved poly (ADP)ribose polymerase (Cell Signaling, Beverly, MA; 1:500), antivinculin (Santa Cruz; 1:1,000), and anti–glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, MA; 1:5,000). Membranes were washed, incubated with horseradish peroxidase–linked secondary antibodies (Amersham, Piscataway, NJ), and detected by chemiluminescence (Amersham) as described (36).

Flow Cytometry Analysis

DNA content was evaluated after propidium iodide staining. Trypsinized HeLa cells were resuspended in 0.5 mL PBS and fixed by adding 5 mL ice-cold ethanol. Cells were next washed twice with PBS with 1% bovine serum albumin fraction V (1% bovine serum albumin/PBS; Sigma) and were resuspended in 0.24 mL of 1% bovine serum albumin/PBS. After adding 30 μL of 500 μg/mL propidium iodide (Sigma) in 38 mmol/L sodium citrate (pH 7.0) and 30 μL of 10 mg/mL DNase-free RNase A (Sigma), samples were incubated at 37°C for 30 minutes. DNA content was based on a measurement of 50,000 cell counts by FACScan (Becton Dickinson, San Jose, CA), and data were analyzed by the CellQuest and Modfit LT programs (Becton Dickinson).

Results

High-Throughput Assay for Inhibitors of the FA/BRCA Pathway

To identify inhibitors of the FA/BRCA pathway, we established a high-throughput screen for the assembly of FANCD2 foci, a critical downstream event in the FA/BRCA pathway. For this purpose, a fusion cDNA was generated that encodes EGFP fused at the amino terminus

of the full-length FANCD2 protein (Fig. 1A). To ascertain whether this protein would function like the wild-type protein, this cDNA was transfected into the FANCD2-deficient (PD20) cell line, which expresses no endogenous FANCD2 protein (Fig. 1B). The EGFP-FANCD2 protein is a higher molecular mass than FANCD2, as predicted, and exhibits DNA damage–inducible monoubiquitination (*lanes 6–9*). When the EGFP-FANCD2 protein was expressed in an FA-A (FANCA deficient) fibroblast line,
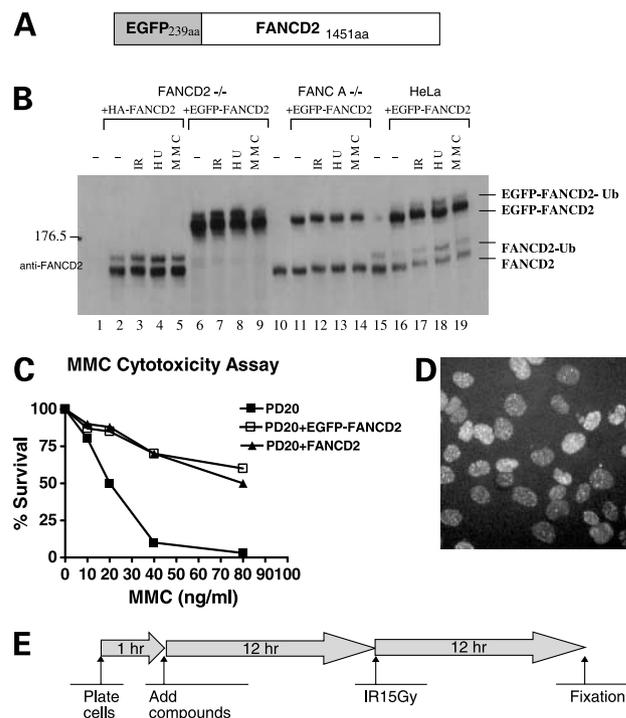

Figure 1. Establishment of a mammalian cell line stably expressing functional EGFP-tagged FANCD2. **A,** a cDNA clone was generated encoding the indicated EGFP-FANCD2 fusion protein. **B,** FANCD2-deficient human fibroblasts (PD20F) were either untransfected (*lane 1*), transfected with hemagglutinin-tagged wild-type FANCD2 cDNA (*lanes 2–5*), or transfected with the cDNA encoding the EGFP-FANCD2 fusion protein (*lanes 6–9*) as indicated. Alternatively, FA-A fibroblasts (GM6914, *lanes 11–14*) or HeLa cells (*lanes 16–19*) were transfected with the cDNA encoding the fusion protein. Cells were either untreated or exposed to genotoxic stress (ionizing radiation) as indicated. Whole cell lysates were run on SDS-PAGE (gels), and cellular proteins were immunoblotted with antisera to FANCD2. **C,** the EGFP-FANCD2 fusion protein functionally complements the mitomycin C hypersensitivity of FANCD2-deficient human fibroblasts. The transfected PD20F cells, stably expressing EGFP-FANCD2 protein, were analyzed for survival in variable concentrations of mitomycin C. **D,** isolation of a PD20F fibroblast clone, expressing a physiologic level of the EGFP-FANCD2 protein and suitable for high-throughput drug screening. A subclone (clone 7) was isolated, by limiting dilution, that expresses EGFP-FANCD2 protein. Activation of this clone with ionizing radiation (*IR*; 15 Gy, followed by a 12-h incubation period) resulted in the assembly of bright green foci, readily detectable in the background of the irradiated cell nucleus. **E,** schematic protocol for the identification of small-molecule inhibitors and agonists of the FA/BRCA pathway. Human fibroblasts (clone 7), stably expressing EGFP-FANCD2, were plated in 384-well plates, pretreated with compounds from chemical libraries, and exposed to ionizing radiation as indicated. Photomicrographs were taken for each well, and chemical inhibitors of green foci assembly were identified.

