Combining Doublecortin-Like Kinase Silencing and Vinca Alkaloids Results in a Synergistic Apoptotic Effect in Neuroblastoma Cells

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ABSTRACT

Microtubule-destabilizing agents, such as vinca alkaloids (VAs), are part of the treatment currently applied in patients with high-risk neuroblastoma (NB). However, the development of drug resistance and toxicity make NB difficult to treat with these drugs. In this study we explore the combination of VAs and doublecortin-like kinase (DCLK) gene by using short interference RNA (siRNA). We examined the effect of VAs and DCLK knockdown on the microtubule network by immunohistochemistry. We performed dose-response studies on cell viability and proliferation. By combining VA with DCLK knockdown we observed a strong reduction in the EC_{50} to induce cell death: up to 7.3-fold reduction of vincristine and 21.1-fold reduction of vinblastine.

Using time-lapse imaging of phosphatidylserine translocation and a terminal deoxynucleotidyl transferase dUTP nick-end labeling-based assay, we found a significant increase of apoptosis by the combined treatment. Induction of caspase-3 activity, as detected via cleavage of N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin, showed a 3.3- to 12.0-fold increase in the combined treatment. We detected significant increases in caspase-8 activity as well. Moreover, the multidrug dose effect calculated by using the median effect method showed a strong synergistic inhibition of proliferation and induction of apoptosis at most of the combined concentrations of siRNAs and VAs. Together, our data demonstrate that the silencing of DCLK sensitizes NB cells to VAs, resulting in a synergetic apoptotic effect.

Introduction

Neuroblastoma (NB) is the most common extracranial solid neoplasm in children under 5 years of age. Despite available therapies, patients with the high-risk disease present an overall survival rate lower than 50% (Maris et al., 2007). Chemotherapeutic drugs used for high-risk NB therapy include platinum compounds, alkylating agents, topoisomerase II inhibitor, anthracycline antibiotics, and vinca alkaloids (VAs), among others (George et al., 2010). VAs, such as vincristine (VCR) and vinblastine (VBL), are antimitotic drugs that disrupt microtubule stability and induce cell death (Jordan et al., 1991). VAs bind to important components of the cytoskeleton, such as β-tubulin subunits of...
α/β-tubulin heterodimers (Don et al., 2004). At micromolar concentrations, VAs depolymerize microtubules, disrupt mitotic spindles, and induce formation of tubulin paracrystals, and G2/M block occurs (Jordan et al., 1992; Pourroy et al., 2004). However, at nanomolar concentrations, microtubule dynamics are suppressed with no effect on microtubule depolymerization, with a postmitotic G2 arrest identified (Pourroy et al., 2004). Treatment with both micromolar and nanomolar concentrations lead to apoptosis. The mechanism by which VAs induce apoptosis is complex, involving protein kinase signaling pathways (Fan et al., 2001). Mitochondria also seem to play a key role in this process (Groninger et al., 2002).

Despite the therapeutic efficacy of VAs, disadvantages have been associated with the use of these drugs in NB therapy in humans. VAs lead to toxicity [e.g., neurotoxicity (greater for VCR), bone marrow suppression (greater for VBL), and vascular complication] (Doll et al., 1986; Lobert et al., 1998) and development of resistance (Don et al., 2004). The development of resistance toward VAs might be caused by the altered expression of the neuronal-associated microtubule protein class III β-tubulin (Don et al., 2004). The increased expression of P-glycoprotein, a multidrug resistance protein, has also been detected in NB cells treated with VAs and may be related to resistance to VA treatment as well (Burkhart et al., 2001). To overcome these disadvantages, efforts have been made to find more specific therapies, and the combination of low doses of VAs with other compounds, such as rapamycin, is also under investigation (Bostrom et al., 2001). Marimpietri et al. (2007).

Several molecular targets have been proposed for NB therapy (Wagner and Danks, 2009; George et al., 2010). These targets play crucial roles in at least one of the tumorigenic processes, such as proliferation, angiogenesis, invasion, and/or metastasis. We have recently proposed doublecortin-like kinase (DCLK) as a novel molecular target for NB therapy (Verissimo et al., 2010). The DCLK gene encodes for several proteins, including the microtubule-associated proteins (MAPs) DCL and DCLK-long (Vreugdenhil et al., 2007). These MAPs are highly expressed in neuroblasts (Shu et al., 2006; Vreugdenhil et al., 2007) and NB tumors (Verissimo et al., 2010). They are known to regulate neurogenesis, neural migration, and transport along microtubules (Koizumi et al., 2006; Shu et al., 2006; Vreugdenhil et al., 2007; Fitzsimons et al., 2008). We and others have shown that DCLK-derived MAPs are involved in microtubule stabilization and the regulation of mitotic spindle formation (Shu et al., 2006; Vreugdenhil et al., 2007). It has been proposed that the mechanism of stabilization of microtubules by DCLK-derived MAPs is similar to the highly homologous MAP doublecortin (DCX) (Shu et al., 2006). MAPs of the DCX family have homologous microtubule-binding domains (doublecortin domains) that are involved in tubulin polymerization and microtubule bundling (Cierpicki et al., 2006). The interaction of doublecortin domains with microtubules is regulated by phosphorylation and dephosphorylation (LoTurco, 2004).

We and others have shown the essential role of DCLK in the proliferation and survival of neuroblast cells. Knockdown of DCL/DCLK-long leads to mitotic spindle disruption and cell cycle arrest (Shu et al., 2006; Vreugdenhil et al., 2007). Moreover, we recently demonstrated that silencing of DCLK-derived MAPs induces apoptosis in NB cells (Verissimo et al., 2010). Gene expression profiling revealed that genes related to oxidative stress and oxidative phosphorylation were differentially affected after knockdown of MAPs encoded by the DCLK gene, and mitochondria were the most affected cellular components (Verissimo et al., 2010).

Because both silencing of DCLK and treatment with VAs result in the disruption of mitotic spindles and induction of apoptosis in NB cells, we hypothesized that they may synergize each other’s effects. Our results indeed show that there is a synergetic effect resulting in a significantly lower dose of VAs required for the induction of caspase-dependent apoptosis in NB cells.

