Combining doublecortin-like kinase silencing and vinca alkaloids results in a synergistic apoptotic effect in neuroblastoma cells

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Abbreviations: AMC, 7-amido-4-methylcoumarin; AnxV, annexin V; AUC, area under the curve; BSA, bovine serum albumin; CI, combination index; DC-domain, doublecortin domain; DCL, doublecortin-like; DCLK, doublecortin-like kinase; DCX, doublecortin; DIC, differential interference contrast; DMSO, dimethyl sulfoxide; Dox, doxycycline; DRI, dose reduction index; KD, knockdown; MAP, microtubule-associated proteins; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NB, neuroblastoma; NC, negative control; PBS, phosphate-buffered saline; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; siRNA, short interference RNA; VA, vinca
alkaloid; VBL, vinblastine; VCR, vincristine; Veh, vehicle; Z-IETD-FMK, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp(O-Met) fluoromethyl ketone

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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, development of drug resistance and toxicity make NB difficult to treat with these drugs. In this study we explore the combination of VAs (vincristine or vinblastine) with knockdown of the microtubule-associated proteins (MAPs) encoded by the doublecortin-like kinase (DCLK) gene using siRNAs. 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 EC50 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 TUNEL-based assay we found a significant increase of apoptosis by the combined treatment. Induction of caspase-3 activity, as detected via cleavage of Ac-DEVD-AMC, showed a 3.3 to 12.0 fold increase in the combined treatment. We detected significant increases in caspase-8 activity as well. Moreover, multi-drug dose-effect calculated using the median effect method showed a strong synergistic inhibition of proliferation and induction of apoptosis at most of the different combined concentrations of siRNAs and VAs. Together, our data demonstrate that 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 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 compounds 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, induce formation of tubulin paracrystals and G2/M block occurs (Jordan et al., 1992; Pourroy et al., 2004). However, at nanomolar concentrations, microtubule dynamics is suppressed with no effect on microtubule depolymerization, being a postmitotic G1 arrest identified (Pourroy et al., 2004). Both treatment with micro- and treatment with nanomolar concentrations lead to apoptosis. The mechanism by which VAs induce apoptosis is complex, involving protein kinase signaling pathways (Fan et al., 2001), and also 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 towards VAs might be due to 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., 1984; Marimpietri et al., 2007).

Several molecular targets have been proposed for NB therapy (George et al., 2010; Wagner and Danks, 2009). 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) doublecortin-like (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 (Fitzsimons et al., 2008; Koizumi et al., 2006; Shu et al., 2006; Vreugdenhil et al., 2007). We, and others, have shown that DCLK-derived MAPs are involved in microtubule stabilization and 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 (DC domains) involved in tubulin polymerization and microtubule bundling (Cierpicki et al., 2006). The interaction of DC domains with microtubules is regulated by phosphorylation and de-phosphorylation (LoTurco, 2004).
We, and others, have shown the essential role of DCLK in proliferation and survival of neuroblast cells. Knockdown of DCL/DCLK-long results in 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 differently expressed after knockdown of MAPs encoded by DCLK gene, and mitochondria were the most affected cellular components (Verissimo et al., 2010).

Since both silencing of DCLK and VAs result in 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. Vincristine (VCR) and vinblastine (VBL) were obtained from Sigma-Aldrich Chemie B.V (Zwijndrecht, The Netherlands). VBL and VCR were dissolved in ultra-purified water (Milli-Q, Millipore, the Netherlands) to 10 mg/ml stock and stored at 4ºC or -20ºC, 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 (Zwijndrecht, The Netherlands), the secondary antibody anti-mouse-HRP from Tube-bio B.V. (Bevelandseweg, The Netherlands), the goat anti-mouse IgG conjugated to Alexa fluor 594 dye was purchased from Molecular Probes (Leiden, The Netherlands), and the anti-rabbit-HRP from Santa Cruz (Tebu-Bio, Heerhugowaard, The Netherlands). We also used a recently developed primary rabbit antibody targeting the QRDLYRPLSSDDLDSVG-C sequence, which
is specifically present in DCL (Saaltink et al. submitted). The 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemie B.V
(Zwijndrecht, The Netherlands). 5 mg/ml MTT in phosphate-buffered saline (PBS) was freshly
prepared before each determination. Annexin V-Alexa 488 was prepared as previously described
(Puigvert et al., 2010). The caspase inhibitor N-benzyloxy carbonyl-Val-Ala-Asp(0-Met)
fluoromethyl ketone (z-VAD-fmk) was purchased from Bachem CH (Weil am Rhein, Germany),
the caspase substrate Ac-DEVD-AMC was obtained from Enzo Life Science (Raamsdonksveer,
The Netherlands) and 7-amido-4-methylcoumarin (AMC) from Invitrogen (Breda, The
Netherlands).

**Cell culture, transfection and drug treatment.** Human SH-SY5Y and mouse N1E-115
neuroblastoma cells were cultured as previously described (Molenaar et al., 2008; Vreugdenhil et
al., 2007). For microscopy assays, both cell lines were grown at 70% confluence in 24-well
plates with glass bottom or 96-well μ clear black plates (Greiner Bio-One BV, Alphen aan den
Rijn, The Netherlands) coated with 100 ng/μl 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 previously described (Verissimo et al., 2010; Vreugdenhil et
al., 2007). N1E-115 cells were transfected with synthetic RNA oligonucleotides siDCL-2
(CAAGAAGACGGCUCACUCCTT-sense and 5’-GGAGUGAGGCCGUCUUCUUGTT-
antisense) or siDCL-3 (GAAAACCAAGAAGGUUCGATT-sense and
TCAACCUUCUUGCCUUUCC-antisense) obtained from Eurogentec Nederland B.V.
(Maastricht, The Netherlands) (Vreugdenhil et al., 2007). SH-SY5Y neuroblastoma cells were
transfected with siDCLK-4 (GCCCACUGCAGCUUCACCTT-sense and
GGUAGGAAGCUUGGGCAGTT-antisense) or siDCLK-5
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 of siDCLK-4 or siDCLK-5 siRNAs (Verissimo et al., 2010) and N1E-115 cells were transfected with 12.5, 25, 50 or 100 nM of siDCL-2 or siDCL-3 siRNAs (Vreugdenhil et al., 2007). Transfection was performed using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) following the manufacturer’s instructions. As negative control (NC) we used the AllStars Negative Control siRNA from Qiagen Benelux B.V. (catalog number: 1027281) (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 ± 5% was obtained, which was determined by using a non-targeting siRNA conjugated to Alexa Fluor 488 (Qiagen Benelux B.V., Venlo, The Netherlands). Cells were treated with different concentrations (0.01, 0.1, 1, 10 or 100 μM) of VCR or VBL.

