Combination of Vorinostat and Flavopiridol Is Selectively Cytotoxic to Multidrug-Resistant Neuroblastoma Cell Lines with Mutant TP53

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Abstract

As p53 loss of function (LOF) confers high-level drug resistance in neuroblastoma, p53-independent therapies might have superior activity in recurrent neuroblastoma. We tested the activity of vorinostat, a histone deacetylase inhibitor, and flavopiridol, a pan-Cdk inhibitor, in a panel of multidrug-resistant neuroblastoma cell lines that included lines with wild-type (wt) and transcriptionally active TP53 (n = 3), mutated (mt), and LOF TP53 (n = 4) or p14ARF deletion (n = 1). The combination of vorinostat and flavopiridol was synergistic and significantly more cytotoxic (P < 0.001) in cell lines with p53-LOF and in the clones stably transfected with dominant-negative p53 plasmids. Cell cycle analysis by flow cytometry showed prominent cell-cycle arrest in G2/M (37%) for a cell line with wt TP53 (CHLA-90) slipped into sub-G1 at 6 to 24 hours (25%–40% specific cell death). The morphological hallmarks of mitotic cell death, including defective spindle formation and abnormal cytokinesis, were detected by confocal microscopy after the treatment with vorinostat plus flavopiridol combination in CHLA-90. The combination caused reduction in the expression of G2/M proteins (cyclin B1, Mad2, MPM2) in 2 cell lines with mt TP53 but not in those with wt TP53. Plk1 expression was reduced in all treated lines. Small interfering RNA knockdown of Mad2 and cyclin B1 or Plk1 synergistically reduced the clonogenicity of CHLA-90 cells. The combination of HDAC inhibitor and flavopiridol may be a unique approach to treating neuroblastomas with p53 LOF, one that evokes induction of mitotic failure. Mol Cancer Ther; 9(12); 3289–301. ©2010 AACR.

Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood (1). Myeloablative cytotoxic therapy and 13 cis-retinoic acid (2) or anti-GD2 immunotherapy (3) has improved the outcome for high risk patients; however, most of these patients develop progressive disease that is refractory to additional cycles of chemotherapy. We have previously shown that the mechanism of such resistance is often through the loss of p53 function due to mutations in TP53 or to MDM2 amplification (4).

The tumor suppressor p53 is the most frequently altered gene in a wide variety of tumors. Inactivation of p53 protein occurs through mutations in the p53 gene or through alterations in genes whose products regulate p53, such as genomic amplification of the negative regulator MDM2, or through inactivation of downstream cell-cycle regulator p14ARF. Alteration in p53 activity contributes to an aggressive chemotherapy- or radiotherapy-resistant phenotype, and consequently, p53 is a target of research aimed at the development of novel anticancer treatments. Among the chief functions of p53 are DNA damage-induced checkpoints in G1 and G2 phases of the cell cycle (5). The mechanisms by which p53 regulates the G2/M transition involve regulation of Cdk1 and transcriptional regulation of Cdc25C, 14-3-3, cyclin B1, and Cdk1 genes. Moreover, p53 has been implicated as an upstream regulator of BubR1 (6) and Mad1 (7). Blastocysts with simultaneous deletion of Mad2 and p53 show accelerated progression through mitosis (8). Mad1, Mad2, and BubR1 are components of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. It has also been shown that Plk1 associates with the DNA-binding domain of p53 (9) and that Plk1 depletion in systems with p53 loss of function (LOF) results in cell death (10). However, the role of p53 in Plk1 inactivation...
remains to be clarified (11). Plk1 is a serine/threonine kinase with an indispensable set of functions regulating mitosis: centrosome maturation, bipolar spindle formation, activation of the Cdk1/cyclin B1 complex, and execution of cytokinesis.

Vorinostat (Zolinza, suberoylanilide hydroxamic acid, or SAHA; Fig. 1A) is an inhibitor of histone deacetylases (HDAC). The antitumor activity of vorinostat occurs through induction of differentiation, growth arrest, or apoptosis, as documented in a broad range of cancer cell lines. Antitumor activity has also been shown in a number of in vivo models. Vorinostat received Food and Drug Administration approval in 2006 for the treatment of advanced cutaneous T-cell lymphoma.

Flavopiridol (NSC 649890; Fig. 1B) is a novel cyclin-dependent kinase inhibitor (12) that has exhibited potent growth-inhibitory activity against a number of human tumor cell lines, both in vitro and in xenografts. Flavopiridol as a single agent has produced 40% durable partial responses in patients with refractory chronic lymphocytic leukemia in a phase I trial given as a 30-minute loading dose followed by 4 hours of infusion weekly for 4 weeks (13, 14).

Flavopiridol and HDAC inhibitors have been shown to synergize in leukemia, breast, lung, and esophageal cancer, and pleural mesothelioma models (15–20). Synergistic cell kill has been attributed to flavopiridol-mediated transcriptional repression of the antiapoptotic genes p21,
Mcl-1, XIAP, and NF-xB. We tested the antitumor activity of the vorinostat and flavopiridol combination in representative multidrug-resistant neuroblastoma cell lines with mutant (mt) or wild-type (wt) TP53.