**Materials and Methods**

**Reagents and Antibodies.** VCR and VBL were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). VBL and VCR were dissolved in ultra-purified water (Milli-Q; Millipore, Amsterdam, The Netherlands) to 10 mg/ml stock and stored at 4 or –20°C, respectively. At the time of use, VCR and VBL were freshly diluted in culture medium. The mouse monoclonal antibody against α-tubulin was obtained from Sigma-Aldrich Chemie B.V.; the secondary antibody anti-mouse-horseradish peroxidase was from Tubbio B.V. (Bevelandseweg, The Netherlands), the goat anti-mouse IgG conjugated to Alexa Fluor 594 dye was purchased from Invitrogen (Leiden, The Netherlands), and the anti-rabbit-horseradish peroxidase was from Santa Cruz Biotechnology (Tebu-Bio, Heerhugowaard, The Netherlands). We also used a recently developed primary rabbit antibody targeting the QRDLRRPLSSDDLSVG-C sequence, which is specifically present in DCL (D. J. Saaltink, B. Havik, P. Luccassen, C. S. Verissimo, and E. Vreugdenhil, unpublished work). The 3′-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemie B.V. MTT (5 mg/ml) in phosphate-buffered saline was freshly prepared before each determination. Annexin V (Axx-V)-Alexa 488 was prepared as described previously (Puigvert et al., 2010). The caspase inhibitor N-benzoyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-fmk) was purchased from Bachem CH (Weil am Rhein, Germany); the caspase substrate Ac-DEVD-7-amido-4-methylcoumarin (AMC) was obtained from Enzo Life Science (Raamsdonksveer, The Netherlands); and AMC was from Invitrogen.

**Cell Culture, Transfection, and Drug Treatment.** Human SH-SY5Y and mouse N1E-115 neuroblastoma cells were cultured as described previously (Vreugdenhil et al., 2007; Molemaar et al., 2008). For microscopy assays, both cell lines were grown at 70% confluence in 24-well plates with glass bottoms or 96-well clear black plates (Greiner Bio-One B.V.; Alphen aan den Rijn, The Netherlands) coated with 100 ng/ml poly-L-lysine for N1E-115 or 100 μg/ml poly-D-lysine for SH-SY5Y cells. SH-SY5Y and N1E-115 cells were transfected with specific siRNAs against DCLK-derived MAPs as described previously (Vreugdenhil et al., 2007; Verissimo et al., 2010). N1E-115 cells were transfected with synthetic RNA oligonucleotides siDCL-2 (CAAGAAGAGGCUCVUCCUTT-sense and 5′-GGAGUGAGGCGCUUCUUUGT-antisense) or siDCL-3 (GAAGCGAAGGGUGGAT-sense and TCGAACCUUCUUCCG- UUUCTT-antisense) obtained from Eurogentec Nederland B.V. (Leiden, The Netherlands) (Vreugdenhil et al., 2007). SH-SY5Y neuroblastoma cells were transfected with siDCLK-4 (GGCGAGGGACCUAACCTT-sense and GGUGAACUGCUGGAGGGTTT-antisense) or siDCLK-5 (UGGAGAACAGAUGAACUGTT-sense and CAUUCUGGUGCUCUCCATT-antisense) siRNAs purchased from Qiagen (Venlo, The Netherlands) (Verissimo et al., 2010). Here, when we mention DCLK knockdown, we are referring to silencing of DCLK-derived MAPs. SH-SY5Y cells were transfected with 50, 100, 200, or 400 nM siDCLK-4 or siDCLK-5 siRNAs (Verissimo et al., 2010) and NIE-115 cells were transfected with 12.5, 25, 50, or 100 nM siDCL-2 or siDCL-3 siRNAs (Vreugdenhil et al., 2007). Transfection was performed.
by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. As negative control (NC) we used the AllStars Negative Control siRNA from QIAGEN Benelux B.V. (Venlo, The Netherlands). This NC siRNA is a commercial synthetic oligonucleotide that has no homology to any known mammalian gene, and several assays have been performed to ensure minimal nonspecific effects on gene expression and phenotype. A transfection efficiency of 95 ± 2% was obtained, which was determined by using a nontargeting siRNA conjugated to Alexa Fluor 488 (QIAGEN Benelux B.V.). Cells were treated with different concentrations (0.01, 0.1, 1, 10, or 100 μM) of VCR or VBL.

Quantitative Real-Time PCR. Quantitative real-time PCR was performed by using ABI Prism 7900HT Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s specifications. The RNA concentration and purity were determined by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. As negative control we used the N1E-115 NB cell line that expresses the shRNA with the sequence 5’-ACAAGAAGCGAGACGACC-3‘, and the shDCL-3 cell line expresses the shRNA with the sequence 5’-ACACAGAAGCGAGACGACC-3‘. As a negative control cell line we used the N1E-115 NB cell line that expresses DCLK-derived MAP DCL. The sequence of the shRNA expressed in the shDCL-2 cell line is 5’-ACACAGAAGCGAGACGACC-3‘. The RNA concentration and purity were determined by using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE).

Time-Lapse Imaging of Phosphatidylserine Translocation. N1E-115 cells were transfected with different concentrations of siDCL-3 or NC siRNAs (12.5, 25, 50, and 100 nM) in 96-well clear black plates (Greiner Bio-One B.V.). Forty eight hours later, different doses of VCR or VBL (0.01, 0.1, 1, 10, and 100 μM) were added to the cells. Subsequently, annexin V conjugated to Alexa Fluor 488 was added, and time-lapse imaging of phosphatidylserine translocation was performed by using a BD Pathway 855 imager (BD Biosciences, San Jose, CA) under controlled conditions (37°C, 5% CO2). Quantitative image analysis was performed as described previously (Puigvert et al., 2010). The total fluorescent intensity per image and the total cell area were quantified by using Image Pro (Media Cybernetics, Inc., Bethesda, MD).

TUNEL-Based Assay. N1E-115 cells were grown on coverslips and transfected with different doses of siDCL-3 (12.5, 25, 50, and 100 nM) for 48 h to induce DCLK KD, or they were transfected with 100 nM NC siRNA. Subsequently, different doses of VAs (0.01, 0.1, 1, and 10 μM) were added to the cells, and, 15 h later, the cells were fixed with 4% paraformaldehyde (Added Pharma B.V., Oss, The Netherlands). Apoptotic cells with fragmented DNA were detected by using ApoTag in situ technology, a TUNEL-based assay (Millipore B.V., Amsterdam, The Netherlands) following the manufacturer’s instructions. Cells were counterstained with Hoechst and viewed by fluorescence microscopy. Five images per condition of two independent experiments were obtained with a Leica (Wetzlar, Germany) DM 400 M microscope.

