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A New Bis-Indole, KARs, Induces Selective M Arrest with Specific Spindle Aberration in Neuroblastoma Cell Line SH-SY5Y

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ABSTRACT

KARs, new semisynthetic antitumor bis-indole derivatives, were found to be inhibitors of tubulin polymerization with lower toxicity than vinblastine or vincristine, used in chemotherapy. Here, we compare the effect of KARs with those of vinblastine and vincristine on cell viability, cell proliferation, and cell cycle in neuroblastoma cell line (SH-SY5Y). At concentrations of the different compounds equivalent in causing 50% of inhibition of cell growth, KARs induced a complete arrest in the G_2/M phase, whereas vinblastine and vincristine induced a partial arrest in both G_0/G_1 and G_2/M. Moreover, a combination of KAR-2 and W13 (an anticalmodulin drug) qualitatively caused a similar arrest in both G_0/G_1 and G_2/M than vinblastine. Levels of cyclin A and B1 were higher in KARs-treated cells than in vinblastine- or vincristine-treated cells. Cdc2 activity was much higher in KAR-2 than in vinblastine-treated cells, indicating a stronger mitotic arrest. The effect of KAR2 and vinblastine on microtubules network was analyzed by immunostaining with anti-tubulin antibody. Results indicated that KAR-2-induces the formation of aberrant mitotic spindles, with not apparent effect on interphase microtubules, whereas vinblastine partially destroyed interphase microtubules coexisting with normal and aberrant mitotic spindles.

Antimitotic drugs are widely used in chemotherapy. They usually target the tubulin/microtubule network of the cytoskeleton, which is formed by an assembly of cytoplasmic tubulin dimers. Several synthetic and natural compounds interact specifically with tubulin and microtubules, fundamentally destroying their dynamic character and leading to cell death. A large number of these agents are plant-derived (Lin et al., 1988). Two Vinca alkaloids from *Catharanthus roseus*, vinblastine, and vincristine are widely used in cancer therapy (Rowinsky and Donehower, 1997). Both drugs inhibit the self-assembly of tubulin into microtubules at substoichiometric concentrations by forming a tubulin-drug complex at the end of a growing microtubule and thus blocking self-assembly (Wilson et al., 1976; Margolis et al., 1980). In addition, it is known that vinblastine induces G_2/M arrest and subsequent apoptosis in different cell lines (Fan et al., 2001). Although the primary target of these Vinca alkaloids is the microtubular network, we recently reported that they also bind to calmodulin and suspend its modulating effect (Molnár et al., 1995; Vértessy et al., 1998). Calmodulin is a ubiquitous Ca^{2+} receptor that is involved in cell proliferation and in the regulation of the cell cycle (Rasmussen and Means, 1989; Lu and Means, 1993), but also in other essential cell processes (Cohen and Kee, 1988). The addition of specific anticalmodulin drugs, such as W13, to cell cultures inhibits re-entry of growth-arrested cells into the cell cycle (G_0/G_1 transition), progression into and through the S phase, and entry and exit from mitosis (Sasaki and Hidaka, 1982; Chafouleas et al., 1984; Agell et al., 1998).

Numerous semisynthetic derivatives of Vinca alkaloids have been synthesized, in response to the extensive need for potent antimitotic agents in clinical chemotherapy, because vinblastine and vincristine have undesired side effects. We recently reported (Orosz et al., 1997a; Vértessy et al., 1998) that the new semisynthetic bis-indole derivative KAR-2, has high anti-microtubular and anti-tumoral activities and lower toxicity than the Vinca alkaloids used in chemotherapy. Moreover, it interacts with calmodulin in vitro but, in con-

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ABBREVIATIONS: KAR-2, 3′-(β-chloroethyl)-2′,4′-dioxo-3,5′-spiro-oxazolidino-4-deacetoxy-vinblastine; KAR-3, 3′-(β-chloroethyl)-2′,4′-dioxo-3,5′-spiro-oxazolidino-4-deacetoxyvincristine; KAR-4, 3′-allyl-2′,4′-dioxo-3,5′-spiro-oxazolidino-4-deacetoxy-vinblastine; PBS, Dulbecco's phosphate-buffered saline; FACS, fluorescence-activated cell sorting; PI, propidium iodide; FITC, fluorescein isothiocyanate; W13, *N*-[4-aminobutiyl]-5-chloro-2-naphthalenesulfonamide; Cdk, cyclin dependent kinase.

trast to vinblastine and vincristine, it does not exhibit anticalmodulin activity in vitro enzymatic assays (e.g., phosphofructokinase) (Orosz et al., 1997a,b; Orosz et al., 1999). In addition, we reported the synthesis of other bis-indole derivatives, KAR-3 and KAR-4, which are analogs of vinblastine and vincristine, respectively (see Fig. 1). These compounds are also powerful antimicrotubular agents with lower anticalmodulin activity and toxicity than vinblastine or vincristine, although they are more toxic and no more powerful than KAR-2. (Orosz et al., 1999).