Materials and Methods

Cell lines

We used 11 neuroblastoma cell lines: CHLA-15, CHLA-20, SK-N-BE(1), SK-N-BE(2), SK-N-RA, LA-N-6, CHLA-136, CHLA-79, CHLA-119, CHLA-90, and CHLA-172. The neuroblastoma origin of these cell lines has been confirmed previously (21).

SK-N-RA cells were maintained in complete medium consisting of RPMI (Mediatech, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Omega Scientific). All other cell lines were cultured in complete medium consisting of Iscove’s modified Dulbecco’s medium (Bio Whittaker), supplemented with 3 mmol/L of l-glutamine (Gemini Bioproducts, Inc.); 5 μg/mL of insulin, 5 μg/mL of transferrin, and 5 ng/mL of selenious acid (ITS Culture Supplement; Collaborative Biomedical Products); and 20% heat-inactivated FBS. All neuroblastoma cell lines were used in this study before passage 40. The cell lines were cultured without antibiotics in a humidified incubator (95% air–5% CO2) at 37°C. All cell lines tested negative for mycoplasma. Cell lines were not selected for drug resistance in vitro. The identities of all cell lines were confirmed by the short tandem repeat (STR) assay. Unique STRs were identified for all cell lines, except between CHLA-15 and CHLA-20 and between SK-N-BE(1) and SK-N-BE(2), and pairs of cell lines that were derived from the same patient and had identical STRs, as expected.

Drugs, chemicals, antibodies, and plasmids

Vorinostat and flavopiridol were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute. Fluorescein diacetate was purchased from Eastman Kodak Company, and eosin Y was from Sigma Chemical Co. Antibodies were purchased from various manufacturers: anti-p53 (DO7) from BDPramingen; anti-Plk1 (208G4), caspase-9 mouse mAb, and caspase-3 rabbit mAb (8G10) from Cell Signaling Technology; anti-p21 (C-19) and anti-Mad2 (sc-28261) from Santa Cruz Biotechnology, Inc.; anti-TP53 (DO7) and anti-53 (DO7) from BDPharmingen; anti-Plk1 (208G4), caspase-9 mouse mAb, and caspase-3 rabbit mAb (8G10) from Cell Signaling Technology; anti-p21 (C-19) and anti-Mad2 (sc-28261) from Santa Cruz Biotechnology, Inc.; anti-TP53 (DO7) and anti-53 (DO7) from BDPharmingen; and horse radish peroxidase–conjugated secondary antibodies from Santa Cruz Biotechnology, Inc. Plasmids containing a mutant p53 R175H or R273H gene were a kind gift from Dr. B. Vogelstein (The Johns Hopkins Medical Institute and Howard Hughes Medical Institute).

Cytotoxicity assay

We determined the cytotoxicity of vorinostat and flavopiridol alone or in combination, using DMSSCAN, a semiautomated fluorescence-based digital image microscopy system that quantifies viable cells in tissue culture multiwell plates on the basis of their selective accumulation of fluorescein diacetate. DMSSCAN is capable of measuring cytotoxicity over a 4-log dynamic range by quantifying total fluorescence per well, which is proportional to the number of viable, clonogenic cells after eliminating background fluorescence with digital thresholding and eosin Y quenching.

To conduct formal quantitative analysis of the interaction between vorinostat and flavopiridol, we employed a fixed-ratio analysis for combination cytotoxicity assays; drugs were tested on a linear scale at concentration ranges that included clinically achievable blood plasma levels. A fixed ratio of 5:1 was chosen after selecting the maximally tested drug concentrations of vorinostat and flavopiridol. Cells were exposed to 0 to 2 μmol/L of vorinostat because it was reported to be in the clinically achievable range when administered orally (22). The mean steady-state concentrations of flavopiridol attainable in patients and inhibitory to Cdk4 were tested (0–0.4 μmol/L) in our experiments.

Cell lines were seeded into 96-well plates in 150 μL of complete medium (3,000–5,000 cells per well) and incubated overnight. Vorinostat (at various concentrations) in 100 μL of complete medium was added to each well (12 replicate wells for each concentration of vorinostat). Flavopiridol (0–0.4 μmol/L) was added to individual wells 24 hours later. Each drug concentration was tested in 12 replicate wells. Cell lines were incubated in the presence of flavopiridol for 5 days, after which fluorescein diacetate in 50 μL of 0.5% eosin Y (final concentration of fluorescein diacetate 10 μg/mL) was added to each well and cells were incubated for an additional 25 minutes at 37°C. Total fluorescence was then measured using the DMSSCAN system and results were expressed as surviving fractions of treated cells compared with control cells.

Transfections

pR175H and pR273H contain 1.8 kilobases of full-length p53 cDNA cloned into pCMV-Neo-Bam under transcriptional control of the immediate early enhancer/promoter of human cytomegalovirus. pR175H and pR273H encode mutant forms of p53 and differ from wt TP53 by a single point mutation at codons 175 and 273, respectively. CHLA-15 (passage 22) and CHLA-20 (passage 23) cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with 4 μg of DNA from the control pCMV-Neo-Bam vector or the pR175H and pR273H vectors. After selection in serum-free IMDM with 300 μg/mL of G418 (Gemini Bio-Products), mass cultures were established and expanded in selective medium.