it was not monoubiquitinated following exposure to DNA damage (Fig. 1B, *lanes 11–14*). Taken together, these results indicate that EGFP-FANCD2 behaves similarly to the wild-type FANCD2 protein.

Next, we determined whether EGFP-FANCD2 is capable of correcting mitomycin C hypersensitivity of FANCD2-deficient (PD20) cells. Expression of EGFP-FANCD2, like wild-type FANCD2, restored normal mitomycin C resistance (Fig. 1C).

Previous studies have shown that ionizing radiation activates the monoubiquitination of FANCD2, allowing FANCD2-Ub to assemble in subnuclear foci. These foci are believed to be sites of DNA repair. The PD20 cells, expressing EGFP-FANCD2, were plated, and individual subclones were isolated. One subclone (clone 7) expressed EGFP-FANCD2 protein diffusely in its nuclei. Following exposure to ionizing radiation, those cells formed bright, green foci in the nucleus (Fig. 1D), similar to the results seen when endogenous FANCD2 is probed by immuno-fluorescence (44). Thus, these cells provide a useful tool for high-throughput screening of inhibitors of the FA/BRCA pathway.

Use of EGFP-FANCD2–Expressing Fibroblasts to Identify Small-Molecule Inhibitors of the FA/BRCA Pathway

The general strategy of the screening assay is outlined in Fig. 1E. A robotic compound distributor, designed to deliver compounds in a 384-well format, was used to deliver a series of compounds to the EGFP-FANCD2–corrected PD20 fibroblasts. Cells were plated in 384-well tissue culture plates. The final concentration of each compound was ~ 40 μmol/L, and the cells were incubated with these compounds for 12 hours. The cells were then irradiated, as indicated, to activate EGFP-FANCD2 foci. Inhibitors of the FA/BRCA pathway were predicted to block EGFP-FANCD2 foci formation.

The specific details of the assay are described in the Materials and Methods. An important feature of the protocol was the use of secondary screens. Any compound initially found to inhibit FANCD2 foci formation was subsequently screened in lower dose ranges (1–20 μmol/L range) by two assays: (*a*) immunofluorescence, to confirm the ability of the compound to block formation of FANCD2 foci and (*b*) Western blot analysis to examine possible inhibition of FANCD2 monoubiquitination. Any compound that passed these secondary screens was then examined for its ability to chemosensitize HeLa cells to the cytotoxic effects of cisplatin or ionizing radiation (14).

Identification of Specific Compounds that Inhibit the FANCD2 Pathway

Initially, two libraries were screened identifying 27 small molecules that inhibited the formation of FANCD2 foci in the micromolar concentration range. Secondary screening narrowed the candidates down to four compounds.

Three of the agents that blocked FANCD2 foci were known kinase inhibitors (wortmannin, H-9, and alsterpaul-lone) and the fourth agent was the natural compound, curcumin.

We have previously shown that wortmannin is a potent inhibitor of the FA/BRCA pathway, through its inhibition of the ATR kinase (27). Although wortmannin was predicted to be an inhibitor of FANCD2 based on these studies, wortmannin is a broadly acting phosphatidylino-sitol 3-kinase inhibitor with significant toxic effects *in vivo*, such as hemorrhage and necrosis (45). H-9 is known to inhibit several kinases, including protein kinase A, protein kinase G, protein kinase C, calcium/calmodulin–dependent protein kinase, and myosin light chain kinase (46, 47). H-9 inhibited the formation of FANCD2 foci in the 50 to 100 μmol/L range in HeLa cells (Fig. 2A).

Alsterpaullone is known to inhibit cyclin-dependent kinase 1/cyclin-dependent kinase B, glycogen synthase kinase-3B, and cyclin-dependent kinase 5 (48, 49). Alster-paullone inhibited the formation of FANCD2 foci at a concentration of 10 μmol/L in HeLa cells (Fig. 2A).