In vertebrates, cell cycle is regulated by a family of Cdks formed by a catalytic subunit and a regulatory subunit called cyclin. Those kinases act sequentially during the cell cycle. Cdk4,6/cyclin D and Cdk2/cyclin E are essential during G_1 and G_1/S transition, Cdk2/cyclin A is active during S phase whereas Cdc2/cyclin A and Cdc2/cyclin B act during mitosis. Cylin A accumulates during S and G_2 phases and cyclin B during G_2 phase. During G_2 both cyclins bind to Cdc2. At G_2/M transition the complexes formed are suddenly activated by the Cdc25 phosphatase, which eliminates two inhibitory phosphates from the catalytic subunit. Cdc2 is responsible for nuclear lamina phosphorylation; consequently, nuclear envelope is disorganized during prophase. Cdc2 also phosphorylates histone H1 leading to chromatin condensation, which occurs at mitosis (Norbury and Nurse, 1992; Reed, 1992; Sherr, 1994; Morgan, 1997). Degradation of cyclin A and cyclin B during metaphase and anaphase, respectively, leads to Cdc2 inactivation and consequently to mitosis exit. When mitotic spindle is incorrectly formed, a checkpoint is activated that inhibits Cdc2 inactivation; consequently, mitosis exit and cell cycle progression. This checkpoint usually works by inhibiting degradation of mitotic cyclins A and B (Gong et al., 1995; Rudner and Murray, 1996). The objective of the present article is to characterize the effect of KARs at cellular levels compared with that of vinblastine and vincristine to asses the origin of the distinct behaviors manifesting in in vitro and in vivo conditions.

The fact that KARs are powerful antimicrotubular agents with lower anticalmodulin activity and toxicity than vinblastine or vincristine makes them promising antitumoral agents, especially KAR-2, which is the less toxic (Orosz et al., 1999). In this article, we compare the effects of KAR2, with those of some other bis-indole derivatives, on human neuroblastoma cell line (SH-SY5Y) at different levels. Analysis of DNA content by flow cytometry showed that treatment with vinblastine and vincristine caused a partial arrest in both G_0/G_1 and G_2/M phases, whereas KAR-2 specifically arrested cell cycle at G_2/M phase. Analysis of chromosome condensation and phospho-H3 (Hendzel et al., 1997), cyclin A and cyclin B1 expression levels, Cdc2 activity and microtubular network, showed that KAR-2-treated cells were arrested in mitosis with a majority of abnormal mitotic spindles. Interestingly, KAR-2 had no effect on interphase microtubules, whereas vinblastine partially destroyed the microtubular network of interphase cells on neuroblastoma cell line.

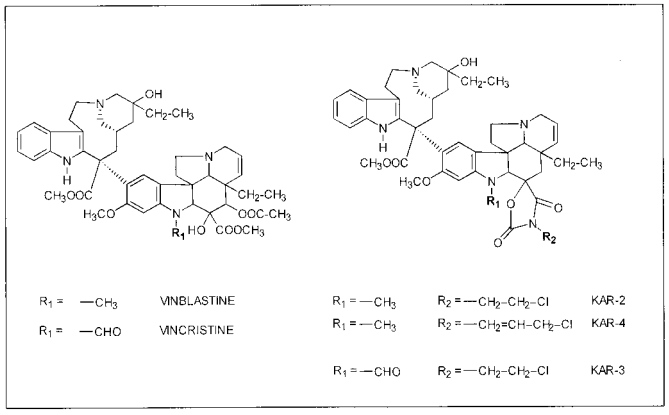

Fig. 1. Structural formula of vincristine, vinblastine, and their bis-indole derivatives KARs.

Materials and Methods

Cell Culture. Human neuroblastoma SH-SY5Y cells (a generous gift from Dr. Jacint Boix, University of Lleida, Spain) were cultured in Dulbecco's modified Eagle's medium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum (Biological Industries), and antibiotics: 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were grown at 37°C under an atmosphere of 5% CO_2. SH-SY-5Y cells were seeded in six-well plates, 4×10^5 cells/well, for all the experiments except where indicated. The final volume in all the dishes was 2 ml. Compounds to be tested were added to cultures 1 day after seeding to ensure uniform attachment of cells at the beginning of the experiments. The cell line used in this study was free of mycoplasma infection as shown by Gen-Probe Mycoplasma Tissue Culture NI Rapid Detection System (Fisher Scientific, Pittsburgh, PA)

Bis-Indole Derivative Treatment. The final concentrations of compounds used were: KAR-2 at 316 nM, KAR-3 at 316 nM, KAR-4 at 355 nM, vincristine at 14 nM, and vinblastine at 5 nM. These concentrations of each compound have been determined by microculture tetrazolium assay as equivalent in causing 50% of inhibition of cell growth in a previous study in 72 h (Orosz et al., 1999). Stocks were dissolved in phosphate-buffered saline (PBS) at 1 mg/ml for each compound and stored at −20°C. On the day of the experiment, compounds were diluted in culture medium. .

Inhibition of Cell Growth and Cell Cycle Analysis. Plasma membrane integrity was estimated by FACS analysis using two different methods. In the PI (Sigma Co, St. Louis, MO) staining method without cell permeabilization, cells were incubated with 18 μg/ml PI and 5 μg/ml RNase (Roche Molecular Biochemicals, Mannheim, Germany). The fluorescence of cells was analyzed by flow cytometry using an Epics XL flow cytometers (Beckman Coulter, Fullerton, CA). The second methods was FITC-Annexin V/PI double staining (Genzime, Cambridge, MA) (see *Assessment of Apoptosis*).