Gene knockdown by siRNA

We used Lipofectamine 2000 reagent (Invitrogen) to transfect cells with small interfering RNAs (siRNA) targeted against Plk1 (Qiagen; cat. no. SI0222837), Mad2 (Santa Cruz Biotechnology, Inc. Cat. #sc-35837), and cyclin B1 (Thermo Scientific) genes. For cyclin B1...
silencing, the mixture of 2 target sequences was used (1:1 ratio): CAACAUUACCUGUCAUAUA and UGCACUA-
GUUCAAGAUUU. Control cells were transfected with an
siRNA containing a scrambled sequence (Qiagen). For
Mad2 and cyclin B1 or Plk1 cotransfection experiments,
5 × 10⁵ CHLA-90 cells were seeded into 6-well plates; gene
silencing of transfected cells was confirmed by immuno-
 blotting of cell lysate assayed at 48 hours and clonogeni-
city was assayed at 72 hours, using the DIMSCAN assay.

Cell-cycle analysis by flow cytometry
Prior to drug exposures, cells were synchronized in
PBS-free medium for 24 hours. Flow cytometry was done
on a BD LSR II flow cytometer (BD Biosciences) and data
were acquired and analyzed using BD FACSDiva soft-
ware. Cell-cycle phases were quantified using MultiCycle
software (Phoenix Flow Systems).

Apoptosis
The APO-DIRECT kit (cat. no. 556381, BD Pharmingen)
was used to identify DNA fragmentation by 2-color flow
cytometry using propidium iodide and FITC-dUTP
added to DNA fragments by terminal deoxynucleotidyl-
transferase (TdT). Staining with the APO-DIRECT kit was
done according to the manufacturer’s instructions. The
percentage of cells showing TdT-mediated fluorescent
staining was determined using band pass filters of 525 ±
25 nm for FITC and 610 ± 10 nm for propidium iodide.

The pan-caspase inhibitor BOC-d-fmk was used to block
the apoptotic component of cell death in CHLA-
90 cells. Cells were preincubated with 40 μmol/L of BOC-
d-fmk 2 hours prior to the addition of 2 μmol/L of vorinostat,
0.2 μmol/L of flavopiridol, or their combination, and an additional 40 μmol/L was added 24 hours
later.

Immunoblotting
Twenty micrograms of total protein per lane was frac-
tionated on 4% to 20% Tris-glycine gels (Invitrogen),
transferred to PVDF filter paper (Invitrogen), and probed
with primary and then HRP-labeled secondary antibo-
dies. Proteins were visualized using SuperSignal West
Pico Chemiluminescent Substrate (Thermo Scientific).

Immunofluorescence
A total of 10,000 CHLA-90 cells were seeded on cham-
er slides and treated with 2 μmol/L of vorinostat
(36 hours), 0.2 μmol/L of flavopiridol (12 hours), or their
combination. Cells were then fixed in 3.65% formaldehyde
in PBS for 20 minutes, permeabilized with 0.25% Triton-X,
and blocked with 3% bovine serum albumin in PBS for
1 hour at room temperature. Next, coverslips were
labeled with 5 μg/mL of anti-tubulin and 0.1 μg/mL
of 4',6-diamino-2-phenylindole (DAP; Invitrogen) for
1 hour at room temperature, washed in PBS, and air-
dried. Coverslips were mounted with Prolong® Gold
antifade reagent (Invitrogen). Images were visualized
using the Axiovert 200 microscope (Carl Zeiss Micro-
maging, Inc.) equipped with 100× Zeiss Plan-Apochro-
mat objective lens and Hamamatsu C4742-80 camera.
IPlab 4.0.8 (BD Biosciences Bioimaging) software was
used for image acquisition, processing, measurement,
and analysis.

Statistical analysis
LC₉₀ (i.e., the drug concentration that was lethal for
90% of the cell population) values were calculated using a
previously described algorithm.

One-way ANOVA was used to investigate the differ-
ence in cytotoxicity of each drug or drug combinations
(flavopiridol, vorinostat, vorinostat + flavopiridol) in
neuroblastoma cell lines with wt TP53 or p₅₃ LOF.
For each drug or drug combinations, the mean number
of logs of cell kill was calculated and then compared
between cell lines with p₅₃ LOF and cell lines with
wt TP53. Analysis was conducted at the 2 highest doses
tested in the experiments. Analyses were done using SAS,
Version 9.1, software (SAS Institute, Inc.).