Analysis of Combined Treatment. The effect of the combination of DCLK silencing and VAs was determined by analyzing the data obtained with MTT assay, time-lapse imaging of phosphatidylserine translocation, and TUNEL-based assay using CalcuSyn software (Biosoft, Ferguson, MO) (Chou and Talalay, 1984). The quantitative representation of a two-treatment pharmacologic interaction is given by the combination index (CI). The equation for mutually nonexclusive drugs that have independent modes of action is: $CI = (D_1/D_{100}) + (D_2/D_{100}) + (D_1/D_{100})(D_2/D_{100})$. For a certain effect x, $(D_1)$ and $(D_2)$ are the concentrations needed by each compound alone to achieve the same effect as concentrations $(D_{100})$ and $(D_{100})$, respectively. The calculation of CI takes into account the potency [median effect of the dose $(D_{50})$ or $(IC_{50})$ and the

excitation 488 nm/emission 615 nm). Live cells stain only with the Cyto-dye (green), and dead cells stain with both Cyto-dye and propidium iodide (red), resulting in a yellow fluorescence. We performed differential interference contrast (DIC) and fluorescence imaging with a Nikon TE-2000 E system under controlled conditions (37°C, 5% CO2). Five images were taken per well with two wells per treatment, and two independent experiments were performed. Image analysis and cell counting were performed by using ImageJ software (Abramoff et al., 2004).

MTT Assay. The effects of DCLK knockdown and VCR or VBL on cell proliferation and survival were determined by using the colorimetric MTT assays described by Mosmann (1983) with some modifications. Both the N1E-115 and SH-SY5Y cell lines were seeded in 96-well culture plates at a seeding density of 6000 and 10 000 cells/well, respectively. N1E-115 cells were transfected with 12.5, 25, 50, or 100 nM siDCL-2 or siDCL-3, and SH-SY5Y NB cells were transfected with 50, 100, 200, or 400 nM siDCL-4 or siDCL-5. Forty eight hours after transfection, the NB cells were treated with VCR or VBL (0.01, 0.1, 1, 10, and 100 μM). After 15-h incubation with the drugs, 0.83 mg/ml MTT solution was added to the medium, and the cells were incubated for 4 h at 37°C, 5% CO2. Subsequently, the medium was removed, and 100 μl of dimethyl sulfoxide was added to each well for solubilization of formazan crystals. The optical density was measured with a FLUOstar Optima plate reader (BMG Labtech GmbH, Offenburg, Germany) by using 540-nm wavelength and a reference wavelength of 630 nm.
shape of each dose-effect curve. CI value is <1, 1, or >1, indicating synergism, additive effect, or antagonism, respectively. CalcuSyn allows the determination of the dose reduction index (DRI) as well. DRI corresponds to the dose of each compound in a synergetic combination that can be reduced at a certain effect level compared with the doses of each treatment alone (Chou and Talalay, 1984).

Caspase-3 Activity Assay. We performed a caspase-3 activity assay as described previously (Qin et al., 2012) with modification. In brief, N1E-115 cells were grown in 96-well clear black plates and transfected with siDCL-3 or NC siRNA. Forty eight hours later, cells were treated with different concentrations of VCR or VBL (0.01, 0.1, 1, and 10 μM) for 15 h. Then, cells were lysed on ice for 1 h with 5× lysis buffer [250 mM HEPES, pH 7.4, 25 mM CHAPS, and protease inhibitor cocktail (1 tablet for 10 ml; Roche Diagnostics, Mannheim, Germany)]. After the caspase-3 substrate Ac-DEVD-AMC was added to the cells to a final concentration of 25 μM, the fluorescence was measured for 40 min at 37°C, using an excitation filter of 360 nm and an emission filter of 460 nm on a FLUOstar Optima plate reader (BMG Labtech GmbH). The caspase-3 substrate has a fluorescent AMC probe, and caspase-3 activity was expressed as picomoles of activity per minute per milligrams of protein by using the fluorescent AMC as standard. Protein concentration was determined by using the Pierce BCA protein assay (Thermo Fisher Scientific, Landsmeer, The Netherlands).

Caspase-8 Activity. The activity of caspase-8 was determined by using the Caspase-Glo 8 assay following the manufacturer’s instructions. In brief, N1E-115 neuroblasts were seeded in 96-well plates and transfected with 50 nM siDCL-3 or NC siRNA. Forty eight hours later, cells were treated with VCR or VBL (0.01, 0.1, 1, or 10 μM) for 15 h. Subsequently, 75 μl of Caspase-Glo 8 reagent with MG-132 inhibitor was added per well to equal the volume of medium with or without 2 μM caspase-8 inhibitor Z-IETD-FMK (Enzo Life Sciences Antwerpen, Belgium). Purified caspase-8 enzyme (10 units/ml; Enzo Life Sciences) was used as positive control. After 30-min incubation, caspase-8 activity was assessed by using a Centro XS³ Microplate Luminometer LB 960 (Berthold Technologies, Vilvoorde, Belgium), and results were normalized to the total amount of protein, which was determined by using the Pierce BCA protein assay (Thermo Fisher Scientific). Two independent experiments were performed.

ATP Bioluminescence Assay. ATP levels were assessed by luminescence using ELITEN Luciferase/Luciferin Reagent (Promega Benelux, Leiden, The Netherlands), according to the manufacturer’s instructions, and the microplate luminometer CentroXS³ LB 960 (Berthold Technologies). Results were normalized to the amount of protein that was determined by performing the Pierce BCA protein assay (Thermo Fisher Scientific).

Statistical Analysis. Results presented are representative of three independent experiments run in triplicate, unless otherwise indicated. Student’s t test and two-way analysis of variance were performed by using Prism 4.00 (GraphPad Software Inc., San Diego, CA) and SPSS statistical software version 17.0 (SPSS Inc. Chicago, IL). When appropriate, significant interactions were further analyzed by post hoc comparisons using Tukey’s LSD test. p values < 0.05 were considered statistically significant. Results are expressed as mean ± S.E.M.