**Neuroblatoma doxycycline-inducible stable cell lines.** The development of the NB doxycycline-inducible stable cell lines from N1E-115 was described by Verissimo *et al.* 2010 (Verissimo et al., 2010). In the presence of doxycycline (Dox), the cells express a shRNA against DCLK-derived MAP DCL. The sequence of the shRNA expressed in shDCL-2 cell line is 5’-ACAAGAUGCAGGAUGACCAGC-3’ and the shDCL-3 cell line expresses the shRNA with the sequence 5’-ACAACAAGAUGCAGGAUGACC-3’. As negative control cell line we used the N1E-115 NB cell line that expresses DCLK-derived MAPs. The cells were treated with Dox or
vehicle (milli-Q water) for 72 hours. Subsequently, the cells were harvested for RNA isolation or ATP bioluminescence assay.

**RNA isolation.** Cells were harvested and the RNA was isolated using TRIzol reagent (Invitrogen BV) according to the manufacturer’s specifications. The RNA concentration and purity were determined using a NanoDrop spectrophotometer (NanoDrop products, Wilmington, DE).

**Quantitative real-time PCR.** Quantitative real-time PCR (RT-qPCR) was performed using ABI Prism 7900HT Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RT-qPCR was carried out with a target-specific assay (Assays-on-demand Gene expression system; Applied Biosystems). Messenger RNA expression of ATP5e (Mm00445969_m1) and Ndufa2 (Mm00477755_g1), were analyzed. The expression levels were normalized to 18S rRNA (Hs99999901_s1, Applied Biosystems).

**Western blot analysis.** Protein lysates, SDS-PAGE, and western blotting were performed as previously described (Vreugdenhil et al., 2007). DCL expression was normalized to α-tubulin. Analysis and quantification of the relative optical densities were performed using ImageJ software (Abramoff et al., 2004).

**Immunohistochemistry.** Neuroblastoma N1E-115 cells were transfected with 50 nM siDCL-2, siDCL-3 or NC siRNA for 48 hours and subsequently treated with 50 μM VCR, VBL or vehicle (milli-Q water) for 6 hours. Then, cells were fixed with methanol-EGTA (97:3) and permeabilized with TBP (1% Triton-x-100, 1% bovine serum albumin (BSA) in PBS) for 1 hour at room temperature. To check the effect of the drug and the combination of drug and DCLK knockdown on the microtubule network, we stained microtubules using mouse anti α-tubulin antibody and Alexa-594 conjugated secondary antibody (Molecular Probes, Leiden, The...
Netherlands). 1 μg/ml Hoechst 33258 (Molecular Probes, Leiden, The Netherlands) was used to visualize the nuclei. Confocal images were obtained with a Nikon TE-2000 E system. The percentage of cells with tubulin paracrystals or with all tubulin reduced in small globular paracrystals was determined based on previous methods (Jordan et al., 1992; Muller et al., 1990). Per treatment, a minimum of 100 cells were counted in triplicate from two independent experiments.

**Live/dead double staining, image analysis and cell counting.** N1E-115 and SH-SY5Y NB cell were cultured in 24-well plates with glass bottom (Greiner Bio-One BV, Alphen aan den Rijn, The Netherlands). Forty-eight hours after transfection with siRNAs and subsequent incubation with VCR or VBL (0.1, 1, 10, 100 and 1000 μM) for 6 hours (N1E-115) or 15 hours (SH-SY5Y), the live/dead double staining assay (Calbiochem, San Diego, CA, USA) was performed as described in the manufacturer’s protocol. In short, we provided to the cells a cell-permeable fluorescent Cyto-dye (Ex/Em 488/518) and propidium iodide (Ex/Em 488/615). Live cells stain only with the Cyto-dye (green) and the 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 on a Nikon TE-2000 E system under controlled conditions (37ºC, 5% CO2). Five images were taken per well with two wells used per treatment and two independent experiments were performed. Image analysis and cell counting were performed 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 using the colorimetric MTT assays described by Mosmann (Mosmann, 1983) with some modifications. Both N1E-115 and SH-SY5Y cell lines were seeded in 96-well culture plates at a seeding density of 6000 and 10000 cells/well, respectively. N1E-115 cells
were transfected with 12.5, 25, 50 or 100 nM of siDCL-2 or siDCL-3 and SH-SY5Y NB cells were transfected with 50, 100, 200 or 400 nM of siDCLK-4 or siDCLK-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 hours incubation with the drugs, 0.83 mg/ml MTT solution was added to the medium, and the cells were incubated for 4 hours at 37°C, 5% CO₂. Subsequently, the medium was removed and 100 μl of DMSO 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) using 540 nm wavelength and a reference wavelength of 630 nm.