Dose–response curves of the vorinostat + flavopiridol
combination for parental cells with wt TP53 versus clones
transfected with mt p₅₃ were compared. The difference in
the cytotoxic effect was examined by comparing the area
between the dose–response curve and the 100% survival
line. Statistical analysis assumed that the difference
between the untreated controls and the highest drug
concentration was 1. The fixed ratio design of the cyto-
toxicity experiments allowed the concentration incre-
ments to be quantitated at 1/8, 1/4, 1/2, and 1. The
area between the survival curves for each treatment
group and the 100% survival line was viewed as the
sum of areas of 4 trapezoids, computed as follows:

\[ \text{Area} = \frac{1}{16} \times (\bar{Y}_0 + \bar{Y}_1) + \frac{1}{8} \times (\bar{Y}_1 + \bar{Y}_2) + \frac{1}{8} \times (\bar{Y}_2 + \bar{Y}_3) + \frac{1}{2} \times (\bar{Y}_3 + \bar{Y}_4) \]

\[ = \frac{1}{16} \times (\bar{Y}_0 + \bar{Y}_1 + 3\times\bar{Y}_1 + 3\times\bar{Y}_2 + 3\times\bar{Y}_3 + \frac{1}{4}\times\bar{Y}_4) \]

where \( \bar{Y}_0, \bar{Y}_1, \bar{Y}_2, \bar{Y}_3, \bar{Y}_4 \) were the mean numbers of
logs of cell kill at the 0th, 1st, 2nd, 3rd, and 4th
drug conditions. Finally, the contrast was computed
as follows:

\[ \frac{1}{16} \times (\bar{Y}_{0B} - \bar{Y}_{0A}) + \frac{1}{8} \times (\bar{Y}_{1B} - \bar{Y}_{1A}) + \frac{3}{16} \times (\bar{Y}_{2B} - \bar{Y}_{2A}) +
\frac{3}{8} \times (\bar{Y}_{3B} - \bar{Y}_{3A}) + \frac{1}{4} \times (\bar{Y}_{4A} - \bar{Y}_{4B}) \]

and tested the difference between the 2 dose–response
curves, where \( \bar{Y}_{0A}, \bar{Y}_{1A}, \bar{Y}_{2A}, \bar{Y}_{3A}, \bar{Y}_{4A} \) were the mean
number of logs of cell kill at each concentration
of the parental cells, and \( \bar{Y}_{0B}, \bar{Y}_{1B}, \bar{Y}_{2B}, \bar{Y}_{3B}, \bar{Y}_{4B} \) were the
mean number of logs of cell kill for cells transfected
with mt TP53. The significance was determined using
contrast analysis in ANOVA, with P values corrected by
the Bonferroni method. Data were analyzed with SAS, Version 9.1, software.

Poisson regression analysis was used to compare the difference in the number of abnormal mitoses between control and the vorinostat + flavopiridol treated groups as evaluated by confocal microscopy.

To compare difference in the fluorescence scores for cells expressing Mad2, cyclin B1, Plk1, Mad2 + cyclin B1, or Mad2 + Plk1 siRNAs, a multiple linear regression model was first fit using data from all the groups and then the difference in fluorescence scores between 2 different groups was evaluated by testing the appropriate linear combinations using the STATA LINCOM command. The fluorescence scores were transformed to the base 10 logarithmic scale before analysis was done.

Results

Cytotoxicity of the vorinostat and flavopiridol combination

Vorinostat and flavopiridol were tested sequentially at the fixed ratio 5:1 in 1 drug-sensitive [SK-N-BE(1)] and 8 multidrug-resistant cell lines [SK-N-RA, CHLA-79, CHLA-136, LA-N-6, SK-N-BE(2), CHLA-90, CHLA-119, and CHLA-172; Table 1, Fig. 1A; Supplementary Fig. 1]. The drug concentrations lethal for 90% or 99% of treated cells (LC90 and LC99) were calculated from dose-response curves, using the DIMSCAN assay, which provides a 4 log dynamic range. Synergy (CI ≤ 0.7), assayed at LC90 and LC99, was observed in 1 cell line with wt TP53 (CHLA-136) and in all 4 cell lines with mt TP53 [SK-N-BE(2), CHLA-90, CHLA-119, and CHLA-172]. We have previously shown these cell lines to be resistant to the commonly used chemotherapeutic drugs melphalan, carboplatin, and/or etoposide (21). Our panel included a pair of cell lines, one member of which was established from a patient at diagnosis [SK-N-BE(1)] and the other member established later from the same patient after disease progression following multiagent chemoradiothy [SK-N-BE(2); ref. 21]. Interestingly, combination of vorinostat + flavopiridol was more active in the SK-N-BE(2) cell line than in drug-sensitive SK-N-BE(1) cells (Supplementary Fig. 1; Table 1). In addition to sequential drug exposures, vorinostat and flavopiridol were administered simultaneously in CHLA-90 cells, but no synergy was observed with this timing schedule (data not shown).

We conducted one-way ANOVA to determine the difference in the cytotoxic effect of the vorinostat + flavopiridol combination in cell lines with wt TP53 versus mt TP53. The analysis was conducted for 2 drug concentrations: vorinostat (1 µmol/L) + flavopiridol (0.2 µmol/L) and vorinostat (2 µmol/L) + flavopiridol (0.4 µmol/L). Statistically significant cytotoxicity was confirmed in cell lines with wt or mt TP53 at the 2 highest tested concentrations (P < 0.0001). However, vorinostat + flavopiridol was significantly more cytotoxic in cell lines with mt TP53 than in cell lines with wt TP53 (P < 0.001).