Results

Combined DCLK Knockdown and Treatment with VAs Potentiates Microtubule Disruption. We first investigated the effect of the combined treatment with VAs (VCR or VBL) and DCLK knockdown (VCR + DCLK KD or VBL + DCLK KD) on the microtubule network (Fig. 1). In NB cells transfected with a nontargeting siRNA (NC) and treated

Fig. 1. N1E-115 neuroblastoma cells treated with VAs, siRNA to silence DCLK, and the combination of VAs and DCLK knockdown. Nuclei are stained in blue (Hoechst stain), and α-tubulin is stained in red (the Alexa Fluor 594 goat anti-mouse IgG was used as secondary antibody). Cells were transfected with 50 nM siRNA for 48 h and subsequently treated with 50 μM VBL, VCR, or vehicle (Mill-Q) for 6 h. A, cells were transfected with NC siRNA and treated with vehicle. B, percentage of cells without paracrystals (white bars), cells with tubulin paracrystals (gray bars), or cells with all tubulin reduced to globular paracrystals of tubulin (black bars). C, cells were transfected with siDCL-2 and treated with vehicle. D, cells were transfected with siDCL-3 and treated with vehicle. E, cells were transfected with NC siRNA and treated with VCR. F, cells were transfected with NC siRNA and treated with vehicle. G, cells were transfected with siDCL-2 and treated with vehicle. H, cells were transfected with siDCL-3 and treated with vehicle. I, cells were transfected with siDCL-2 and treated with VCR. J, cells were transfected with siDCL-3 and treated with VCR. Arrows indicate examples of tubulin paracrystals, and + indicate examples of globular paracrystals of tubulin. To determine the percentage of cells with paracrystals, a minimum of 100 cells was counted in triplicate per treatment from two independent experiments. The images were obtained with a magnification of ×120. Scale bars, 15 μm.
with the vehicle used to dissolve VAs a normal arrangement and organization of the microtubule network was observed (Fig. 1A). Mitotic cells also presented a normal phenotype. In HeLa cells treatment with VBL doses higher than 10 μM results in the formation of tubulin paracrystals (Jordan et al., 1992). Likewise, NB cells treated with 50 μM VBL showed the presence of tubulin paracrystals and cells became rounded (Fig. 1, B and E). Similar results were obtained with 50 μM VCR (Fig. 1, B and F). Cell rounding was also observed in cells transfected with previously described siRNAs targeting DCLK (Vreugdenhil et al., 2007), siDCL-2, or siDCL-3 alone (Fig. 1, C and D). In these cells the microtubules looked less organized compared with NB cells transfected with negative control siRNA (Fig. 1A). In addition, in mitotic cells the morphology of the mitotic spindles was altered, in line with previous results (Shu et al., 2006; Vreugdenhil et al., 2007). Tubulin paracrystals were not found in cells treated with siRNAs alone, and no differences were found between cells transfected with siDCL-2 or siDCL-3 alone (Fig. 1, B–D). In the combined treatment groups (VBL + siDCL-2, VBL + siDCL-3, VCR + siDCL-2, or VCR + siDCL-3), we detected that the microtubules were reduced to small globular paracrystals in approximately 40% of the cells, and the other cells presented tubulin paracrystals (Fig. 1, B and G–J). The observed and registered cells that were under combined treatment showed a higher disruption of microtubules compared with cells incubated with VAs (VCR or VBL) or DCLK knockdown (siDCL-2 or siDCL-3 transfection) alone. A similar phenotype, microtubules reduced to small globular paracrystals, was detected by Jordan et al. (1992) in HeLa cells treated with 100 μM VBL. We did not detect significant differences between VCR + DCLK KD and VBL + DCLK KD (Fig. 1, B and G–J).

**Combination of DCLK Knockdown with VAs Increases Cell Death in NB Cells.** We performed dose-response studies (Fig. 2) in mouse (N1E-115) and human (SH-SY5Y) NB cell lines transfected with siRNAs (siDCL-2 or siDCL-3 for N1E-115 and siDCLK-4 or siDCLK-5 for SH-SY5Y) that induced DCLK knockdown, and 48 h later the cells were treated with VCR or VBL. A live/dead double-staining assay was used to detect the effect of the treatment (Verissimo et al., 2010) (Fig. 2; Tables 1 and 2). We did not detect a significant difference between cells treated with nontargeting siRNA (NC) and nontransfected cells (Supplementary Fig. 1). We found that the concentration of VCR needed to induce cell death in N1E-115 cells can be reduced significantly (p < 0.001) in cells transfected with siDCL-2 (5.6-fold reduction) or siDCL-3 (7.3-fold reduction) (Fig. 2, A and B; Table 1). The effect of the combination with VBL was even more pronounced: a significant (p < 0.001) 21.1-fold reduction was found when cotreated with siDCL-2 and a 12.5-fold reduction was found when cotreated with si-DCL-3.

**TABLE 1**

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<tr>
<th>Treatment</th>
<th>EC₅₀ μM ± S.E.M.</th>
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<tr>
<td>NC + VCR</td>
<td>182.390 ± 117.627</td>
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<tr>
<td>siDCL-2 + VCR</td>
<td>32.434 ± 1.345*</td>
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<tr>
<td>siDCL-3 + VCR</td>
<td>25.061 ± 4.857**</td>
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<tr>
<td>NC + VBL</td>
<td>238.781 ± 63.117</td>
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<tr>
<td>siDCL-2 + VBL</td>
<td>11.298 ± 4.285*</td>
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<tr>
<td>siDCL-3 + VBL</td>
<td>19.099 ± 6.728*</td>
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NC, negative control siRNA; siDCL-2 and siDCL-3, siRNAs that induce DCLK silencing in N1E-115 cells.

* Used as reference.

**p < 0.05; **, p < 0.01.
In SH-SY5Y human NB cells, we observed a significant (p < 0.001), although less pronounced, difference in EC_{50} values between VCR-treated cells with siDCLK-4 (2.09-fold reduction) and siDCLK-5 (3.47-fold reduction) compared with the negative control (Table 2). Although a reduction of EC_{50} for VBL treatment with both siDCLK-4 and siDCLK-5 was detected, no significant difference was obtained compared with VBL with nontargeting siRNA (VBL + NC) treatment.