**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 μclear black plates (Greiner Bio-One BV, Alphen aan den Rijn, The Netherlands). Forty-eight hours later, different doses of VCR or VBL (0.01, 0.1, 1 and 10 μM) were added to the cells. Subsequently, Annexin V conjugated to Alexa fluor 488 was added and time-lapse imaging of phosphatidylserine (PS) translocation was performed as previously described (Puigvert et al., 2010) for a period of 15 hours. Cells incubated with the caspase inhibitor z-VAD-fmk (100 μM) were used as negative control. DIC and fluorescence imaging were performed using BD Pathway™ 855 imager (Becton Dickinson) under controlled conditions (37°C, 5% CO₂). Quantitative image analysis was performed as described before (Puigvert et al., 2010). The total fluorescent intensity per image and the total cell area were quantified using Image Pro (Media Cybernetics, 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 forty-eight hours to induce DCLK KD or 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 hours later, the cells were fixed with 4% paraformaldehyde (Added Pharma B.V., Oss, The Netherlands). Apoptotic cells with fragmented DNA were detected by ApopTag® 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 microscope Leica DM 400 M.

**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 the 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 non-exclusive drugs that have independent modes of action is: $CI = (D_1)/(D_{x1}) + (D_2)/(D_{x2}) + (D_1)(D_2)/(D_{x1})(D_{x2})$. 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 $(D_{x1})$ and $(D_{x2})$ when the compounds are used in combination. The calculation of CI takes into account the potency (median effect of the dose $(D_m)$ or IC50) and the 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 synergistic combination that can be reduced at a certain effect level when compared with the doses of each treatment alone (Chou and Talalay, 1984).

**Caspase-3 activity assay.** We performed a caspase-3 activity assay as previously described (Qin et al., 2011) with modification. In brief, N1E-115 cells were grown in 96-well μclear 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 hours. Then, cells were lysed on ice for 1 hour with 5x lysis buffer (250 mM Hepes pH 7.4, 25 mM CHAPS and protease inhibitor cocktail (1 tablet for 10 ml, Roche Diagnostics GmbH)). After caspase-3 substrate Ac-DEVD-AMC was added to the cells to a final concentration of 25 μM, the fluorescence was measured for 40 minutes 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, Offenburg, Germany). The caspase-3 substrate has a fluorescent 7-amino-4-methylcoumarin (AMC) probe and the caspase-3 activity was expressed as pmol activity/min/mg protein using the fluorescent AMC as standard. Protein concentration was determined using the Pierce BCA protein assay (Thermo Fisher Scientific, Landsmeer, The Netherlands).

**Caspase-8 activity.** The activity of caspase-8 was determined using Caspase-Glo® 8 assay and following the manufacturing instructions. In brief, N1E-115 neuroblastoma cells were seeded in 96 well-plates and transfected with 50 μM 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 hours. Subsequently, 75 μl of Caspase-Glo 8 reagent with MG-132 inhibitor was added per well to equal volume of medium with or without 2 μM caspase-8 inhibitor Z-IETD-FMK (Enzo Life Sciences Antwerpen, Belgium). 10 units/ml purified caspase-8 enzyme (#BML-SE172, Enzo Life Sciences, Antwerpen, Belgium) was used as positive control. After 30 minutes incubation, caspase-8 activity was assessed using Centro XS³ Microplate Luminometer LB 960 (Berthold Technologies, Vilvoorde, Belgium) and results were normalized to total amount of protein, which was determined using the Pierce BCA protein assay (Thermo Fisher Scientific, Landsmeer, The Netherlands). Two independent experiments were performed.
ATP bioluminescence assay. ATP levels were assessed by luminescence using ELITEN® rLuciferase/Luciferin Reagent (Promega Benelux, Leiden, The Netherlands) according to the manufacture’s instruction and using the microplate luminometer CentroXS³ LB 960 (Berthold Technologies, Vilvoorde, Belgium). Results were normalized to the amount of protein that was determined performing Pierce BCA protein assay (Thermo Fisher Scientific, Landsmeer, The Netherlands).

Statistical analysis. Results presented are representative of three independent experiments run in triplicates, unless otherwise indicated. Student’s t-test and two-way ANOVA were performed using GraphPad Prism 4.00 (GraphPad software, La Jolla, USA) and SPSS statistical software version 17.0 (SPSS Inc. Chicago, IL, USA). When appropriate, significant interactions were further analyzed by post-hoc comparisons using Tukey’s LSD test. P values smaller than 0.05 were considered statistically significant. Results are expressed as mean ± standard error of the mean (SEM).

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 non-targeting siRNA (negative control, NC) and treated 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). Similarly, 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 using 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 as compared to NB cells transfected with negative control siRNA (Fig. 1A). Additionally, in mitotic cells the morphology of the mitotic spindles was altered, being 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 with 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 around 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 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. in HeLa cells treated with 100 μM VBL (Jordan et al., 1992). 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) inducing DCLK knockdown and 48 hours later 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; Table 1 and 2). We did not detect a significant difference between cells treated
with non-targeting siRNA (NC) and non-transfected cells (Supplemental 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 co-treated with siDCL-2 and a 12.5 fold reduction when co-treated with si-DCL-3. In SH-SY5Y human NB cells, we observed a significant (p < 0.001) though less pronounced difference in EC50 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 EC50 for VBL treatment with both siDCLK-4 and siDCLK-5 was detected, no significant difference was obtained when compared to VBL with non-targeting 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 hours with different doses of VCR or VBL and different concentrations of siRNAs as described in the Material and Methods section. Fig. 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 no treated cells (Supplemental Fig. 2). The determination of siRNA doses was based on results obtained previously, which demonstrated that 50 nM and 200 nM siRNA was enough to induce significant knockdown in N1E-115 and SH-SY5Y cells, respectively (Verissimo et al., 2010). Since the present (Fig. 1 and 2; Table 1 and 2) and previous studies (Verissimo et al., 2010) show no significant differences between the two siRNAs used to induce DCLK knockdown in N1E-115
and in SH-SY5Y cells, we decided to proceed 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 using the MTT assay (Mosmann, 1983). The data was analyzed by means of CalcuSyn software (Chou and Talalay, 1984). This software provided combined index (CI) values based on multi-drug dose-effect calculations using the median method, as described in Material and Methods section. Synergism (CI lower than 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, synergism was consistently detected at higher doses of siRNA and VAs than in N1E-115 cells (Table 3).