Effect of p53 mutations on the activity of the vorinostat + flavopiridol combination

To examine whether the vorinostat + flavopiridol combination is predominantly active in neuroblastomas carrying mt p53, we transfected CHLA-15 and CHLA-20 cells with the pCMV-Neo-Bam empty vector as a control or with mt p53 (pR175H or pR273H) expression vectors, and 2 G418-resistant clones were selected from each cell line. The clones were designated as CHLA-15175A, CHLA-15175B, CHLA-20273C, and CHLA-20273D. Compared with other neuroblastoma cell lines, CHLA-15 and CHLA-20 clump to a lesser extent and thus were chosen for these experiments to yield higher transfection efficiency. The sensitivities of mt p53–transduced clones, parental cell lines, and empty vector-transduced controls to the vorinostat + flavopiridol combination were compared using the DIMSCAN assay (Fig. 1B, Table 1). Examining the activity of mt p53 in transfected clones with the p53-specific monoclonal antibody DO7, we detected increased basal levels of p53 protein in 3 of 4 clones transfected with mt p53 expression vector. In addition, reduced p53 activity was confirmed by the lack of induction of p21 in etoposide-challenged samples in clones CHLA-15175A and CHLA-20273D and modest induction of p21 in clones CHLA-15175A and CHLA-20273C by using immunoblotting (Fig. 1C). Transfection with p53 mutant expression vectors sensitized both cell lines to the vorinostat + flavopiridol combination (Fig. 1B). LC90 values of CHLA-15 clones (CHLA-15175A and CHLA-15175B) were 1.6 to 3 times lower relative to empty vector controls (designated as CHLA-15EV). Similarly, LC90 values of the CHLA-20 clones were greater than 2 times lower relative to CHLA-20EV (Table 1).

Contrast analysis of variance, corrected with the Bonferroni method, showed statistically significant sensitivity (P < 0.001) for clones expressing mt p53 relative to empty vector controls in response to the tested combination.

Induction of apoptosis by the vorinostat + flavopiridol combination

Addressing whether apoptosis has a role in cell death induced by the combination, we detected increased apoptosis by the TUNEL assay in mt TP53 CHLA-90 cells (Fig. 2A). Flavopiridol and especially the vorinostat + flavopiridol combination increased apoptosis, which was partially reversed by pretreatment with the pancaspase inhibitor BOC-d-fmk (Fig. 2A). In addition, activation of caspase-3 and -9 was measured by immunoblotting in 2 cell lines with wt TP53 (CHLA-136 and CHLA-15) as well as 2 cell lines with mt TP53 (CHLA-90 and CHLA-172; Fig. 2B; Supplementary Fig. 2). Activation of caspase-3 and -9 was sustained throughout exposure to the combination in CHLA-136 and CHLA-15.
cell lines. In CHLA-90 and CHLA-172 cell lines, caspase-3 and -9 activation was detectable for 12 hours. Collectively, our data suggest that this drug combination induces cell death that may be both caspase dependent and caspase independent.

**Effect of vorinostat + flavopiridol on cell-cycle distribution**

Various HDAC inhibitors and flavopiridol have been shown to modulate cell-cycle progression. HDAC inhibitors of various classes induce G1 and/or G2/M arrest, or premature sister chromatid separation (24), through alterations in BubR1 (25), p55Cdc/Cdc20 (26), p21, cyclins D1, E, and A, cdc25A (27), and other targets. Treatment with flavopiridol in mitosis can result in premature mitotic exit (28). As predominant cytotoxicity in our cell lines was seen in the presence of mt TP53, and because p53 controls G1 and G2 checkpoints, we measured cell cycle in CHLA-90 (mt TP53) and SK-N-RA (wt TP53) lines treated with the drug combination at regular time intervals for 24 hours (Fig. 3). Cells were pretreated with or without 2 μmol/L of vorinostat and exposed to 0.2 μmol/L of flavopiridol. Accumulation in G2 was prominent in wt TP53 SK-N-RA cells while mt TP53 CHLA-90 cells slipped into sub-G0/G1 after treatment with the combination (Fig. 3). This observation suggested that cell death mediated by the tested combination may occur through failed mitosis in neuroblastoma lines with mt TP53, due to abrogation of the G2/M checkpoint in mutant cells.