The natural compound, curcumin, also caused a decrease in FANCD2 foci formation in the screening assay at a range of 5 to 10 μmol/L (Fig. 2A). Curcumin is a widely studied compound and has been shown to cause a reduction in the size of solid tumors in animal models by inducing apoptosis.

Although all four of the candidate compounds were biologically active, we found that alsterpaullone and H-9 were toxic to HeLa cells as single agents in the dosage range required to inhibit FANCD2 foci formation ($IC_{50} < 1$ and 25 μmol/L, respectively), whereas curcumin was significantly less toxic at the range required to inhibit FANCD2 monoubiquitination, with an IC_{50} of 15 μmol/L (Fig. 2B). We surmised that low-dose curcumin (5–10 μmol/L) would be a reasonable candidate for cisplatin sensitization as it inhibited the FA/BRCA pathway with minimal toxic effects on cells. Moreover, curcumin has been evaluated in human subjects, demonstrating no significant toxicity even when dosed at 8 g/d (50).

Curcumin Inhibits the FA/BRCA Pathway

To further characterize the activity of curcumin as an FA/BRCA pathway inhibitor, we used immunocytochem-istry with a FANCD2-specific antibody to visualize FANCD2 foci following increasing doses of curcumin. As described above, we observed decreased foci forma-tion (Fig. 2A). Western blot analysis confirmed that curcumin inhibits the FA/BRCA pathway by inhibiting FANCD2 monoubiquitination (Fig. 3A). Cisplatin treat-ment caused an increase in FANCD2 monoubiquitination (compare Fig. 3A, *lanes 1–5* and *6–11*). Curcumin caused a dose-dependent decrease in FANCD2 monoubiquitina-tion in the cisplatin-treated cells (*lanes 7–11*) but did not cause decreased monoubiquitination in untreated cells until the 20 um dose range (*lanes 1–5*). Densitometry was used to compare the monoubiquitinated form of FANCD2 (the long form) with the unubiquitinated form (short form) and showed that there is a 30% decrease in the long form of FANCD2 when 5 μmol/L curcumin is added to the cisplatin-treated cells, whereas there is virtually no change when curcumin is added to untreated cells. Because FANCD2 monoubiquitination occurs primarily
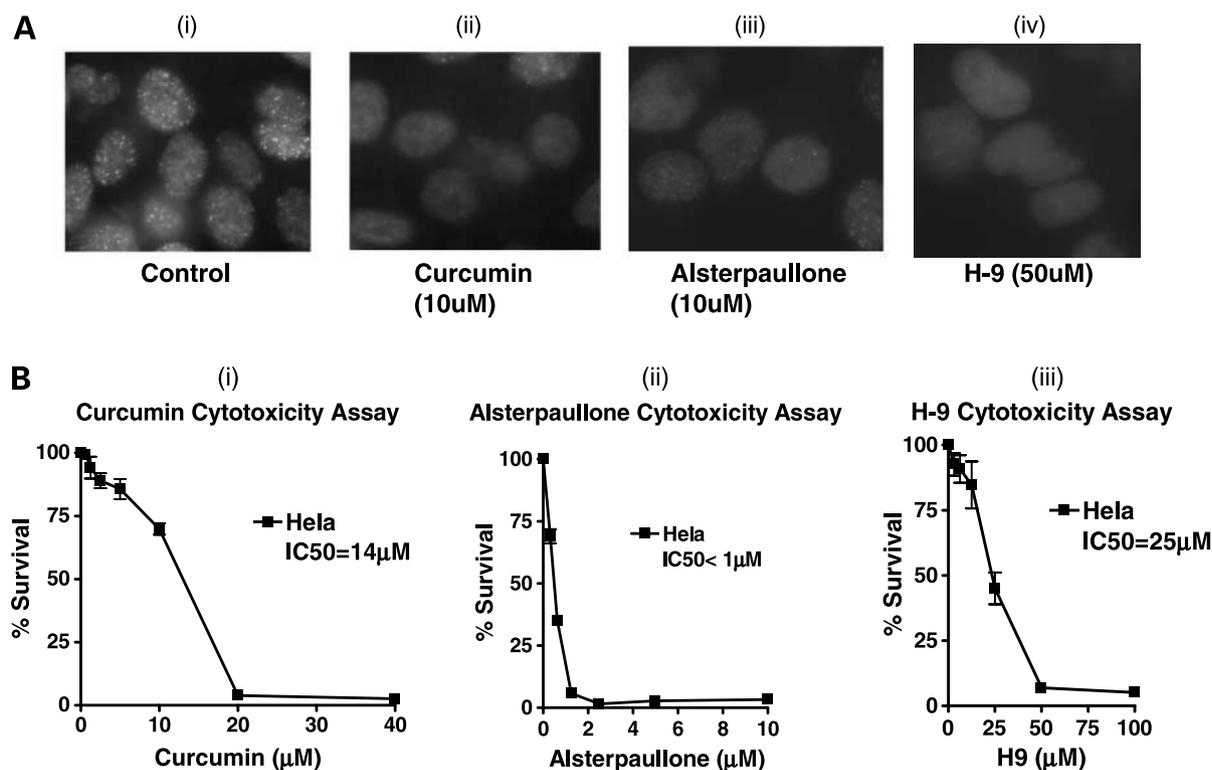
Figure 2. Immunofluorescence and toxicity profiles of the three novel FANCD2 inhibitors. **A,** inhibition of foci by the three novel FANCD2 inhibitors. **i,** HeLa control cells damaged with ionizing radiation, no experimental compounds, IF for FANCD2 foci. **ii,** same as above with 10 μmol/L curcumin. **iii,** same as above with 10 μmol/L alsterpaullone. **iv,** same as above with 50 μmol/L H-9. **B. i,** crystal violet survival assay was done with HeLa cells exposed to increasing doses of curcumin from 0.3 to 40 μmol/L. Representative of three independent experiments. *Points,* percentage survival; *bars,* SE. **ii,** crystal violet assay done with H-9, with a dose range of 200 to 1.5 μmol/L. Representative of three independent experiments. *Points,* percentage survival; *bars,* SE. **iii,** crystal violet assay done to assess the cytotoxicity of alsterpaullone with HeLa cells. Representative of three independent experiments. *Points,* percentage survival; *bars,* SE.