We also determined the dose-reduction index (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 of VBL from 5.1 to 146.4. In SH-SY5Y, 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 siRNAs 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 if the cells die via an apoptotic process, as previously observed with independent DCLK knockdown (Verissimo et al., 2010) and VAs (Marimpietri et al., 2007; Pourroy et al., 2004), we performed four different apoptotic assays (Fig. 4 and 5; Supplemental Fig. 3).
115 NB cells express DCLK while 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, as 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 in the Material and Methods section and studied with time-lapse imaging of phosphatidylserine translocation (Fig. 4 and Supplemental Movie) and a TUNEL-based assay (Supplemental Fig. 3 and 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 ANOVA 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 ($p \leq 0.001$). 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 non-targeting siRNA and treated with VBL (see Fig. 4B). For a more clear 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). Since 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 multi-drug dose effects to determine if there was synergism between the effect of DCLK knockdown and VAs (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 hours combined treatment (VBL + DCLK KD) onwards, strong and very strong synergism was detected. At earlier time points we observed no synergism, which may be due to the limited time for the action of VBL. At high concentrations of siRNA/VBL (50 nM/1 μM), synergism was detected from 10 hours treatment onwards. For low concentrations (12.5 nM/0.01 μM or 25 nM/0.1 μM) synergism was identified from 5 hours onwards. These results indicate that the synergism is dose and time dependent. At high concentrations the effect of the compounds alone is probably too strong to detect the additive or synergetic combined effect. However, a synergistic effect becomes clear at longer time exposure of the combined treatment (Supplemental Table 1).

The occurrence of a synergistic apoptotic effect was further investigated 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 combined index (CI) using CalcuSyn software (Chou and Talalay, 1984). We obtained CI values lower than 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 less TUNEL-positive cells comparing to 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 a 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 if an extrinsic apoptotic pathway is involved the synergistic apoptotic process in NB cells treated with VAs (VCR or VBL) and DCLK knockdown.

N1E-115 NB cells were treated separately or in combination with different concentrations of VAs (VCR or VBL) and DCLK knockdown (siDCL-3 transfection). A non-targeting siRNA was used as negative control (NC) for transfection (see Material and Methods section). As in non-transfected 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 VAs 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 if the extrinsic apoptotic pathway is activated in treated NB cells, we have 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 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 to VAs alone. The induction of caspase-8 activity was inhibited using the inhibitor Z-IETD-FMK.

**DCLK silencing induces changes in mitochondria activity.** We have previously 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). Since changes in mitochondria activity due to DCLK knockdown might contribute to the synergistic apoptotic process detected with the combined treatment (DCLK KD + VAs), we have explored the effect of DCLK silencing on mitochondria activity. We checked the mRNA expression levels of two genes, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 (Ndufa2) and ATP synthase subunit epsilon (ATP5e), which play a crucial role on 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 to 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 Material 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 VAs 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 in this work permit a significant reduction in VAs doses required to effectively kill neuroblastoma cells in vitro.

VAs are important chemotherapeutic agents used in NB therapy. However, there is evidence that treatment of NB with VAs results in toxicity (Lobert et al., 1998) and in 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 investigate the combination of the VAs treatment with knocking down of the microtubule-stabilizing proteins encoded by DCLK gene by RNA interference technology. The novelty of this combined treatment has several aspects: 1) Since 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 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) Since VAs lead to the development of drug resistance through an increase of microtubule-stabilizing proteins (Don et al., 2004; Lobert et al., 1998), 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 highly expressed in human NBs (Verissimo et al., 2010); 5) Silencing of DCLK leads to disruption of mitotic spindles and induces apoptosis (Shu et al., 2006; Verissimo et al., 2010; Vreugdenhil et al., 2007). Therefore, the combined VAs + DCLK KD approach might be more effective than pre-existing approaches for NB.

Our data indicate that silencing the expression of DCLK sensitizes NB cells for microtubules-destabilizing agents VCR and VBL. This sensitizing effect was more effective in N1E-115 than in SH-SY5Y NB cells. This difference might be due to the fact that 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 (Deuel et al., 2006; Koizumi et al., 2006). Future studies are needed to investigate whether or not the expression of DCX in SH-SY5Y compensates for DCLK knockdown and provides resistance of these cells towards VAs. An alternative explanation might be related to the achieved knockdown levels of DCLK, as 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 activation of caspase-3 (Fan et al., 2001) and silencing of DCLK leads to 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 to 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 pro-apoptotic Bax and inactivation of anti-apoptotic Bcl-2. In WSU-CLL cells, VCR and VBL induced Bcl2 phosphorylation (Moon and Lerner, 2002). DCLK knockdown leads to activation of the intrinsic apoptotic process in NB cells as well, with up-regulation of Bax and down-regulation of Bcl2 (Verissimo et al., 2010). Therefore, our results suggest that the combined treatment results in 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, the 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 VAs-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 slowing or blocking of cell-cycle at metaphase-anaphase transition and induction of apoptosis (Jordan and Wilson, 2004). The mitotic arrest at low concentrations is due to alterations in microtubule dynamics rather than microtubule depolymerization (Jordan and Wilson, 2004). We detected less synergism at higher concentrations of VAs, probably due to 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).

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 homologue DCX (Shu et al., 2006). DCLK, like DCX, has two microtubule-binding domains (DC-domains) and a Ser/Pro-rich region (Vreugdenhil et al., 2007). Each DC-domain binds tubulin and microtubules in a different way (Kim et al., 2003). Since one DC-domain interacts with tubulin dimers and the other domain 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 to enhance longitudinal interactions, which is an outstanding way for crosslinking protofilaments and increasing microtubule stability (Moores et al., 2004). Thus, 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 the disruption of the electron transport, oxidative phosphorylation, and adenosine triphosphate (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) in changes in mitochondria activity and in apoptosis (Groninger et al., 2002; Moon and Lerner, 2002; 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. Future in vivo studies are needed to confirm the efficacy of the combined treatment and to establish its significance as potential therapeutic approach.