Neuroblastoma cells were harvested and stained in Tris-buffered saline containing 50 μg/ml PI, 10 μg/ml ribonuclease A (Sigma), and 0.1% Igepal CA-630 (Sigma) for 1 h at 4°C. DNA content was analyzed by FACS. Data from 12,000 cells were collected and analyzed using Multicycle program (Phoenix Flow Systems, San Diego, CA). All experiments were performed in triplicate

All statistical analysis was done using the Mathematica 3.0 program (Wolfram Research, Inc., Champaign, IL). We applied the parametric, unpaired, two-tailed independent sample t test with 95% confidence intervals [$\mu \pm 2.58$ (S.D.)], and $p < 0.05$ (*) was considered to indicate a significant difference.

Assessment of Apoptosis. Cells were seeded in six-well plates, 25×10^3 cells/well and, after a pre-equilibration period of 24 h, they were exposed to the bis-indole derivatives to be tested for a period of 24 or 48 h. Double-staining for FITC-Annexin V binding and for cellular DNA using PI was performed according the product insert. Cells were processed by flow cytometry. Approximately 3×10^4 cells were measured for each histogram.

Gel Electrophoresis, Immunoblotting, Immunoprecipitation, and Protein Kinase Assay. Cyclin A and cyclin B1 were analyzed using electrophoresis and immunoblotting, as described previously (Taulés et al., 1998). Monoclonal antibodies against cyclin

A (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:100 dilution, antibodies against cyclin B1 (Upstate Biotechnology, Lake Placid, NY) at 1:500 dilution. As a control of protein loading the blot membrane was also hybridized with polyclonal anti-Cdk4 at 1:500 dilution. The reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega, Madison, WI). The experiment was done at 24 h of incubation with the tested compounds. To determine Cdc2 activity levels, immunoprecipitations were performed as described previously (Taulés et al., 1998), except that 3 μg of anti-Cdc2 antibodies (Upstate Biotechnology) were used and 2 μg of histone H1 was used as substrate.

Immunocytochemistry. Cells were grown on glass coverslips coated with poly(D-lysine) (50 μg/ml at final concentration) (Sigma). For double-labeling, anti-β-tubulin (Roche) and PI were fixed in cold methanol for 2 min. After three washes in sterile PBS, cells were incubated 1 h at room temperature in a humidified atmosphere, with the specific monoclonal antibody anti-β-tubulin (1:50 dilution) with 1% ovalbumin. Coverslips were then washed three times (5 min each) in PBS and incubated for 1 h at room temperature in the dark with FITC-coupled anti-mouse-IgG secondary antibody (1:100 dilution; Promega). After three washes in PBS, RNase was added at a final concentration of 100 μl/ml for 30 min at room temperature in the dark and DNA stained with PI (1 μg/ml) for 1 min at room temperature in the dark. Cells were washed once in 0.1% PBS with Triton X-100 and twice in PBS. Finally, coverslips were mounted on glass slides with mowiol (Calbiochem, San Diego, CA).

For 33342 Hoechst staining, after three washes in PBS, cells were fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 15 to 30 min at room temperature and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at 4°C. After two washes in PBS, the coverslips were incubated with 33342 Hoechst (Sigma) at a final concentration of 5 μM in PBS, washed, and mounted on glass slides with mowiol. Cells were visualized using a confocal microscope (Leica TCSNT; Leica Lasertechnik, Heidelberg, Germany) and an Zeiss Axioskop optical microscope (Carl Zeiss GmbH, Jena, Germany).

For double labeling with anti-phospho- H3 and PI, cells were fixed in 95% (v/v) ethanol/5% (v/v) acetic acid for 5 min. After three washes in sterile PBS, cells were incubated 1 h at room temperature in a humidified atmosphere, with 8% (w/v) bovine serum albumin in PBS in the dark to block nonspecific sites. After three washes in PBS, coverslips were incubated overnight at 4°C with the specific monoclonal antibody anti-α-phospho-H3 (at a final concentration of 5 μg/ml; Upstate Biotechnology) with 1% bovine serum albumin. Coverslips were then washed three times (5 min each) in PBS and incubated for 1 h at room temperature in the dark with FITC-coupled goat anti-rabbit IgG secondary antibody (1:100 dilution; Sigma). After three washes in PBS, RNase was added at a final concentration of 100 μl/ml for 30 min at room temperature in the dark and DNA stained with PI (1 μg/ml) for 3 min at room temperature in the dark. Finally, coverslips were mounted on glass slides with Immuno Fluore mounting medium (ICN Biomedicals, Costa Mesa, CA). Cells were visualized using a laser scanning cytometer (LSC; CompuCyte, Cambridge, Massachusetts).