in G_1-S phase, we did flow cytometry with propidium iodide staining to test whether cell cycle arrest in the G_2-M phase could explain the observed decrease in FANCD2 monoubiquitination. Consistent with published data (51), low-dose curcumin did not affect the cell cycle in HeLa cells treated with cisplatin (Fig. 3B). These data suggest that curcumin may have an inhibitory effect upstream in the FA pathway, before monoubiquitination of FANCD2.

Curcumin Sensitizes Ovarian and Breast Tumor Cells with a Functional FA/BRCA Pathway to Cisplatin

The FA/BRCA pathway is required for the response to cisplatin-mediated DNA damage. To determine whether curcumin is a cisplatin sensitizer, we analyzed two ovarian tumor lines—the cisplatin-sensitive, FANCF-deficient parental 2008 line and the 2008 line corrected with the *FANCF* cDNA (2008 + FANCF). The parental 2008 line is deficient in FANCF owing to epigenetic silencing of the *FANCF* gene (14). Using dose-inhibition studies, we showed that curcumin enhanced the cytotoxicity of cisplatin in the *FANCF* cDNA-corrected cells, but not in the parental 2008 cells, from an IC_{50} of 2 to 1 μmol/L cisplatin in 2008-corrected cells (Fig. 4A, i, ii).

This result indicates that this compound is a potential cisplatin sensitizer that works through inhibition of the FA/BRCA pathway. Curcumin sensitized the cells in a dose range of 3 to 20 μmol/L (Fig. 4A, iii), corresponding to the curcumin dose range required for inhibition of the FA/BRCA pathway.

Because recurrent breast cancers are also generally resistant to cisplatin we next determined whether there are synergistic effects of curcumin with cisplatin in breast cancer cell lines. We used dose-inhibition studies to test whether curcumin could sensitize a breast cancer cell line, MCF7, to cisplatin (Fig. 4B). Consistent with the ovarian cancer cell line data, MCF7 showed increased cisplatin sensitivity (from an IC_{50} of 10 to 2 μmol/L) with the addition of 5 μmol/L curcumin.

Western blot analysis confirmed that curcumin inhibits FANCD2 monoubiquitination in both MCF7 and 2008 + FANCF cells in the presence of cisplatin (Fig. 4C). Specifically, curcumin caused a dose-dependent decrease in FANCD2 monoubiquitination in the range of 1 to 20 μmol/L curcumin (Fig. 4C, *lanes 3–6* and *15–18*). We repeated these experiments using pretreatment with curcumin for 6 hours followed by washout and the

addition of platinum. Dose-response curves were similar to those observed with continuous curcumin exposure (data not shown). These results indicate that sequential curcumin and platinum administration may be as effective as combination treatment.