In summary, the silencing of DCLK, which is highly expressed 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 due to the independent action of VAs and DCLK knockdown inducing mitotic spindle disruption and alteration on the mitochondria activity. Future in vivo studies would be of high interest to validate the decrease of tumorigenicity.

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Authorship Contributions

Participated in research design: Carla S. Verissimo, A. Vroon, Carlos P. Fitzsimons and Erno Vreugdenhil

Conducted experiments: Carla S. Verissimo and S. Cheng

Contributed new reagents or analytic tools: Jordi C. Puigvert, Y. Qin, L. Price, Erik H. J. Danen, Bob van de Water

Performed data analysis: Carla S. Verissimo and S. Cheng

Wrote or contributed to the writing of the manuscript: Carla S. Verissimo, J. van Deutekom, Carlos P. Fitzsimons and Erno Vreugdenhil
References


**Footnotes**

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Legends for Figures

Fig. 1. N1E-115 neuroblastoma cells treated with vinca alkaloids (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 hours and subsequently treated with 50 μM vinblastine (VBL), vincristine (VCR) or vehicle (milli-Q) for 6 hours. A, cells transfected with negative control (NC) siRNA and treated with vehicle; B, percentage of cells without paracrystals (white bars), cells with tubulin paracrystals (grey bars) or cells with all tubulin reduced to globular paracrystals of tubulin (black bars); C, cells transfected with siDCL-2 and treated with vehicle; D, cells transfected with siDCL-3 and treated with vehicle; E, cells transfected with NC siRNA and treated with VBL; F, cells transfected with NC siRNA and treated with VCR; G, cells transfected with siDCL-2 and treated with VBL; H, cells transfected with siDCL-3 and treated with VBL; I, cells transfected with siDCL-2 and treated with VCR; J, cells transfected with siDCL-3 and treated with VCR. Arrows, examples of tubulin paracrystals; *, examples of globular paracrystals of tubulin. To determine the percentage of cell with paracrystals, a minimum of 100 cells were counted in triplicate per treatment from two independent experiments. The images were obtained with a magnification of 120x. Scale bars, 15 μm.

Fig. 2. Dose-response of N1E-115 and SH-SY5Y neuroblastoma cells transfected with siRNAs for 48 hours to induce DCLK knockdown and treated with different concentrations of vincristine (VCR) or vinblastine (VBL) ranging from 100 nM to 1000 μM. A, N1E-115 cells transfected with 50 nM siDCL-2, siDCL-3 or negative control (NC) siRNAs and treated with VCR for 6
hours. B, N1E-115 cells transfected with 50 nM siDCL-2, siDCL-3 or NC and treated with VBL for 6 hours. C, SH-SY5Y cells transfected with 200 nM siDCLK-4, siDCLK-5 or NC siRNAs and treated with VCR for 15 hours. D, SH-SY5Y cells transfected with 200 nM siDCLK-4, siDCLK-5 or NC and treated with VBL for 15 hours. These results were obtained using the live/dead double staining assay and show the mean of percentage of dead cells detected in one representative experiment from two performed independently (N=10). Bars, SEM. ***, P < 0.001.

**Fig. 3.** Expression of DCLK and α-tubulin in N1E-115 and SH-SY5Y neuroblastoma cells transfected for 48 hours with siDCL-3, siDCLK-4 or negative control (NC) siRNAs. A, western blotting results of N1E-115 cells transfected with different concentrations (12.5, 25, 50 or 100 nM) of siDCL-3 or with 100 nM NC siRNA. B, quantification and normalization of DCLK expression to α-tubulin in N1E-115 cells. C, DCLK and α-tubulin expression in SH-SY5Y transfected with 50, 100, 200 or 400 nM siDCLK-4 or 400 nM NC siRNA. D, quantification and normalization of the DCLK expression to α-tubulin in SH-SY5Y. DCLK, DCLK-derived MAP of 40 kDa (DCL). A and C, representative gels of two run independently; columns, mean of four measurements of the relative optical density in the representative gel shown (N=4); bar, SEM. *, p<0.05. ***, P<0.001.

**Fig. 4.** Apoptosis studies in N1E-115 neuroblastoma cells transfected with siDCL-3 or negative control (NC) siRNAs for 48 hours and treated with vincristine (VCR), vinblastine (VBL) or vehicle (milli-Q water) using time-lapse imaging of phosphatidylserine translocation. Time-lapse imagining was started immediately after adding VBL, VCR or vehicle (Veh). A, time-lapse
imaging of phosphatidyl-serine 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 hours after starting the assay. The images were taken at 30 minutes intervals (see Supplemental Movie). B, ratio of Annexin V (AnxV) by the total cell area over time of cells treated with 0.1 μM VBL or vehicle. C, area under the curve (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; siDCL-3, cells transfected with siDCL-3 and treated with vehicle; VBL, cells transfected with NC siRNA and treated with VBL; VCR, cells transfected with NC siRNA and treated with VCR; siDCL-3 + VBL, cells transfected with siDCL-3 and treated with VBL; siDCL-3 + VCR, cells transfected with siDCL-3 and treated with VCR; siDCL-3 + VBL + Casp. Inhib., cells transfected with siDCL-3, treated with VBL and caspase inhibitor z-VAD-fmk; siDCL-3 + VCR + Casp. Inhib., cells transfected with siDCL-3, treated with VCR and caspase inhibitor z-VAD-fmk. 20x magnification. Scale bars, 500 μm.

Data points and columns, mean of two technical replicates within one representative experiment of two independent experiments; bars, SEM. *P<0.05; **P<0.01.