Results

Inhibition of the Cell Growth by the New Vinca Alkaloids, KARs. Cell cultures with the same number of cells were treated separately with vincristine (14 nM), vinblastine (5 nM), KAR-2 (316 nM), KAR-3 (316 nM), and KAR-4 (355 nM). These concentrations caused 50% inhibition of cell growth as determined by microculture tetrazolium assay (Orosz et al., 1999). The number of cells was counted at different times (8, 24, 48, and 72 h) after treatment and plotted against the percentage of initial number of cells (0 h). It was observed that all the compounds induced a similar time-dependent decrease in cell growth (Fig. 2). The effects of all these compounds became significant 24 h after the beginning of the treatment. Between 24 and 48 h, the decrease in cell growth was approximately 50%, and less than 13% of the cells survived after 72 h (Fig. 2). These results corroborate that the different concentrations of each compound selected for this study cause an equivalent mortality of cells at different times. Thus, these concentrations (which we will refer to as IC_{50} values) are suitable for the comparative studies reported in the following sections.

Treatment with KAR-2, KAR-3 and KAR-4 Completely Arrested Neuroblastoma Cells in G_2/M Phase of the Cell Cycle, with a Low Percentage of Apoptosis. The experiment was performed with asynchronously growing cells. In this population (0 h), most cells were in G_1 phase (48.7% ± 0.8%) or S phase (41.4% ± 1.1%), whereas 9.9% ± 0.5% were in the G_2/M phase (n = 37; mean ± S.E.M). To examine the effects of the cytotoxic agents at their IC_{50} values on cell cycle distribution, neuroblastoma cells, treated with each compound for 8, 24, and 48 h, were analyzed by

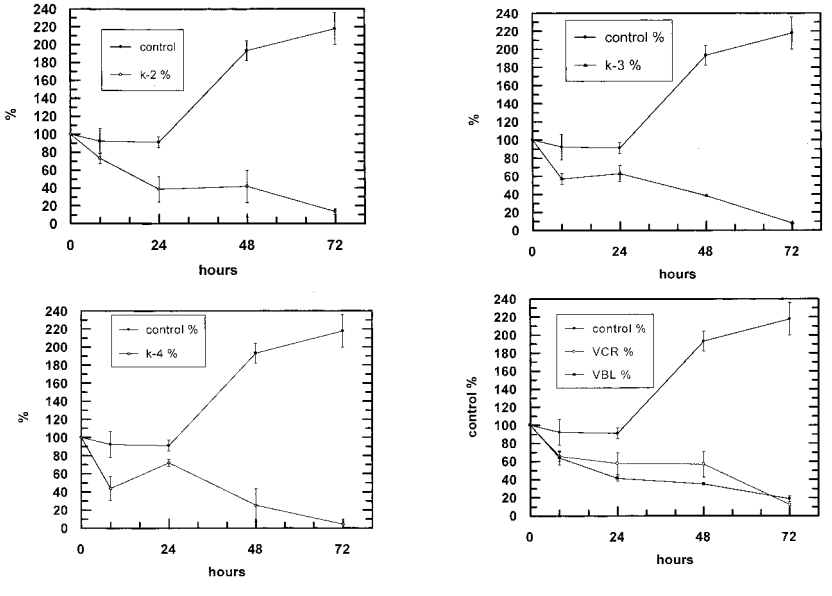

Fig. 2. Time-dependent antiproliferative effects of vinblastine (VBL), vincristine (VCR), and the new KAR bisindole derivatives on neuroblastoma SH-SY5Y cells. Cells (4×10^5 cells/well) were incubated for 0 to 72 h with the bis-indole derivatives. Every 8, 24, 48, and 72 h, the wells were trypsinized and cells were counted in a XL Coulter FACS. Cell numbers represent only viable cells, as measured by the exclusion of PI and the percentage is relative to the initial cell number. Results are given as mean ± S.E.M. of three independent experiments performed in triplicate. Control, ●; KAR-2, 316 nM (○); KAR-3, 316 nM (▲); KAR-4, 355 nM (◇); VBL, 5 nM (■); VRC, 14 nM (□). A, control and KAR-2. B, control and KAR-3. C, control and KAR-4. D, control, vincristine and vinblastine.

FACS (Fig. 3). Treatment with vincristine or vinblastine led to a different pattern of cell cycle distribution than treatment with KAR-2, KAR-3, or KAR-4. After 8 h of treatment, KAR-2, KAR-3, and KAR-4, similarly to vincristine and vinblastine, induced a small but significant decrease in the percentage of cells in G_1 phase and a small increase in percentage of cells in G_2/M and S phases with respect to nontreated cells. At 24 h of treatment with KARs, 65% of treated cells were in G_2/M phase, whereas less than 8% of cells remained in G_1. The percentage of cells in S phase increased with respect to control cells, except in the case of KAR-2–treated cells, which were not significantly different from control cells. In contrast, when cells were treated 24 h with vincristine or vinblastine, less than 50% of cells were in G_2/M and 36% of cells remained in G_1. The percentage of cells in S phase decreased for vinblastine and remained statistically unchanged with respect to control for vincristine.