Curcumin Chemosensitizes Tumor Cells to Cisplatin but not to Paclitaxel

Next, we used dose-inhibition studies to test whether the chemosensitizing activity of curcumin was specific to DNA-damaging agents, such as cisplatin, or whether curcumin could also sensitize cells to an antimicrotubule agent, paclitaxel. We compared the effects of curcumin with paclitaxel on both the 2008 + FANCF ovarian tumor cell line and the MCF7 breast cancer cell line. In contrast to the results seen for cisplatin, curcumin had no effect on the dose-dependent paclitaxel cytotoxicity profile of these cells (Fig. 4D). Therefore, curcumin seems to specifically sensitize cells to cisplatin-mediated DNA damage rather than microtubular damage. Consistent with these studies, the FA/BRCA pathway does not seem to play a role in

cellular sensitivity to paclitaxel as evidenced by the lack of sensitivity of the FA cell line 2008 in these dose-inhibition studies (Fig. 4D, i).

Curcumin Does Not Inhibit ATR-Mediated Phosphorylation of CHK1

Cisplatin has previously been reported to activate ATR, possibly through stalling of DNA replication forks. We have previously reported that the DNA-damage response kinase ATR is required for activation of the FA/BRCA pathway following DNA damage (27). We therefore investigated the effect of curcumin on the activation of ATR. To do this, we studied phosphorylation of the ATR target CHK1 by immunoblotting with a phosphospecific antibody following 24 hours of cisplatin treatment in the presence of 1, 5, 10, and 20 μmol/L curcumin. CHK1 phosphorylation was not decreased after 24 hours, with increasing doses of curcumin. Taken together, these results suggest that curcumin does not interfere with ATR signaling and functions differently from other compounds isolated in the screen (data not shown).
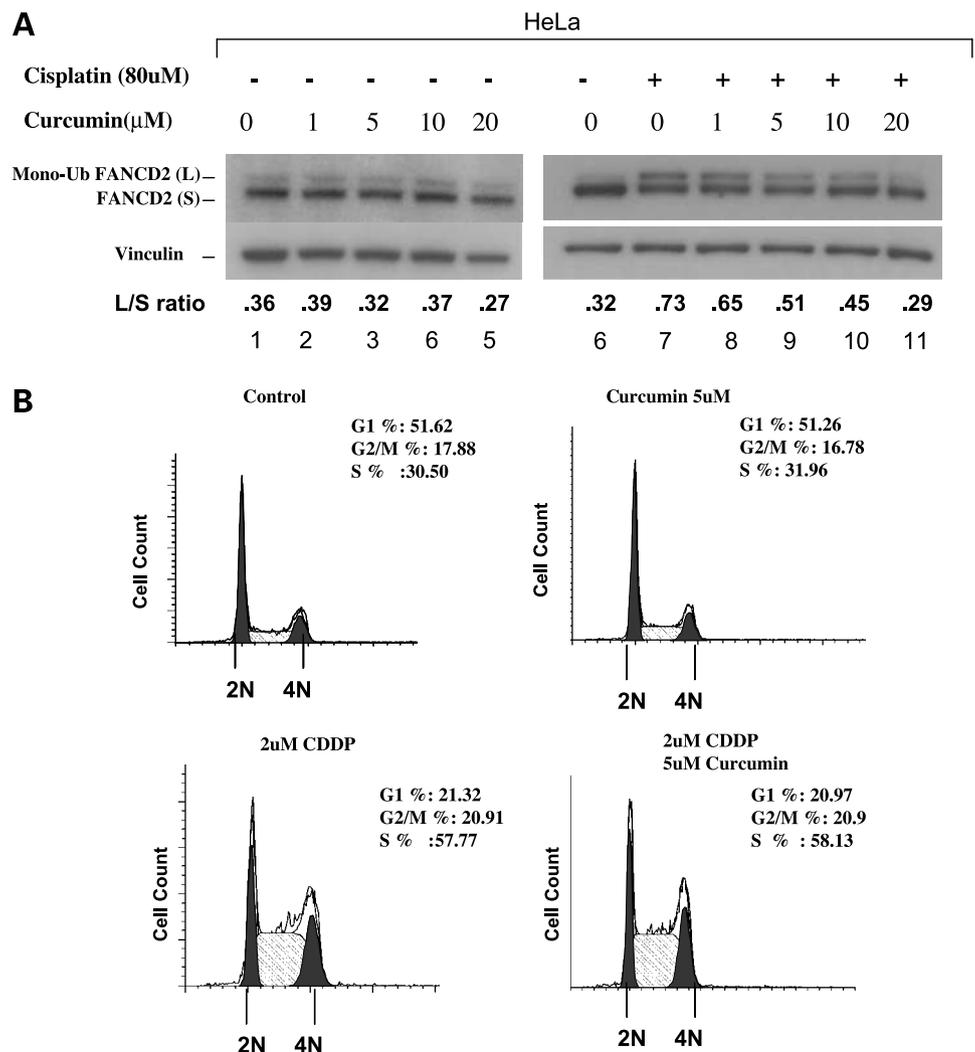