The effect of VAs + DCLK KD was further investigated for a possible synergistic effect. For this study, we treated the cells for 15 h with different doses of VCR or VBL and different concentrations of siRNAs as described under Materials and Methods. Figure 3 shows that the different concentrations of siRNA induced different levels of DCLK knockdown, as measured by Western blot analysis. No significant difference in DCLK expression was detected between cells transfected with different concentrations of NC siRNA (12.5, 25, 50, and 100 nM), cells with mock transfection (treated with Lipofectamine only), and untreated cells (Supplemental Fig. 2). The determination of siRNA doses was based on results obtained previously, which demonstrated that 50 and 200 nM siRNA was enough to induce significant knockdown in N1E-115 and SH-SY5Y cells, respectively (Verissimo et al., 2010). Because the present (Figs. 1 and 2; Tables 1 and 2) and previous studies (Verissimo et al., 2010) showed no significant differences between the two siRNAs used to induce DCLK knockdown in N1E-115 and SH-SY5Y cells, we decided to proceed by using a single siRNA: siDCL-3 for N1E-115 and siDCLK-4 for SH-SY5Y cells.

We investigated the effect of the different combined treatments on cell proliferation and induction of cell death by using the MTT assay (Mosmann, 1983). The data were analyzed by means of CalcuSyn software (Chou and Talalay, 1984). This software provided CI values based on multidrug dose-effect calculations using the median method, as described under Materials and Methods. Synergism (CI < 1) was observed with all combined treatments, except with the combination of 50 nM siDCLK-4 and 0.1 μM VBL in SH-SY5Y cells (Table 3). In N1E-115 cells, synergism was detected even at 12.5 nM, which already induces 43.08 ± 3.32% DCLK knockdown (Fig. 3). In SH-SY5Y cells, synergism was consistently detected at higher doses of siRNA and VAs than in N1E-115 cells (Table 3).

We also determined the DRI, which estimates how much the dose of VAs or siRNAs can be reduced by the combined treatment (Table 3). In N1E-115 cells, the DRI of VCR ranges from 1.6 to 4.6, and the DRI of VBL ranges from 5.1 to 146.4. In SH-SY5Y cells, DRI analysis indicated that the concentration of VCR and VBL could be reduced 86.8 and 14.8 times, respectively, when 10 μM of the VA was combined with 200 nM of siDCLK-4 (Table 3). As indicated in Table 3, the combined treatment would allow the reduction of siRNA concentration as well. The DRI of siDCL-3 ranges from 1.9 to 8.0, and the DRI of siDCLK-4 is between 1.7 and 15.1.

**The Synergistic Effect of DCLK Knockdown and VAs Involves An Apoptosis Pathway.** To explore whether the cells die via an apoptotic process, as observed previously with

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<th>Treatment</th>
<th>EC_{50} ± S.E.M.</th>
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<tr>
<td>NC + VCR*</td>
<td>114.815 ± 17.008</td>
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<tr>
<td>siDCLK-4 + VCR</td>
<td>54.702 ± 11.138*</td>
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<tr>
<td>siDCLK-5 + VCR</td>
<td>33.113 ± 11.021*</td>
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<tr>
<td>NC + VBL*</td>
<td>56.702 ± 17.517</td>
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<tr>
<td>siDCLK-4 + VBL</td>
<td>29.107 ± 8.950</td>
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<tr>
<td>siDCLK-5 + VBL</td>
<td>28.840 ± 8.950</td>
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*NC, negative control siRNA. siDCLK-4 and siDCLK-5, siRNAs that induce DCLK knockdown in SH-SY5Y.

* Used as reference.

*, P < 0.05.
independent DCLK knockdown (Verissimo et al., 2010) and VAs (Pourroy et al., 2004; Marimpietri et al., 2007), we performed four different apoptotic assays (Figs. 4 and 5; Supplemental Fig. 3). N1E-115 NB cells express DCLK, whereas DCX expression is below detection levels. In contrast, SH-SY5Y cells express both DCLK and DCX (Verissimo et al., 2010). Therefore, we performed this study in N1E-115 NB cells, because DCX might compensate for the silencing of DCLK (Deuel et al., 2006; Koizumi et al., 2006).

Forty eight hours after transfection with siRNA targeting DCLK (DCL-3) N1E-115 NB cells were treated with different doses of VAs (VCR or VBL) as indicated under Materials and Methods and studied with time-lapse imaging of phosphatidylserine translocation (Fig. 4; Supplemental Movie) and a

**Fig. 4.** Apoptosis studies in N1E-115 neuroblastoma cells transfected with siDCL-3 or NC siRNAs for 48 h and treated with VCR, VBL, or vehicle (Milli-Q water) using time-lapse imaging of phosphatidylserine translocation. Time-lapse imaging was started immediately after adding VBL, VCR, or Veh. A, time-lapse imaging of phosphatidylserine translocation in cells treated with 0.1 μM VBL. The images shown are the overlap of DIC and fluorescent imaging at 0, 7.5, and 15 h after starting the assay. The images were taken at 30-min intervals (see Supplemental Movie). Magnification, ×20. Scale bars, 500 μm. B, ratio of AnxV by the total cell area over time of cells treated with 0.1 μM VBL or vehicle. C, AUC of the ratio AnxV/total cell area in cells treated with different concentrations of VCR (0.01, 0.1, 1, and 10 μM). D, AUC of the ratio AnxV/total cell area in cells treated with different concentrations of VBL (0.01, 0.1, 1, and 10 μM). NC, cells transfected with NC siRNA and treated with vehicle; siDCLK-3, cells transfected with siDCLK and treated with vehicle; VCR, cells transfected with NC siRNA and treated with VCR; siDCLK-3 + VCR, cells transfected with siDCLK-3 and treated with VCR; siDCLK-3 + VBL, cells transfected with siDCLK-3 and treated with VBL; siDCLK-3 + VCR + Casp. Inhib., cells transfected with siDCLK-3, treated with VBL and caspase inhibitor z-VAD-fmk; siDCLK-3 + VCR + Casp. Inhib., cells transfected with siDCLK-3, treated with VCR and caspase inhibitor z-VAD-fmk. Data points and columns indicate the mean of two technical replicates within one representative experiment of two independent experiments; bars indicate S.E.M. *, p < 0.05; **, p < 0.01.