**Effect of vorinostat + flavopiridol on mitosis**

To confirm our hypothesis that the combination mediates cell death of mt TP53 neuroblastomas through failed mitosis, CHLA-90 cells were examined by immunofluorescence to visualize α-tubulin and DNA for abnormal spindle formation and DNA condensation (Fig. 4A). Confocal microscopy of the stained cells showed that, while DMSO-treated cells go through various phases of mitosis without any abnorm-

<table>
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<tr>
<th>Cell lines</th>
<th>LC90, μmol/L</th>
<th>CI IC90</th>
<th>LC99, μmol/L</th>
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<tr>
<td></td>
<td>V</td>
<td>FL</td>
<td>V + FL</td>
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<td><strong>Wild-type TP53</strong></td>
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<td>SK-N-BE(1)</td>
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<td>0.2</td>
<td>0.7 ± 0.1</td>
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<td>&gt;2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;0.4</td>
<td>&gt;2.0 + &gt;0.4</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CHLA-79</td>
<td>&gt;2.0</td>
<td>0.4</td>
<td>1.4 ± 0.3</td>
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<tr>
<td>CHLA-136</td>
<td>&gt;2.0</td>
<td>0.4</td>
<td>1.3 ± 0.3</td>
<td>0.7 ± 0.2</td>
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<td><strong>Mutant TP53</strong></td>
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<td>SK-N-BE(2)</td>
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<td>1.1 ± 0.2</td>
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<td>CHLA-119</td>
<td>&gt;2.0</td>
<td>0.3</td>
<td>0.6 ± 0.1</td>
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<td>CHLA-90</td>
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<td>0.8 ± 0.2</td>
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<td>CHLA-15&lt;sub&gt;EV&lt;/sub&gt;</td>
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Abbreviations: V, vorinostat; FL, flavopiridol; combination, V + FL.
<sup>a</sup> If 90% or 99% cytotoxicity was not achieved at the tested concentrations, then LC90 or LC99 values were reported as "greater than the highest tested concentration". The highest concentrations tested were 2 μmol/L for vorinostat and 0.4 μmol/L for flavopiridol. Similarly, if 90% or 99% cytotoxicity was achieved at concentrations lower than tested, then LC90 or LC99 values were reported as less than the lowest tested concentration. The lowest concentrations tested were 0.025 μmol/L for vorinostat and 0.05 μmol/L for flavopiridol.
<sup>b</sup> The combination index (CI) correlates the cytotoxicity of drug combinations to the single-agent activities. Standard deviations for CI values are also included.
<sup>c</sup> CI was not determined (ND) if cytotoxicity was not achieved within the concentration range tested. Interpretation of CI values: 0.1–0.3, strong synergism; >0.3–0.7, synergism; >0.7–0.9, slight synergism; >0.9–1.0, additive effect; >1.1, antagonism.
ality, cells treated with the combination exhibited profound abnormalities in spindle formation and cytokinesis (Fig. 4B).

G2 checkpoint activation by the vorinostat + flavopiridol combination

To characterize activation of the combination-induced G2 checkpoint, we examined the time-kinetic expression of Cdk1, pCdk1 Thr14/Tyr15, and cyclin B1 by immunoblotting in 2 cell lines with mt TP53 (CHLA-136) or mt TP53 (CHLA-90). Drug effects were determined according to activation of caspase-3 to a cleaved 19-kDa form and caspase-9 to a cleaved 35 kDa form. Equal loading of protein was confirmed by β-actin expression. Cells were pretreated with 2 μmol/L of vorinostat (V) for 24 hours and then 0.2 μmol/L of flavopiridol (Fl) was added for additional 6, 9, 12, 16, 18, or 24 hours. Abbreviation: C, vehicle-treated controls.

We observed dramatic downregulation of cyclin B1 in response to flavopiridol and more so to the combination in cell lines with mt TP53 (in CHLA-90, 3-fold by flavopiridol and >7-fold by the combination; in CHLA-172, 3-fold with either flavopiridol or the combination), compared with those with wt TP53 (in CHLA-136 induction by flavopiridol, 1.3-fold downregulation by the combination; in SK-N-RA, 1.5-fold by flavopiridol and 1.8-fold by the combination).

The vorinostat + flavopiridol combination affects mitosis exit markers in mt TP53 neuroblastoma cell lines

We measured MPM2, Mad2, and Plk1 in protein lysates obtained from cells treated with vorinostat and/or flavopiridol at frequent time intervals by immunoblotting (Fig. 5B). These studies were carried out on 2 cell lines with mt TP53 (CHLA-90 and CHLA-172) and 2 cell lines with wt TP53 (CHLA-136 and SK-N-RA).
During normal mitosis, MPM2 staining is intense in prophase and diminishes during interphase; weakly positive MPM2 expression in interphase is associated with recognition of centriolar proteins (29). MPM2 expression is considered a marker of mitosis (30), and disappearance of MPM2 is commonly used to assay mitotic exit. In wt TP53 CHLA-136 and SK-N-RA cells, MPM2 expression was maintained throughout the exposure to vorinostat + flavopiridol combination. In contrast, mt TP53 CHLA-90 and CHLA-172 cells exhib-
Our data indicate that cells with mt TP53 are induced to exit mitosis when treated with the drug combination.