Figure 3. Curcumin inhibits FANCD2 monoubiquitination. **A,** Western blot analysis of FANCD2 monoubiquitination after 24-h exposure to increasing concentrations of curcumin. HeLa cells were exposed to the indicated concentrations of curcumin (*lanes 1–5*) or curcumin plus cisplatin (*lanes 6–11*). Whole cell lysates were examined by immunoblotting with the indicated FANCD2 antisera. Densitometry done using the Scion Image program 4.0.3.2 (Scion Corp., Frederick, MD) to measure the ratio of FANCD2-L/FANCD2S is shown below the blots. The blots were reprobed with vinculin as a loading control. **B,** cell cycle distribution analysis of propidium iodide–stained HeLa cells based on 50,000 cell counts (**i**) untreated, (**ii**) treated with 24-h exposure to 10 μmol/L curcumin alone, (**iii**) cisplatin (*CDDP*) alone, and (**iv**) cisplatin and curcumin combined. These profiles represent the repetition of three sets of experiments.

Curcumin and Cisplatin Synergize to Induce Apoptotic Cell Death

To further investigate the mechanism by which curcumin sensitizes cells to curcumin we did a poly(ADP)ribose polymerase cleavage assay (Fig. 5). During the process of apoptosis, caspase-3 cleaves poly(ADP)ribose polymerase, resulting in an 85 kDa cleavage product. Treatment of 2008 and 2008 + FANCF cells with single agent curcumin at both 5 and 10 μmol/L, or cisplatin at 10 μmol/L for 24 hours, resulted in more apoptotic cell death in the 2008 cell line than in the corrected cell line. In contrast, the combination of cisplatin and curcumin resulted in marked apoptotic cell death in only the corrected ovarian cancer cell line (2008 + FANCF), as evidenced by increased cleavage of poly(-ADP)ribose polymerase (Fig. 5, *lanes 10–12* versus *lanes 4–6*). Densitometry noted below the figure quantitates this result, reflecting that there is increased apoptosis in the FA/BRCA–corrected cell population. This observation suggests that curcumin-mediated inhibition of the FA/BRCA pathway sensitizes ovarian and breast cancer cell lines to increased apoptotic cell death following cisplatin treatment and that, without an intact FA/BRCA pathway, the cells do not exhibit increased apoptosis to the combination therapy.
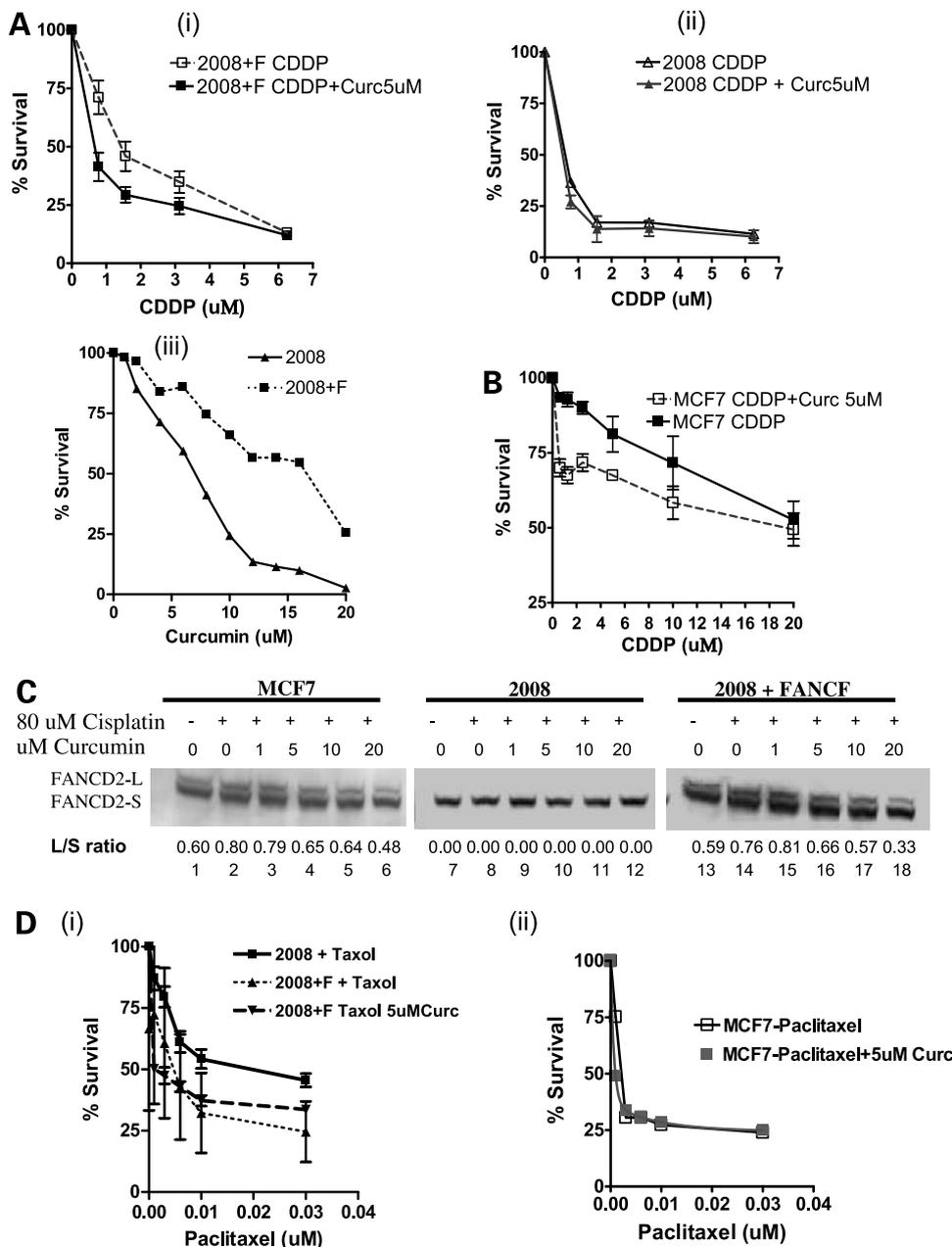