### Table 3

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Combination</th>
<th>Ratio</th>
<th>CI (DRI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1E-115</td>
<td>siDCLK-3/VCR</td>
<td>12.5:0.01</td>
<td>0.424 (synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.0:1</td>
<td>0.343 (synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50:1</td>
<td>0.469 (synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100:10</td>
<td>0.615 (moderate synergism)</td>
</tr>
<tr>
<td></td>
<td>siDCLK-3/VBL</td>
<td>12.5:0.01</td>
<td>0.153 (strong synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.0:1</td>
<td>0.263 (strong synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50:1</td>
<td>0.517 (synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100:10</td>
<td>0.730 (moderate synergism)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>siDCLK-4/VCR</td>
<td>50:0.1</td>
<td>0.066 (very strong synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100:1</td>
<td>0.199 (strong synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200:10</td>
<td>0.256 (strong synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400:100</td>
<td>0.372 (synergism)</td>
</tr>
<tr>
<td></td>
<td>siDCLK-4/ VBL</td>
<td>50:0.1</td>
<td>4.646 (strong antagonism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100:1</td>
<td>0.545 (synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200:10</td>
<td>0.427 (synergism)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400:100</td>
<td>0.576 (synergism)</td>
</tr>
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</table>

CI values and DRI for the combination of DCLK knockdown (siDCLK) with VCR or VBL on the reduction of cell proliferation and survival in two neuroblastoma cell lines (N1E-115 and SH-SY5Y)

The CI is based on the formula: $CI = (D_1/D_2)_{1/2} + (D_2/D_1)_{1/2}$ (Chou and Talalay, 1984). CI can be <1, 1, and >1, indicating synergism, additive effect and antagonism, respectively. DRI corresponds to how much the dose of each compound in a synergetic combination can be reduced at a certain effect compared with the doses of each compound alone (Chou and Talalay, 1984).
TUNEL-based assay (Supplemental Fig. 3; Supplemental Table 2). Time-lapse imaging of phosphatidylserine translocation allowed us to detect the progression of the apoptotic process in real time (Puigvert et al., 2010). After automated imaging, quantitative analysis was performed. Two-way analysis of variance showed a highly significant effect of the treatment ($F_{4,5} = 92.72; p \leq 0.0001$), time ($F_{29,145} = 193.95; p \leq 0.0001$), and an interaction of treatment and time ($F_{116,145} = 10.038; p \leq 0.0001$) (Fig. 4B). Post hoc comparisons showed that the overall effect of the combination of 50 nM siDCL-3 and 0.1 μM VBL (siDCL-3 + VBL) was significantly different from all other treatments ($p \leq 0.0001$). The p value was 0.003 when comparing the overall effect of NB cells with DCLK knockdown and VBL (siDCL-3 + VBL) with cells transfected with a nontargeting siRNA and treated with VBL (see Fig. 4B). For a clearer representation and comparison of the results we determined the area under the curve (AUC) representing the cumulative effect in time of the different treatments (Fig. 4, C and D). As expected, the caspase inhibitor z-VAD-fmk inhibited the apoptotic process induced by
VAs (VCR or VBL) + DCLK KD (Fig. 4, B–D). Because z-VAD-fmk reacts on other enzymatic systems, experiments where the observed effects may be attributed to inhibition of caspases as those described herein should be interpreted carefully (Misaghi et al., 2006).

We observed significant differences ($p < 0.05$) between VA and VA + DCLK knockdown at different VA (VCR or VBL) doses (Fig. 4, C and D). The exception was 0.01 μM VBL (the lowest dose tested) combined with DCLK silencing, which was not significantly different ($p = 0.2608$) from cells treated with 0.01 μM VBL alone (Fig. 4D).

We calculated multidrug dose effects to determine whether there was synergism between the effect of DCLK knockdown and VA (VCR or VBL) treatment in the induction of apoptosis. As shown in Supplemental Table 1, synergism was verified when NB cells were treated with VBL + DCLK KD at the different time points. Although a trend was detected, the increase in apoptosis over time was not synergistic in NB cells treated with VCR + DCLK KD. From 5 h of combined treatment (VBL + DCLK KD) onward, strong and very strong synergism was detected. At earlier time points we observed no synergism, which may be because of the limited time for the action of VBL. At high concentrations of siRNA/VBL (50 nM/1 M), synergism was detected from 10 h of treatment onward. For low concentrations (12.5 nM/0.01 μM or 25 nM/0.1 μM) synergism was identified from 5 h onward. These results indicate that the synergism depends on dose and time. At high concentrations the effect of the compounds alone is probably too strong to detect the additive or synergistic combined effect. However, a synergistic effect becomes clear at longer time exposures of the combined treatment (Supplemental Table 1).

The occurrence of a synergistic apoptotic effect was further investigated by using a TUNEL-based assay (Supplemental Table 2). We determined the percentage of TUNEL-positive cells for the different conditions (different doses of siDCL-3, VAs, and the combination of those). Subsequently, we calculated the CI by using CalcuSyn software (Chou and Talalay, 1984). We obtained CI values $< 1$ for the combination of siDCL-3 and VAs, indicating synergism between the used compounds. In Supplemental Fig. 3, one can observe that the combination of DCLK knockdown and VAs results in a significantly higher percentage of TUNEL-positive cells ($p < 0.05$). Cells incubated with siDCL-3, VAs, and z-VAD-fmk caspase inhibitor presented clearly fewer TUNEL-positive cells compared with cells treated with siDCL-3 plus VAs (Supplemental Fig. 3). This suggests that the detected TUNEL-positive cells were indeed apoptotic cells.