Depletion of G2/M proteins is lethal for a multidrug-resistant neuroblastoma cell line

To confirm that aberrant mitosis can be used as a therapeutic strategy against p53 LOF neuroblastomas, we examined the clonogenicity of CHLA-90 cells that were transfected with Mad2 siRNA in the absence or presence of cyclin B1 or Plk1 siRNA. CHLA-90 cells were chosen for this siRNA transfection experiment, as these cells grow in single suspension and accommodate higher transfection efficiency. Mad2 was chosen for cotransfection experiments with cyclin B1 and Plk1 due to selective downregulation of this protein by the drug combination in mt TP53 cell lines. Transient expression of Mad2, cyclin B1, or Plk1 siRNA produced robust depletion (70% for Mad2, 80% for cyclin B1, and 85% for Plk1) of target proteins as measured at 48 hours by immunoblotting, whereas mock siRNA did not affect levels of any of these proteins. Simultaneous reduction of Mad2 and cyclin B1 or Plk1 levels acted synergistically with significant (P < 0.001) growth inhibition/cell death of CHLA-90 cell line as measured by the DMSCAN assay at 72 hours (Fig. 5D). Our data indicate that the silencing of G2/M genes can sensitize multidrug-resistant neuroblastoma cells with p53 LOF.

Discussion

We have previously shown that relapsed neuroblastomas manifest resistance to multiple drugs that is sustained in vitro (21) and such drug resistance is often caused by loss of TP53 function (4). In addition to mutations, TP53 LOF in neuroblastomas has been ascribed to MDM2 amplification (4) or p14ARF inactivation (32).

TP53, due to its central role in the normal cellular stress response and frequent inactivation in tumors, is an appealing target for drug development. Several approaches are being pursued to target altered TP53: selective expression of replication-deficient viruses that can selectively propagate in p53-deficient cells (33), reconstitution of the wt TP53 gene (34), or restoration of the transactivation function to mt p53 protein with the use of small molecules (35). Alternatively, the defective checkpoint status of cells with inactive TP53 has been exploited with drugs that can inhibit Chk2, Plk1, aurora kinases, Wee1, and other proteins that regulate the G2/M checkpoint (36–38).

Our study was undertaken to determine the in vitro activity of vorinostat and flavopiridol in well-characterized, multidrug-resistant neuroblastoma cell lines (21). Our interest in vorinostat is derived from our previous observation that HDAC1 was highly expressed in multidrug-resistant neuroblastoma cell lines relative to drug-sensitive cell lines, and inhibition of HDAC1 with siRNA or a small molecule, depsipeptide, sensitized highly drug-resistant cell lines to commonly used...
Figure 5. Effect of vorinostat (V) + flavopiridol (Fl) on the expression of G2/M proteins. Representative neuroblastoma cell lines with wt TP53 (CHLA-136) and mt TP53 (CHLA-90) are shown. Cells were pretreated with 2 μmol/L of vorinostat for 24 hours and then 0.2 μmol/L of flavopiridol Fl was added for an additional 6, 9, 12, 16, 18, or 24 hours. A, immunoblot analysis of cyclin B1 (55 kDa), Cdk1 (34 kDa), and pCdk1 Thr15/Tyr15 (34 kDa) protein expression. Equal loading of protein was confirmed by β-actin expression. B, immunoblot analysis of MPM2 (84 kDa), Mad2 (24 kDa), and Plk1 (62 kDa) protein expression. Equal loading of protein was confirmed by β-actin expression. C, quantitative analysis of expression of cyclin B1, MPM2, Mad2, and Plk1 proteins as the ratio to β-actin in CHLA-136 (■) and CHLA-90 (*) cell lines treated with vorinostat + flavopiridol at 6, 9, 12, 16, 18, and 24 hours. Abbreviations: C, vehicle-treated controls; V, treatment with 2 μmol/L of vorinostat. D, CHLA-90 cells were transfected with 200 pmol/L total (100 pmol/L of target siRNA + 100 pmol/L of scrambled siRNA for single targets and 100 pmol/L of each in cotransfection experiments) of scrambled, Mad2, cyclin B1, and Plk1 siRNAs. Forty eight hours later, transfection efficacy was confirmed by immunoblotting (data not shown), and 72 hours later cell clonogenicity was determined using the DIMSCAN assay. The DIMSCAN assay was done in 3 separate experiments. Lanes: 1, control; 2, scrambled siRNA; 3, Mad2 siRNA; 4, cyclin B1; 5, co-expression of Mad2 and cyclin B1 siRNAs; 6, Plk1 siRNA; and 7, co-expression of Mad2 + Plk1 siRNA.
chemotherapeutic agents (39). Vorinostat as a single agent showed limited activity in pediatric in vitro and in vivo models (40); however, due to its broad range of mechanisms of action, vorinostat remains the drug of interest for development with other drugs in combinations and evaluation in children is ongoing (www.clinicaltrials.gov); most recently, a phase I trial of vorinostat and 13-cis-retinoic acid was completed (41). In pediatric patients, phase I clinical trial of flavopiridol has not shown objective responses (42), signifying the importance of identifying active combinations for this drug. Both vorinostat and flavopiridol possess distinct mechanisms of action from traditional chemotherapeutic drugs; consequently, they may prove effective against neuroblastomas that acquired resistance during treatment.