**Fig. 5.** Caspase-3 and -8 activity in N1E-115 neuroblastoma cells transfected for 48 hours with siDCL-3 or negative control (NC) siRNAs and treated for 15 hours with vincristine (VCR), vinblastine (VBL) or vehicle (milli-Q water). A and B, detection of caspase-3 activity by measurement of the cleaved fluorescent AMC from Ac-DEVD-AMC, a caspase-3 substrate. C and D, caspase-8 activity in relative light units (RLU) normalized for the amount of protein (RLU/mg protein). A and C, N1E-115 cells transfected with siRNAs and treated with different concentrations of VBL (0.01, 0.1, 1 and 10 μM). NC, cells transfected with NC siRNA and treated with vehicle; siDCL-3, cells transfected with siDCL-3 and treated with vehicle; VBL, cells transfected with NC siRNA and treated with VBL; VCR, cells transfected with NC siRNA and treated with VCR; siDCL-3 + VBL, cells transfected with siDCL-3 and treated with VBL; siDCL-3 + VCR, cells transfected with siDCL-3 and treated with VCR; siDCL-3 + VBL + Casp. Inhib., cells transfected with siDCL-3, treated with VBL and caspase inhibitor z-VAD-fmk; siDCL-3 + VCR + Casp. Inhib., cells transfected with siDCL-3, treated with VCR and caspase inhibitor z-VAD-fmk. 20x magnification. Scale bars, 500 μm.

Data points and columns, mean of two technical replicates within one representative experiment of two independent experiments; bars, SEM. *P<0.05; **P<0.01.
concentrations (0.01, 0.1, 1 or 10 μM) of VCR. B and D, cells transfected and treated with different concentrations (0.01, 0.1, 1 or 10 μM) of VBL. NC, cells transfected with NC siRNA and treated with vehicle; siDCL-3, cells transfected with siDCL-3 siRNA and treated with vehicle; VBL, cells transfected with NC siRNA and treated with VBL; VCR, cells transfected with NC siRNA and treated with VCR; siDCL-3 + VBL, cells transfected with siDCL-3 and treated with VBL; siDCL-3 + VCR, cells transfected with siDCL-3 and treated with VCR; siDCL-3 + VBL + Inhibitor, cells transfected with siDCL-3, treated with VBL and z-VAD-fmk (A and B) or with z-IETD-fmk (C and D); siDCL-3 + VCR + Inhibitor, cells transfected with siDCL-3, treated with VBL and z-VAD-fmk (A and B) or with z-IETD-fmk (C and D). Columns, mean of two technical replicates within one representative experiment; bars, SEM. *P<0.05; **P<0.01.

**Fig. 6.** Effect of DCLK-derived MAP (DCL) knockdown on mitochondria activity. A, fold-change of Ndufa2 and ATP5e mRNA levels in neuroblastoma stable cell lines with (Dox) or without (Veh) DCLK knockdown; B, fold change of relative light units (RLU) in stable cell lines with (Dox) or without (Veh) DCLK knockdown. The fold-change was calculated by normalizing to the control N1E-115 neuroblastoma cell line that expresses DCLK-derived MAPs treated with Dox or Veh respectively. Two neuroblastoma stable cell lines were used (see Material and Methods). These stable cell lines, in the presence of doxycycline (Dox), express a shRNA (shDCL-2 or shDCL-3) that targets DCLK-derived MAP mRNA (Verissimo et al., 2010). mRNA levels were normalized to 18S rRNA. ATP levels were detected using the ATP luminescence assay and the results were normalized to the total amount of protein. Veh, vehicle (milli-Q water); Dox, doxycycline. Bars, SEM. *, P<0.05. ***, P<0.001.
Fig. 7. Hypothetical model for the consequences of NB treatment with DCLK silencing, vinca alkaloids (VAs) or the combination. Both treatments cause independently the disruption of microtubule spindles and DCLK silencing induces mitochondrial activity changes. The combined treatment results in a synergistic apoptotic process. DCLK (red) binds to α/β-tubulin heterodimers and microtubules, being involved in microtubule polymerization and stabilization. DCLK silencing results in disruption of microtubule spindles. VAs (orange) interact with the microtubules and with β-tubulin of α/β-tubulin heterodimers, blocking microtubule polymerization, inducing the formation of pracrystaline aggregates and disruption of microtubule spindles. The consequences of the combined treatment converge at the disruption of the microtubule spindles and changes in the mitochondria activity, leading to a synergistic caspase-dependent apoptosis process. This figure is based on our results and on the following literature: (Cierpicki et al., 2006; Jordan et al., 1992; Jordan and Wilson, 2004; Moores et al., 2004; Pourroy et al., 2004; Shu et al., 2006; Verissimo et al., 2010; Vreugdenhil et al., 2007).
TABLE 1

DCLK knockdown sensitzes N1E-115 neuroblastoma cells for vinca alkaloids. EC50 values (μM) for dose-response curves shown in Fig. 2A-B

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>EC50 (μM) ± SEM</th>
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<tbody>
<tr>
<td>N1E-115</td>
<td>NC + VCR$^5$</td>
<td>182.390 ± 117.627</td>
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<tr>
<td></td>
<td>siDCL-2 + VCR</td>
<td>32.434 ± 1.345$^*$</td>
</tr>
<tr>
<td></td>
<td>siDCL-3 + VCR</td>
<td>25.061 ± 4.857$^{**}$</td>
</tr>
<tr>
<td></td>
<td>NC + VBL$^5$</td>
<td>238.781 ± 63.117</td>
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<tr>
<td></td>
<td>siDCL-2 + VBL</td>
<td>11.298 ± 4.285$^*$</td>
</tr>
<tr>
<td></td>
<td>siDCL-3 + VBL</td>
<td>19.099 ± 6.728$^*$</td>
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</tbody>
</table>

NC, negative control siRNA.

siDCL-2 and siDCL-3, siRNAs that induce DCLK silencing in N1E-115 cells.

$^5$, used as reference.