**Combining DCLK Knockdown with VAs Induces Apoptosis via Caspase-3 and Caspase-8 Activation.** We performed a modified caspase-3 activity assay in which the caspase-3-like activity was measured by monitoring the production of cleaved fluorescent AMC from the caspase-3 substrate Ac-DEVD-AMC in extracts of treated N1E-115 NB cells (Lazebnik et al., 1994). The study of caspase-8 activity allowed us to investigate whether an extrinsic apoptotic pathway is involved in the synergistic apoptotic process in NB cells treated with VAs (VCR or VBL) and DCLK knockdown (siDCL-3 transfection). A nontargeting siRNA was used as NC for transfection (see Materials and Methods). As in nontransfected cells (data not shown), no caspase-3 activity was detected in cells transfected with NC siRNA (Fig. 5). Cells treated with a combination of VAs and siDCL-3 presented a significantly higher caspase-3 activity than cells treated with VAs or siDCL-3 alone (Fig. 5). The synergistic effect was not significant in cells treated with 0.01 μM VCR, the lowest VA concentration we tested (Fig. 5A). The caspase-3 activity was mostly enhanced ($p < 0.01$) by combining DCLK knockdown in the treatment when cells were treated with 0.1 μM VAs or 1 μM VCR (Fig. 5, A and B). The increase of caspase-3 activity after the combined treatment was completely abolished by using a caspase inhibitor z-VAD-fmk, indicating caspase-dependent apoptosis.

To study whether the extrinsic apoptotic pathway is activated in treated NB cells, we evaluated the activity of caspase-8. No significant difference was detected between NC and cells transfected with siDCL-3 (Fig. 5, C and D), indicating that DCLK knockdown does not induce the activation of caspase-8. However, cells treated with the combination of VAs and siDCL-3 presented higher caspase-8 activity than cells treated with VAs alone. This increase of caspase-8 activity was significant when using 0.1, 1, and 10 μM VCR or 0.01 and 10 μM VBL in combination with DCLK knockdown compared with VAs alone. The induction of caspase-8 activity was inhibited by using the inhibitor Z-IETD-FMK.

**DCLK Silencing Induces Changes in Mitochondria Activity.** We previously had found a high correlation between the expression of DCL and mitochondria-related genes, such as genes involved in the oxidative phosphorylation process (Verissimo et al., 2010). Because changes in mitochondria activity caused by DCLK knockdown might contribute to the synergistic apoptotic process detected with the combined treatment (DCLK KD + VAs), we explored the effect of DCLK silencing on mitochondria activity. We checked the mRNA expression levels of two genes, Ndufa2 and ATP5e, which play a crucial role in the oxidative phosphorylation process (Fig. 6A). In addition, we investigated the effect on ATP synthesis (Fig. 6B). We detected a significant decrease ($p < 0.001$) in the expression of both genes when DCLK is silenced compared with cells that express DCLK (Fig. 6A). In agreement, less ATP synthesis was measured in the neuroblastoma cells with DCLK knockdown (Fig. 6B). We detected an ATP reduction of 55.85 ± 6.05% in shDCL-2 cells and 82.24 ± 8.25% in shDCL-3 cells. shDCL-2 and shDCL-3 stable cell lines are derived from N1E-115 cell line and, in the presence of doxycycline, express a shRNA against DCLK mRNA (see Materials and Methods and Verissimo et al., 2010). The results shown in Fig. 6 were normalized to the control N1E-115 NB cell line that expresses DCLK-derived MAPs.

**Discussion**

In the current study we investigated the effect of combining VA treatment and DCLK knockdown in NB cells. We showed that the combined treatment results in a strong synergistic effect inducing NB cell death. Moreover, we demonstrated that the NB cells die via a caspase-3-dependent apoptosis process. Therefore, this study indicates that the combined treatments tested permit a significant reduction in
VA doses required to effectively kill neuroblastoma cells in vitro.

VAs are important chemotherapeutic agents used in NB therapy. However, there is evidence that the treatment of NBs with VAs results in toxicity (Lobert et al., 1998) and the development of drug resistance (Don et al., 2004). Therefore, the reduction of the dose of these compounds is desired. It has been shown that combining VAs with other drugs is more effective than single-drug treatment, allowing a significant reduction of the doses (Marimpietri et al., 2005). The combined treatment might prevent the activation of compensatory pathways by NB cells, allowing a higher efficiency of the treatment (Petrelli and Giordano, 2008).

Here, we investigated the combination of the VA treatment with knocking down of the microtubule-stabilizing proteins encoded by the DCLK gene by RNA interference technology. The novelty of this combined treatment has several aspects: 1) because both DCLK silencing (Shu et al., 2006; Vreugdenhil et al., 2007) and VAs (Jordan and Wilson, 2004) lead to microtubule destabilization, one would expect an additive or synergistic apoptotic effect from the combined treatment; 2) in the case of synergistic effect, a significant reduction of the compounds may be used and, therefore, a more specific and less toxic therapeutic approach might be obtained; 3) because VAs lead to the development of drug resistance through an increase of microtubule-stabilizing proteins (Lobert et al., 1998; Don et al., 2004), silencing the DCLK might allow that NB cells become more vulnerable and therefore less resistant to treatment with VAs; 4) DCLK-derived MAPs are quite specifically expressed in neuroblasts (Vreugdenhil et al., 2007) and high expressed in human NBs (Verissimo et al., 2010); and 5) silencing of DCLK leads to the disruption of mitotic spindles and induces apoptosis (Shu et al., 2006; Vreugdenhil et al., 2007; Verissimo et al., 2010). Therefore, the combined VAs + DCLK KD approach might be more effective than pre-existing approaches for NBs.

Our data indicate that silencing the expression of DCLK sensitizes NB cells for the microtubule-destabilizing agents VCR and VBL. This sensitizing effect was more effective in N1E-115 than in SH-SY5Y NB cells. This difference might be because the SH-SY5Y cell line also expresses DCX, which may compensate for the silencing of DCLK (Verissimo et al., 2010). Such compensating effects between DCX and DCLK have been described previously (Deuel et al., 2006; Koizumi et al., 2006). Future studies are needed to investigate whether the expression of DCX in SH-SY5Y compensates for DCLK knockdown and provides resistance of these cells toward VAs. An alternative explanation might be related to the achieved knockdown levels of DCLK, because a higher concentration of siRNAs had to be used to obtain knockdown in SH-SY5Y than in N1E-115 cells.