Both vorinostat and flavopiridol are known to have G2/M inhibitory effects and were combined to induce cell death through cell-cycle perturbation in multidrug-resistant cell lines with mt TP53 or wt TP53. In our study, greater cytotoxicity was observed in cell lines with mt TP53 (SK-N-BE(2), CHLA-90, CHLA-119, and CHLA-172), or p14ARF deletion (LA-N-6; ref. 43) than in those with functional p53/wt TP53. We confirmed the requirement for p53 LOF in the activity of the vorinostat + flavopiridol combination by showing enhanced cytotoxicity in clones of wt TP53 cells (CHLA-15 and CHLA-20) stably expressing mt TP53. Moreover, the multidrug-resistant SK-N-BE(2) cell line with mt TP53 was more susceptible to the combination than the exquisitely drug sensitive SK-N-BE(1) line. SK-N-BE(1) and SK-N-BE(2) represent a pair of cell lines, one member of which was established from a patient at diagnosis [SK-N-BE(1)] and carries wt TP53 and another member was established from the same patient after disease progression following multiagent chemoradiotherapy [SK-N-BE(2); ref. 21].

Previous studies of the vorinostat + flavopiridol combination, conducted in breast cancer and leukemia cells of myeloid and/or lymphoid lineage, have shown that the combination can induce apoptosis through marked increase in mitochondrial damage (44) or downregulation of XIAP and Mcl-1 (18, 20). We focused on examining the impact of G2/M perturbation and associated cellular targets as the mechanism of synergy in highly drug-resistant neuroblastoma cell lines that often fail to undergo apoptosis in response to standard and investigational drugs. We showed differential cell-cycle progression for cell lines with wt TP53 (SK-N-RA) or mt TP53 (CHLA-90) when treated with vorinostat + flavopiridol. SK-N-RA cells arrested in G2 phase, whereas CHLA-90 cells failed to arrest following genomic insult and instead proceeded to aberrant mitosis (Fig. 3). Respectively, cell lines with wt TP53 and mt TP53 displayed differential expression of cyclin B1, Mad2, and MPN2 proteins following vorinostat + flavopiridol treatments (Fig. 5A and B; Supplementary Fig. 3) relative to wt TP53 cells.

Mad2 is central to the spindle assembly checkpoint, a cellular mechanism that monitors the accuracy of chromosome segregation. Severely reduced levels of Mad2 provokes extensive cell death due to the suppression of mitotic checkpoint signaling (45, 46), a phenomenon more pronounced in models lacking TP53 (8). However, transient downregulation of Mad2 through the use of Mad2 siRNA did not affect clonogenic survival of mt TP53 neuroblastoma cells (CHLA-90; Fig. 5D). We coexpressed Mad2 with targets also downregulated by the vorinostat + flavopiridol combination: cyclin B1 or Plk1 siRNA. The cyclin B/cdk1 complex is generally thought to prevent activation of the anaphase-promoting complex (APC), whose activity begins to rise during anaphase and thereby initiates mitotic exit (47), although premature degradation of cyclin B can promote early APC activation (45). Coexpression of Mad2 siRNA with cyclin B1 siRNA resulted in significant (P < 0.001) reduction in clonogenic survival relative to single target knockdown (Fig. 5D). Similar results were obtained by combined reduction of Mad2 and Plk1 levels (Fig. 5D).

Plk1 is a member of the polo-like kinases (Plk) family of serine/threonine kinases with fundamental functions in cell-cycle progression such as activation of Cdk1, chromosome segregation, centrosome maturation, bipolar spindle formation, regulation of APC, and execution of cytokinesis. Several investigations using p53-deficient cells have shown that Plk1 depletion was sufficient to induce apoptosis or DNA damage, whereas cells with intact TP53 were less sensitive to Plk1 depletion (10). More recently, the Plk1 inhibitor, BI-2536, was shown to be selectively effective against colorectal cancer cells without wt TP53, both in vitro and in vivo (48). In our studies, the combination of vorinostat + flavopiridol caused Plk1 depletion in all examined cell lines irrespective of their TP53 status. However, transient expression of Plk1 siRNA in neuroblastoma cell lines was predominantly lethal to cells with wt TP53 (CHLA-90) compared with wt TP53 (CHLA-136; data not shown); thus, our data are consistent with the findings of other investigators and affirm that Plk1 can be considered as a therapeutic target in neuroblastomas with p53 LOF.

A recently completed phase I pharmacokinetic study of pulse-dose vorinostat and split-dose flavopiridol did not show objective responses in adults with advanced solid tumors (49). Nonetheless, it was hypothesized that the combination has the potential to be highly active; it is conceivable that, as we have found in neuroblastomas, the vorinostat + flavopiridol combination can be selectively active in tumors with defective TP53 and the lack of objective responses in the study may partly be attributable to the selection of the patient population.

Our data show that the vorinostat + flavopiridol combination is predominantly cytotoxic to neuroblastomas with p53 LOF via TP53 mutations or p14ARF deletion and that such cytotoxicity is mediated through aberrant
**Disclosure of Potential Conflicts of Interest**

No financial or personal relationships to disclose.

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**References**


Combination of Vorinostat and Flavopiridol Is Selectively Cytotoxic to Multidrug-Resistant Neuroblastoma Cell Lines with Mutant $TP53$

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