Figure 4. Curcumin chemosensitizes ovarian and breast tumor cell lines. **A. i,** curcumin sensitizes FA-competent cells. **ii,** curcumin does not sensitize FA-affected cells. **iii,** curcumin dose-response curves for the isogenic pair of FA cells, 2008, and FA-corrected cells, 2008 + FANCF. Dose-inhibition studies evaluating cisplatin response in the 2008 and 2008 + FANCF cell lines with the addition of 5 μmol/L curcumin. 2008 + FANCF (□), 2008 + FANCF with curcumin (■), 2008 (▲), and 2008 + curcumin (△). Representative of three independent experiments. *Points,* percentage survival; *bars,* SE. **B,** curcumin sensitizes breast cancer cells. Dose-inhibition studies of MCF7, with 5 μmol/L curcumin. ■, MCF7; □, MCF7 with curcumin. Representative of three independent experiments. *Points,* percentage survival; *bars,* SE. **C,** Western blot confirming the inhibition of FANCD2 monoubiquitination by curcumin in MCF7 and 2008 + FANCF cell lines. The 2008 cells do not monoubiquitinate FANCD2. *Lanes 1, 7,* and *13,* untreated cells; *lanes 2, 8,* and *14,* cells treated with 80 μmol/L cisplatin for 24 h; *lanes 3* to *6, 9* to *12, 15* to *18,* cells treated with 80 μmol/L cisplatin and increasing doses of curcumin (1, 5, 10, and 20 μmol/L) for 24 h. **D,** curcumin does not synergize with paclitaxel. Dose-inhibition studies evaluating paclitaxel response in the **(i)** 2008 and 2008 + FANCF cell lines and 2008 + FANCF with paclitaxel and 5 μmol/L curcumin, **(ii)** MCF7 cell lines with paclitaxel alone and the addition of 5 μmol/L curcumin. Representative of three independent experiments. *Points,* percentage survival; *bars,* SE.
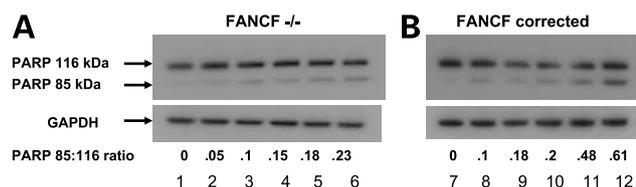
Figure 5. Curcumin synergizes with cisplatin by inducing apoptosis in an FA/BRCA intact cell line. Western blot of (**A**) 2008 (FANCF−/−) and (**B**) 2008-corrected (FANCF+/+) cells treated with curcumin and/or cisplatin for 24 h, evaluating poly(ADP)ribose polymerase (*PARP*) cleavage to the 85 kDa fragment (indicating apoptosis). **A** and **B**, *lanes 1* and *7*, untreated; *lanes 2* and *8*, cells with 5 μmol/L curcumin; *lanes 3* and *9*, 10 μmol/L curcumin; *lanes 4* and *10*, 80 μmol/L cisplatin; *lanes 5* and *11*, 80 μmol/L cisplatin and 5 μmol/L curcumin; and *lanes 6* and *12*, 80 μmol/L cisplatin and 10 μmol/L curcumin. Densitometry indicating the ratio of the 85 kDa protein to the 116 kDa protein is indicated below the blots. Densitometry was done using Scion Image 4.0.3.2 (Scion Corp.). The blots were reprobed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control.