*, p < 0.05; **, p < 0.01
TABLE 2

DCLK knockdown sensitizes SH-SY5Y neuroblastoma cells for vinca alkaloids. EC50 values (μM) for dose-response curves shown in Fig. 2C-D

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>EC50 (μM) ± SEM</th>
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<tr>
<td></td>
<td>NC + VCR 5</td>
<td>114.815 ± 17.008</td>
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<td></td>
<td>siDCLK-4 + VCR</td>
<td>54.702 ± 11.138</td>
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<tr>
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<td>siDCLK-5 + VCR</td>
<td>33.113 ± 11.021</td>
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<tr>
<td>SH-SY5Y</td>
<td>NC + VBL 5</td>
<td>56.702 ± 17.517</td>
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<tr>
<td></td>
<td>siDCLK-4 + VBL</td>
<td>29.107 ± 8.417</td>
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<tr>
<td></td>
<td>siDCLK-5 + VBL</td>
<td>28.840 ± 8.950</td>
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</table>

NC, negative control siRNA.

siDCLK-4 and siDCLK-5, siRNAs that induce DCLK knockdown in SH-SY5Y.

5, used as reference.

*, p< 0.05
TABLE 3

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

<table>
<thead>
<tr>
<th>Ratio</th>
<th>CI</th>
<th>DRI</th>
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<tr>
<td>siDCLK-3/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCR or VBL</td>
<td>(nM/μM)</td>
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<tr>
<td>12.5/0.01</td>
<td>0.424 (Synergism)</td>
<td>3.349 7.965</td>
</tr>
<tr>
<td>25/0.1</td>
<td>0.343 (Synergism)</td>
<td>4.623 7.875</td>
</tr>
<tr>
<td>50/1</td>
<td>0.469 (Synergism)</td>
<td>3.170 6.493</td>
</tr>
<tr>
<td>100/10</td>
<td>0.815 (Moderate synergism)</td>
<td>1.630 4.967</td>
</tr>
<tr>
<td>siDCLK-3/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VBL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/0.01</td>
<td>0.153 (Strong synergism)</td>
<td>146.418 6.858</td>
</tr>
<tr>
<td>25/0.1</td>
<td>0.263 (Strong synergism)</td>
<td>37.975 4.223</td>
</tr>
<tr>
<td>50/1</td>
<td>0.517 (Synergism)</td>
<td>8.407 2.512</td>
</tr>
<tr>
<td>100/10</td>
<td>0.730 (Moderate synergism)</td>
<td>5.139 1.866</td>
</tr>
<tr>
<td>siDCLK-4/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCR or VBL</td>
<td>(nM/μM)</td>
<td></td>
</tr>
<tr>
<td>50/0.1</td>
<td>0.066 (very strong synergism)</td>
<td>2.631E+3 15.151</td>
</tr>
<tr>
<td>100/1</td>
<td>0.199 (strong synergism)</td>
<td>22.630 6.442</td>
</tr>
<tr>
<td>200/10</td>
<td>0.256 (strong synergism)</td>
<td>86.838 4.098</td>
</tr>
<tr>
<td>400/100</td>
<td>0.372 (synergism)</td>
<td>567.143 2.700</td>
</tr>
<tr>
<td>siDCLK-4/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VBL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50/0.1</td>
<td>4.646 (Strong antagonism)</td>
<td>0.221 8.358</td>
</tr>
<tr>
<td>100/1</td>
<td>0.545 (Synergism)</td>
<td>2.936 4.898</td>
</tr>
<tr>
<td>200/10</td>
<td>0.427 (Synergism)</td>
<td>14.823 2.781</td>
</tr>
<tr>
<td>400/100</td>
<td>0.576 (Synergism)</td>
<td>1.440E+3 1.739</td>
</tr>
</tbody>
</table>

**Note:** The combined index (CI) is based on the formula: CI = (D1/(Dx1)+1+D2/(Dx2)+1)1/(D1/(Dx1)+2+D2/(Dx2)+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).
Figure 1
Figure 2
Figure 3

Panel A: Western blot of N1E-115 cells showing the expression of α-Tubulin and DCLK under different concentrations of siDCL-3 (NC, 12.5, 25, 50, 100 nM).

Panel B: Bar graph showing the normalized DCLK expression levels in N1E-115 cells treated with siDCL-3.

Panel C: Western blot of SH-SY5Y cells showing the expression of α-Tubulin and DCLK under different concentrations of siDCL-4 (NC, 50, 100, 200, 400 nM).

Panel D: Bar graph showing the normalized DCLK expression levels in SH-SY5Y cells treated with siDCL-4.
Figure 4
Figure 6
Figure 7

- **DCLK silencing**
- **No treatment**
- **DCLK silencing + Vinca alkaloids**
- **Vinca alkaloids**

Altered expression of mitochondria-related genes

Disruption of microtubule spindles

Changed Mitochondrial activity

Apoptosis

Legend:
- DCLK
- α/β-tubulin heterodimers
- Vinca alkaloid
- Paracrystalline aggregates of tubulin
Combining doublecortin-like kinase silencing and vinca alkaloids results in a synergistic apoptotic effect in neuroblastoma cells

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Supplemental Figures

Supplementary Fig. 1. Dose-response of N1E-115 and SH-SY5Y neuroblastoma cells treated only with vinca alkaloids (vincristine (VCR) or vinblastine (VBL)) or transfected with negative control siRNA (NC) and treated with different concentrations of VCR or VBL (VCR + NC or VBL + NC). The concentration of VCR and VBL ranges from 100 nM to 1000 µM. A, N1E-115 cells treated only with VCR or transfected with 50 nM NC siRNAs and treated with VCR for 6 hours. B, N1E-115 cells treated only with VBL or
transfected with 50 nM NC siRNA and treated with VBL for 6 hours. C, SH-SY5Y cells transfected with 200 nM NC siRNAs and treated with VCR for 15 hours or treated only with VCR. D, SH-SY5Y cells transfected with 200 nM NC siRNA and treated with VBL for 15 hours or treated only with VBL. These results were obtained using the live/dead double staining assay and show the mean of percentage of dead cells detected in one representative experiment from two performed independently (N=10). Bars, SEM.