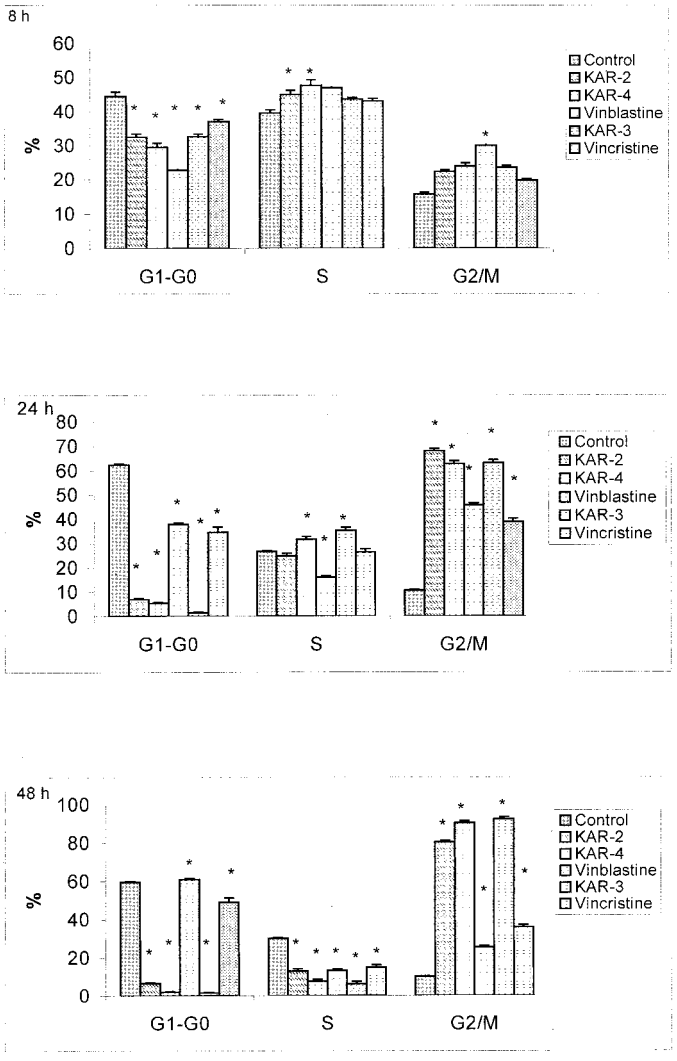

Fig. 3. Cell-cycle phase distribution in neuroblastoma cell line after vinblastine, vincristine, KAR-2, KAR-3, and KAR-4 treatment. Gray bars represent treated control tumor cell cycle frequency distribution (*y*-axis) expressed as percentage of sorting into the G_0-G_1, S, and G_2-M phases. The other bars represent KAR-2 (316 nM), KAR-4 (355 nM), vinblastine (5 nM), KAR-3 (316 nM), and vincristine (14 nM) treatment. After 8 h of treatment, cell populations were decreased in G_0/G_1 phases, whereas there was a small increase in both the S and G_2/M phases [$n = 12$, values represent mean ± SD., *, significantly different ($P < 0.01$)]. After 24 h of treatment, cells treated with KARs, vincristine, and vinblastine exhibited a decrease in G_0/G_1 phase, whereas there was an increase in the G_2/M phase; however, the effect of KARs was significantly different from that of vinblastine and vincristine ($n = 12$; mean ± SD.; *, $P < 0.01$). After 48 hr of treatment, cells treated with KARs, vincristine, and vinblastine exhibited a decrease in G_0/G_1 phase, whereas there was an increase in the G_2/M phase. Although KARs caused an irreversible decrease in G_2/M, the decreases caused by vincristine and vinblastine were reversible ($n = 12$; mean ± S.D.; *, $P < 0.01$).

After 48 h of treatment with KARs, the arrest in G_2/M was higher than 80%, whereas percentages in G_1 and S phases were lower than 7 and 13% respectively. In the case of KAR-3 and KAR-4, the arrest in G_2/M was so strong (more than 90%) that in some of the samples, the G_0/G_1 peak was undetectable. In contrast, after 48 h of treatment with vincristine or vinblastine, the effects observed at 24 h were partially reversed. Therefore, we observed a decrease in the percentage of cells in G_2/M and a higher proportion of cells in G_1 with respect to the values reported at 24 h. Treatments with KARs did not induce polyploidy even after 72 h of treatment (data not shown).

Furthermore, we examined the effect of vinblastine on cell cycle distribution pattern at the same dose as KAR-2 (316 nM) or KAR-4 (355 nM), for comparative purposes. The effect was similar to that of the KARs, although the number of viable cells was reduced drastically. We also checked the effect of these two KARs at the IC_{50} value of vinblastine (5 nM). They had no effect on cell cycle distribution at this concentration