Discussion

In the current study, we devised a high-throughput, cell-based screen to identify inhibitors of the FA/BRCA pathway. The screen was based on the inhibition of assembly of FANCD2 foci, a critical downstream determinant of the pathway. We identified four inhibitors, including three synthetic kinase inhibitors and one natural compound, curcumin. Secondary screens indicated that these four compounds blocked FANCD2 foci formation, blocked FANCD2 monoubiquitination, and synergized with cisplatin in the cytotoxicity of tumor cells in a dose-dependent manner. Cytotoxicity assays characterizing these compounds showed that curcumin was significantly less toxic than the other agents. This observation is in agreement with phase I clinical data showing that curcumin is well tolerated (50) and suggested that curcumin could be a potential therapeutic agent for combination chemotherapy with DNA cross-linking agents, such as cisplatin.

Curcumin (diferuloylmethane), a low-molecular-weight polyphenol derived from the rhizome *Curcuma longa*, is the principal ingredient in the spice turmeric (50). The compound is listed by the Food and Drug Administration as ''generally regarded as safe,'' and extensive studies in animals and humans have shown both anti-inflammatory and antineoplastic activity. Curcumin has antiproliferative activity and inhibits tumor initiation in a variety of tumor models (52, 53). Curcumin is safe in human trials at p.o. doses as high as 8,000 mg/d (50, 54).

Although the precise mechanism of the antineoplastic activity of curcumin remains unknown, possible mechanisms include increased induction of apoptosis in a p53-dependent manner, up-regulation of carcinogen-detoxifying enzymes, such as glutathione *S*-transferases (55, 56), antioxidation (57), and suppression of cyclooxygenase (58). Recent studies also suggest that curcumin may sensitize ovarian tumor lines to cisplatin (59), although the mechanism of this cisplatin sensitization has remained unknown. Further data suggest that curcumin may have activity in the treatment of other human diseases, such as cystic fibrosis (60). In our studies, curcumin sensitized *FANCF*-corrected 2008 cells to cisplatin, but had a lesser effect on the chemosensitization of the parental 2008 cells, suggesting an important role for the FA/BRCA pathway in curcumin sensitization of cisplatin.

Our data suggest that curcumin has an inhibitory effect upstream in the FA/BRCA pathway before the monoubiquitination of FANCD2. The signaling events that lead to FANCD2 monoubiquitination following DNA damage as yet remain unclear, although DNA replication fork arrest and ATR activation seem to be early events (27). Our observation that curcumin fails to inhibit ATR-mediated phosphorylation of CHK1 suggests that curcumin may target another DNA-damage response protein at the replication fork or may function downstream of ATR.

Recently, investigators have determined that curcumin can irreversibly inhibit thioredoxin reductase, an enzyme that is important in regulating thioredoxin, which in turn regulates the redox potential of the cell and assists in DNA synthesis by serving as an electron donor to ribonucleotide reductase (61). Incubation of thioredoxin reductase with curcumin led to covalent alkylation of the active site, likely due to the creation of two curcumin adducts at residues Cys^{496} and Ser^{497}. This raises the possibility that curcumin may inhibit the FA/BRCA pathway through covalent association with one of the FA pathway proteins, although the exact target remains unknown. Accordingly, it will be interesting to determine whether curcumin pretreatment blocks the assembly of the multisubunit FA complex (A/B/C/E/F/G/L/M complex).

The current study shows that curcumin-mediated inhibition of the FA/BRCA pathway sensitizes tumor cells to cisplatin, resulting in apoptotic death of ovarian and breast tumor cell lines. We observed activity of curcumin at a dose range of 3 to 10 μmol/L. Whether these serum concentrations can be achieved *in vivo*, through the p.o. administration of curcumin, or through the use of a more hydrophilic curcumin derivative remains to be determined. Curcumin is composed of several natural derivatives and it will be interesting to determine if any of these specific components contains the inhibitor activity of the FA/BRCA pathway. Based on these findings, further investigation of curcumin as a cisplatin sensitizer is warranted and animal studies are ongoing to study the effect of combination curcumin and cisplatin in mouse models of ovarian cancer. The data also suggest that inhibition of FANCD2 monoubiquitination within tumor tissue may represent a useful biomarker of the curcumin effect.

In conclusion, we have developed a high-throughput assay to identify compounds that inhibit the FA/BRCA pathway and sensitize tumor cell lines to DNA-damaging agents to cause cancer cell death. Using this method, we have identified curcumin as a compound that can sensitize cancer cell lines to cisplatin. This screen will be an important tool in identifying other agents and classes of compounds that may be useful in the sensitization of tumors to chemotherapeutic agents.

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Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway

Deborah Chirnomas, Toshiyasu Taniguchi, Michelle de la Vega, et al.

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