Supplemental Fig. 2. Expression of DCLK and α-tubulin in N1E-115 neuroblastoma cells transfected for 48 hours with different concentrations negative control (NC) siRNA (12.5, 25, 50 and 100nM), treated with lipofectamine only (Mock) or not treated (Not Transf).
Supplemental Fig. 3. Detection of tunnel positive neuroblastoma cells after treatment. N1E-115 neuroblastoma cells treated with vincristine (VCR) or vinblastine (VBL), siDCL-3 to silence DCLK or the combination of vinca alkaloids (VCR or VBL) and siDCL-3. Cells were transfected with 50 nM of siDCL-3 or negative control (NC) siRNA for 48 hours. Subsequently, 0.1 M VCR or VBL was added for 15 hours. A, cells are stained in red for DNA fragmentation (TUNEL+) and counterstained with Hoechst (blue). B, Percentage of positive cells for TUNEL staining, indicating the percentage of apoptotic cells with fragmented DNA. The images were obtained with a magnification of 20x. Scale bars, 100 m. Inhib., cells were treated with z-VAD-fmk caspase inhibitor. Columns, mean of three independent experiments (N=15); bars, SEM. *, P<0.05; **, P<0.01.
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**SUPPLEMENTARY TABLE 1**

Combined index (CI) values and dose-reduction index (DRI) for the combination of DCLK knockdown (siDCL-3) and vinblastine (VBL) on the translocation of phosphatidylserine in N1E-115 neuroblastoma cells at different time points after adding VBL

<table>
<thead>
<tr>
<th>Time after adding VBL</th>
<th>Ratio siDCL-3/ VBL (nM/µM)</th>
<th>CI</th>
<th>DRI VBL</th>
<th>DRI siDCL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>0.295 (Strong synergism)</td>
<td>3.385</td>
<td>2.19E+05</td>
<td></td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>0.128 (Strong synergism)</td>
<td>7.812</td>
<td>4.50E+06</td>
<td></td>
</tr>
<tr>
<td>50/ 1</td>
<td>1.143 (Slight antagonism)</td>
<td>0.875</td>
<td>2.57E+06</td>
<td></td>
</tr>
<tr>
<td><strong>7.5 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>0.351 (Synergism)</td>
<td>2.849</td>
<td>9.03E+04</td>
<td></td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>0.051 (Very strong synergism)</td>
<td>19.708</td>
<td>6.68E+05</td>
<td></td>
</tr>
<tr>
<td>50/ 1</td>
<td>11.67 (Very strong antagonism)</td>
<td>0.086</td>
<td>4.55E+04</td>
<td></td>
</tr>
<tr>
<td><strong>10 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>0.062 (Very strong synergism)</td>
<td>16.852</td>
<td>415.648</td>
<td></td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>8.68E-05 (Very strong synergism)</td>
<td>1.02E+07</td>
<td>1.15E+04</td>
<td></td>
</tr>
<tr>
<td>50/ 1</td>
<td>0.692 (Synergism)</td>
<td>5.326</td>
<td>252.559</td>
<td></td>
</tr>
<tr>
<td><strong>12.5 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>0.002 (Very strong synergism)</td>
<td>1.56E+06</td>
<td>447.995</td>
<td></td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>2.17E-08 (Very strong synergism)</td>
<td>1.17E+40</td>
<td>4.62E+07</td>
<td></td>
</tr>
<tr>
<td>50/ 1</td>
<td>0.001 (Very strong synergism)</td>
<td>2.74E+10</td>
<td>1001.229</td>
<td></td>
</tr>
<tr>
<td><strong>15 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>9.77E-05 (Very strong synergism)</td>
<td>1.31E+04</td>
<td>4.68E+04</td>
<td></td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>9.32E-41 (Very strong synergism)</td>
<td>2.23E+56</td>
<td>1.07E+40</td>
<td></td>
</tr>
<tr>
<td>50/ 1</td>
<td>1.68E-35 (Very strong synergism)</td>
<td>8.99E+47</td>
<td>5.97E+34</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The combined index (CI) is based on the formula: CI = (D\_1/\text{D\_1})+(D\_2/\text{D\_1})+(D\_1/\text{D\_2})+(D\_2/\text{D\_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).
### Supplemental TABLE 2

Combined index (CI) values and dose-reduction index (DRI) for the combination of DCLK knockdown (siDCL-3) with vincristine (VCR) or vinblastine (VBL) on the induction of DNA fragmentation (TUNEL based assay) in N1E-115 neuroblastoma cell line

<table>
<thead>
<tr>
<th>Ratio siDCL-3/ VCR or VBL (nM/µM)</th>
<th>CI</th>
<th>DRI VCR or VBL</th>
<th>DRI siDCL-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>siDCL-3/ VCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>0.305 (Synergism)</td>
<td>3.293</td>
<td>1142.674</td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>0.678 (Synergism)</td>
<td>1.475</td>
<td>1.13E+04</td>
</tr>
<tr>
<td>50/ 1</td>
<td>0.493 (Synergism)</td>
<td>2.03</td>
<td>1.03E+06</td>
</tr>
<tr>
<td>100/ 10</td>
<td>0.514 (Synergism)</td>
<td>1.946</td>
<td>4.61E+07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>siDCL-3/ VBL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5/ 0.01</td>
<td>0.386 (Synergism)</td>
<td>2.596</td>
<td>1393.771</td>
</tr>
<tr>
<td>25/ 0.1</td>
<td>0.698 (Synergism)</td>
<td>1.434</td>
<td>2.03E+04</td>
</tr>
<tr>
<td>50/ 1</td>
<td>0.211 (Strong synergism)</td>
<td>4.747</td>
<td>1.02E+07</td>
</tr>
<tr>
<td>100/ 10</td>
<td>0.689 (Synergism)</td>
<td>1.452</td>
<td>4.61E+07</td>
</tr>
</tbody>
</table>