The fact that vinblastine treatment (at its IC_{50}) caused an arrest in cell cycle in both G_1 and G_2/M phase, whereas KAR treatments caused a practically complete arrest in G_2/M phase, is difficult to explain if we consider that the main effect of these drugs is their anti-microtubular activity. We hypothesized that these differences may be because vinblastine and vincristine show significant anticalmodulin activity in vitro, whereas KARs do not (Orosz et al., 1997a). To test this hypothesis, we treated cells with the anticalmodulin drug W13 (30 μM) alone and in combination with KAR-2 (316 nM), and we compared the effects on cell cycle distribution with those observed after treatments with vinblastine (5 nM) or KAR-2 (316 nM). It has been described previously that W13 inhibits the re-entry of growth-arrested cells into the cell cycle (G_0/G_1 transition) (Rasmussen and Means, 1989); the progression into and through S phase (Chafouleas et al., 1982; López-Girona et al., 1992; Taulés et al., 1998), the initiation of mitosis (G2/M) transition) (Patel et al., 1999) and mitosis exit (Lorca et al., 1993). We observed a similar arrest on G_1 and G_2/M after treatment with vinblastine and with a combination of W13 and KAR-2. In contrast, KAR-2 alone arrested mainly in G_2/M phase, whereas W13 alone caused a typical anticalmodulin effect, a partial stop in G_1/G_0, S, and G_2/M (Fig. 4).

DNA histograms of cells treated with KARs, vinblastine, or vincristine showed a small increase in the number of nuclei with DNA content (<2n) typical of cells undergoing necrosis. Because all these drugs caused arrest in G_2/M, the apoptotic peak would be impossible to identify, because it would coincide with the S phase or G_1/G_0 peaks. Consequently, FACS analysis using annexin V was used to establish whether necrosis or apoptosis is involved in the mechanism of cell death in response to treatments with KARs, vinblastine, and vincristine. Cells exposed to the cytotoxic agents for 48 h were double-stained with PI and FITC-Annexin V. All the

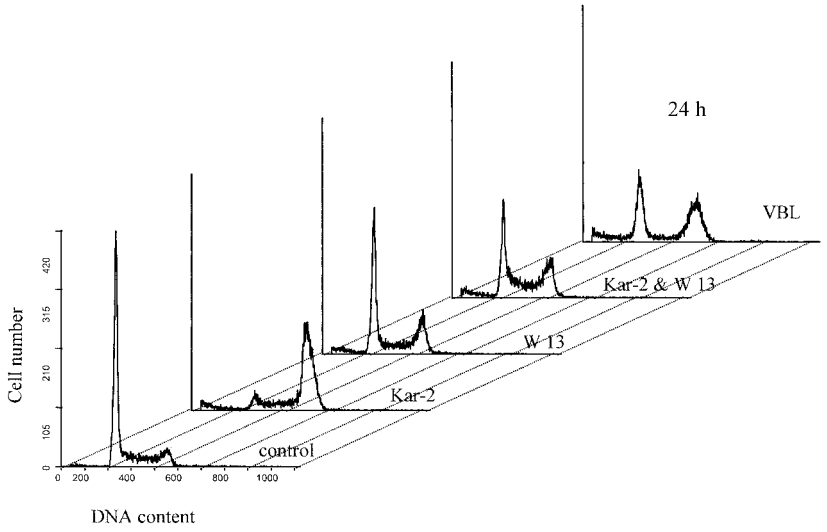

Fig. 4. Cell cycle analysis of neuroblastoma cells untreated or treated with KAR-2 (316 nM), W13 (30 μM), KAR-2 and W13 (316 nM and 30 μM, respectively), and vinblastine (VBL; 5 nM) for 24 h. DNA was stained with PI as described under *Materials and Methods*

compounds at their IC_{50} levels slightly increased the number of apoptotic cells (Annexin V staining only), whereas the proportion of necrotic cells, including late apoptotic cells (Annexin V- and PI- positive), was very low in all cases (Table 1). An increase in the number of apoptotic and necrotic cells was evident in cells exposed to high doses of vincristine (0.1 μM) or KAR-2 (1 μM). Thus, 33 and 22% of apoptotic cells and 7 and 6% of necrotic/late apoptotic cells, respectively, were observed at 48 h.

Treatment with KARs Induced an Increase of the Number of Cells with Condensed Chromosomes and with Phospho-H3. To determine whether cells were stopped in G_2 or in mitosis after treatments, chromatin was visualized by Hoechst staining. Results showed that only 3.4% ± 0.6% of control cells had condensed chromosomes. After 24 h of treatment with the compounds, cells with condensed chromosomes were the following: 46 ± 6% after KAR-3, 41 ± 2% after KAR-2, 36 ± 5% after KAR-4, 34 ± 3% after vincristine, and 18 ± 2% after vinblastine. To confirm that the cells were really stopped in mitosis after the different treatment, we analyzed phosphorylation of H3 with a specific antibody. H3 is phosphorylated at the beginning of metaphase and until telophase. Thus, it can be used as a mitotic marker. After 24 h, phosphorylated H3-positive cells were the following: 10.7 ± 3% without treatment, 46.4 ± 7% after KAR-2, 16.3 ± 1%. These results correlated with the condensed chromosome quantification shown above. These percentages correlate with the results of cell cycle analysis.

TABLE 1

Cytometric analysis of Annexin V-FITC staining and PI accumulation after exposure to the bis-indole derivates

Final concentrations, KAR-2, 316 nM; KAR-4, 355 nM; VBL, 5 nM; VRC, 14 nM; KAR-3, 316 nM for 48 h on neuroblastoma cells. Apoptotic cells (annexin V^+/PT^-), necrotic cells (annexin V^+/PI^+) include late apoptotic cells. 30,000 cells were counted. Data are shown as the mean value ± S.E.M. (Annexin V-AV).