We identified synergism in the inhibition of proliferation and induction of N1E-115 NB cell death by combining DCLK silencing and VAs. Moreover, our results suggest that the combined treatment-induced cell death is mediated via a caspase-3-dependent apoptotic process. In line with this notion are the findings that microtubule-destabilizing agents, such as VAs, cause the activation of caspase-3 (Fan et al., 2001), and silencing of DCLK leads to the activation of caspase-3 as well (Verissimo et al., 2010). Previous studies have shown that VAs induce caspase-8 activation (Bayless and Davis, 2004; Casado-Zapico et al., 2010). We have detected an increase in caspase-8 activity in NB cells treated with the combination of VAs and DCLK knockdown compared with cells treated with VAs alone. The apoptotic mechanism induced by VAs has also been related with mitochondria activity (Moon and Lerner, 2002) with up-regulation of the proapoptotic Bax and inactivation of antiapoptotic Bcl-2. In WSU-CLL cells, VCR and VBL induced Bcl2 phosphorylation (Moon and Lerner, 2002). DCLK knockdown leads to the activation of the intrinsic apoptotic process in NB cells as well, with the up-regulation of Bax and down-regulation of Bcl2 (Verissimo et al., 2010). Therefore, our results suggest that the combined treatment results in the activation of an extrinsic (caspase-8) apoptotic pathway that converges with the intrinsic (mitochondrial) apoptotic pathway at caspase-3.

The synergistic effect between VAs and siRNAs that target DCLK might be related to their distinct and independent effects on the spindle microtubules, changes on the oxidative phosphorylation process, and induction of apoptosis. Therefore, when these compounds are combined, a stronger induction of apoptotic cell death may be obtained than by either compound alone. In Fig. 7 we propose a hypothetical model...
for the consequences of the combined treatment that result in a synergistic apoptotic effect.

VAs bind to β-tubulin subunits of the α/β-tubulin heterodimers, affecting the polymerization, stabilization, and dynamics of the microtubules (Fig. 7). High- and low-affinity VA binding sites in the microtubules have also been found (Mollinedo and Gajate, 2003). At low concentrations, VAs bind at the plus (+) ends of (spindle) microtubules, thereby inhibiting tubulin dimer addition. At high concentrations, VAs are able to bind in addition to the low-affinity sites along the walls of the microtubules, resulting in the separation of the protofilaments and formation of paracrystalline structures (Mollinedo and Gajate, 2003). Treatment with VAs leads to the disruption of microtubules, particularly spindle microtubules, resulting in the slowing or blocking of the cell cycle at metaphase-anaphase transition and induction of apoptosis (Jordan and Wilson, 2004). The mitotic arrest at low concentrations is caused by alterations in microtubule dynamics rather than microtubule depolymerization (Jordan and Wilson, 2004). We detected less synergism at higher concentrations of VAs, probably caused by the distinct action and Wilson, 2004). We detected less synergism at higher concentrations of VAs, probably caused by the distinct action of these agents at different concentrations. At high doses, the induction of apoptosis might also occur without cell cycle arrest (Pourroy et al., 2004).

The silencing of DCLK results in disruption of the spindle microtubules (Shu et al., 2006; Vreugdenhil et al., 2007). The stabilization of the microtubules and regulation of mitotic spindle formation by DCLK might be similar to the homolog DCX (Shu et al., 2006). DCLK, like DCX, has two microtubule-binding domains (doublecortin domains) and a Ser/Pro-rich region (Vreugdenhil et al., 2007). Each doublecortin domain binds tubulin and microtubules in a different way (Kim et al., 2003). Because one doublecortin domain interacts with tubulin dimers and the other domain interacts with the microtubule, a stabilizing role for DC-containing proteins in microtubule nucleation has been suggested (Kim et al., 2003). DCX selectively binds 13 protofilament microtubules over a fenestration in the microtubule wall and it interacts with four tubulin monomers (Moores et al., 2004). Hence, it has the potential to reinforce the lateral connections between protofilaments and enhance longitudinal interactions, which is an outstanding way for cross-linking protofilaments and increasing microtubule stability (Moores et al., 2004). Thus, the silencing of DCLK may result in a destabilized (spindle) microtubule organization that facilitates the access of VAs to the microtubules, leading to an effective microtubule disruption at low doses of VAs. In other words, the effect of the combined treatment may converge at the disruption of (spindle) microtubules and, subsequently, apoptosis (Fig. 7).

Silencing of DCLK resulted in down-regulation in the expression of oxidative phosphorylation genes and reduction of ATP synthesis. In line with this finding, we have previously described a high correlation between DCLK expression and mitochondria-related genes in NB tumors (Verissimo et al., 2010). It is known that disruption of the electron transport, oxidative phosphorylation, and ATP production can induce apoptotic cell death (Green and Reed, 1998). However, ATP is also required for downstream events of the apoptotic process (Green and Reed, 1998).

There are similarities in the mechanism of apoptosis induction by VAs and DCLK knockdown, which might work in a synergistic manner. Both silencing of DCLK and VAs result in cell cycle arrest (Jordan et al., 1992; Pourroy et al., 2004; Shu et al., 2006) and changes in mitochondria activity and apoptosis (Groninger et al., 2002; Moon and Lerner, 2002; Shu et al., 2006; Vreugdenhil et al., 2007; Verissimo et al., 2010).

In this study we show for the first time that combining DCLK silencing and VAs induces a possibly synergistic apoptotic effect in NB cells in vitro. Additional in vivo studies are needed to confirm the efficacy of the combined treatment and establish its significance as a potential therapeutic approach.
pressed in human neuroblastomas and quite specifically expressed in neuroblasts and NBs, sensitizes the NB cells for the microtubule-destabilizing agents VCR and VBL. The combined treatment results in a synergetic caspase-3-dependent apoptotic effect that might be caused by the independent action of VAs and DCLK knockdown inducing mitotic spindle disruption and alteration on the mitochondria activity. Future in vivo studies to validate the decrease of tumorigenicity would be of high interest.

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Authorship Contributions

Participated in research design: Verissimo, Vroon, Fitzsimons, and Vreugdenhil.

Conducted experiments: Verissimo and Cheng.

Contributed new reagents or analytic tools: Puigvert, Qin, Price, Danen, and van de Water.

Performed data analysis: Verissimo and Cheng.

Wrote or contributed to the writing of the manuscript: Verissimo, van Deutekom, Fitzsimons, and Vreugdenhil.

References