Compounds	AV^+ PI^- cells	AV^+ PI^+ cells
	%	
Control	0.6 ± 0.7	0.1 ± 0.1
KAR-2	9.2 ± 0.3	2.6 ± 0.1
KAR-3	11.6 ± 4.7	2.7 ± 0.4
KAR-4	11.5 ± 2.1	2.7 ± 0.1
VBL	17.6 ± 1.3	2.9 ± 0.1
VCR	11.9 ± 1.2	3.7 ± 2.3

VBL, vinblastine; VCR, vincristine.

However, it should be taken into account that the percentage of cells with phospho-H3 or condensed chromosomes is always lower than the percentage of cells arrested in G_2/M (by FACS), because the cells in G_2 do not have condensed chromosomes or phosphorylated H3. Thus, KARs induced a strong arrest of cells in mitosis, which is consistent with their inhibitory effect on tubulin polymerization and mitotic spindle formation.

Effect of KARs on the Levels of Cyclins A and B1 and Cdc2. In cells with normal spindle checkpoint, failure of correct mitotic spindle formation should prevent mitotic exit by inhibition of cyclin A and B1 degradation, and thus inhibition of Cdc2 inactivation. Consequently, we analyzed the levels of cyclins A and B1 and activity of Cdc2 after the various treatments. Western blot analysis showed that all bis-indole derivative-treated cells had higher levels of cyclin A than nontreated cells. Furthermore, the levels of cyclin A were slightly higher after KARs treatment compared with vinblastine or vincristine (Fig. 5.1). Cyclin B1 levels were similar in vinblastine- and vincristine-treated cells than in control, whereas the levels in KAR-2-, KAR-3-, and KAR-4-treated cells were higher. Furthermore, we found that Cdc2 activity was higher in Kar-2 or vinblastine-treated cells than in nontreated cells. The activity after Kar-2 treatment was double that after vinblastine treatment (Fig. 5, 2). These results indicate that the arrest induced by KAR-2 occurred before anaphase-telophase transition and that this arrest was stronger in KAR-2- than in vinblastine-treated cells.

KAR-2 Induced Aberrant Spindle Formation but Did Not Affect Interphase Microtubules. To further characterize the mechanisms associated with KAR-2–induced growth arrest, we examined the effects of KAR-2 at concentrations of 316 nM and 5 nM on spindle and microtubules behavior. The effects were compared with those induced by vinblastine at 5 nM and 316 nM. Immunostaining with anti-β-tubulin antibody revealed marked differences between the control and vinblastine or KAR-2 treated cells (Fig. 6). Mitotic spindles were normal in untreated cells. As shown above, in cells treated with vinblastine at 5 nM, the number of cells with condensed chromosomes and phospho-H3 were higher than in untreated cells. Although some of them had mitotic spindle with normal appearance (approximately 30%

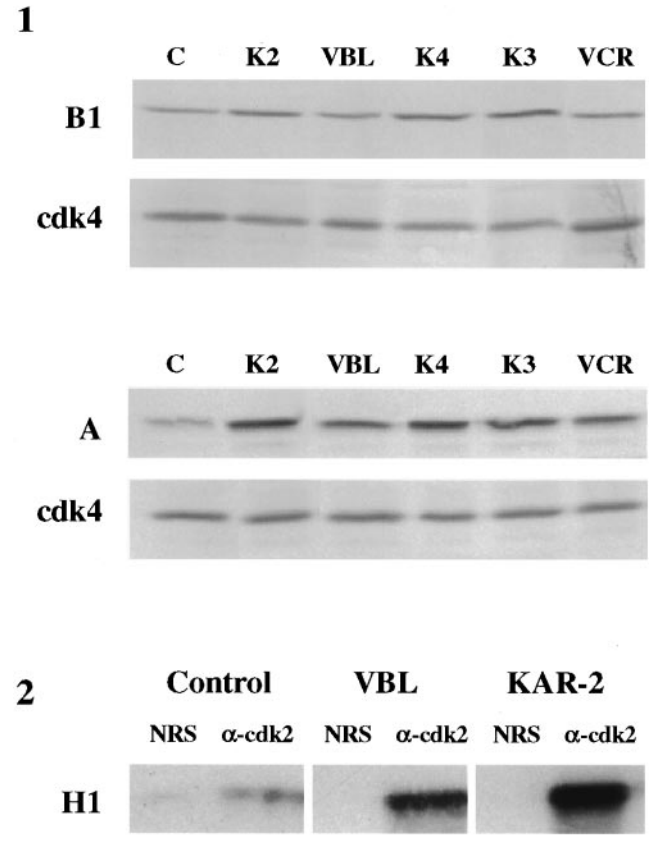

Fig. 5. Effect of vinblastine (VBL), vincristine (VCR), KAR-2 (K2), KAR-3 (K3), and KAR-4 (K4) on the levels of cyclins and Cdc2. 1, determination of cyclin A (A) and cyclin B1 (B1) levels by immunoblotting after treatment with the anti-cancer drugs for 24 h as indicated under *Materials and Methods*. 2, determination of the level of Cdc2 after treatment with VBL or KAR-2. Treated and nontreated cells were lysed at 24 h. Cell lysates were immunoprecipitated with antibody against Cdc2. Immunoprecipitations using normal rabbit serum were performed as controls. To analyze the activity of Cdc2, histone H1 substrate was used.

of mitosis was normal), the microtubule network of most of the interphasic cells was severely damaged. In contrast, KAR-2–treated cells (at 316 nM) had a high number of aberrant mitotic spindles (less than 10% of mitosis were normal), whereas the interphase cells had an apparently normal microtubular network (Fig. 6). KAR-2, at a concentration of 5 nM, had no apparent effect on either the interphase or mitotic microtubules. In contrast, vinblastine, at a concentration of 316 nM induced complete destruction of the microtubular network of interphase cells (data not shown).

Discussion

Bis-indole derivatives extracted from *Catharanthus roseus* such as vincristine and vinblastine have been used extensively as antimitotic drugs in cancer chemotherapy since the 1960s. We reported the synthesis and characterization of novel bis-indole compounds, KAR-2, KAR-3, and KAR-4, which are semisynthetic derivatives of bis-indoles occurring in *Catharanthus roseus* extract in relatively large amounts (Orosz et al., 1997a,b, 1999). The most promising candidate to be used as an antitumoral drug was KAR-2, which has similar or even higher anti-β-tubulin and antitumor activity compared with other bis-indoles but significantly lower toxicity (Orosz et al., 1997b, 1999). We have recently documented that although their antimicrotubular activities in in vitro systems are similar, KAR-2 and vinblastine display distinct effects in neuroblastoma and other cell lines (primary brain cells, PC12, Ehrlich ascites tumor cells) (Orosz et al., 1999) and in mice hosting different tumor cells. Concerning the in vivo effect of KAR derivatives versus vinblastine and vincristine in tumor cells, we have also demonstrated that maximal cytotoxic activities of KARs in mice hosting leukemia P388 or Ehrlich ascites tumor cells are similar to those of vinblastine and vincristine; however, significant prolongation of life span could be reached with KAR derivatives after the administration of a single dose (Orosz et al., 1999). The single dose administration is an important issue in relation to the multidrug resistance problem known in the case of vinblastine and vincristine. In addition, the KAR-2 administration did not induce neurotoxic side effects (e.g., paralysis of bladder or lower extremities) as observed with bis-indoles routinely used in therapy. In this study, we further characterized the effects of KAR-2 and its parent compound in cultured neuroblastoma cell line as a model to better understand the cellular mechanism of KARs compared with vinblastine and vincristine.

FACS analysis showed that KARs completely inhibit cell cycle progression in G_2/M phases, in contrast to vincristine and vinblastine, which caused partial arrest in G_0/G_1 and G_2/M phases. The results obtained on cell cycle arrest with vincristine and vinblastine are consistent with those obtained with these drugs in other cell lines (Jordan et al.,

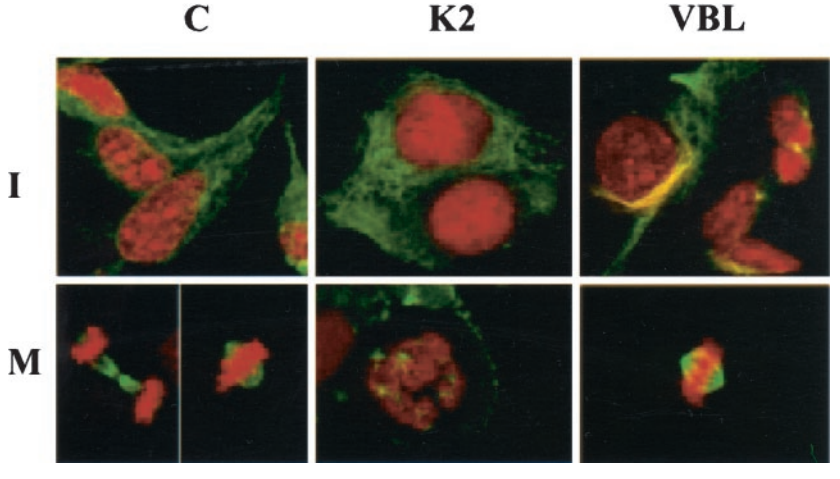

Fig. 6. Confocal Microscope images of control (C), KAR-2- (K2), and vinblastine- (VBL) treated cells at 316 and 5 nM, respectively. Cells were subcultured on coverslips. After 24 h of treatment, cells were fixed as described under *Materials and Methods*. Tubulin was visualized by immunofluorescence after treatment with anti-β-tubulin (primary) and FITC-labeled (secondary) antibodies (green). DNA was visualized by PI staining (red). I, interphasic cells; M, mitotic cells; example of normal anaphase (left) and metaphase (right). K2, example of the predominant mitotic spindle; VBL, example of normal mitotic spindle.

1992; Wilson and Jordan, 1994; Molnár et al., 1995; Petru et al., 1995). It has been reported that calmodulin participates in the regulation of the G_0/G_1 transition (Chafouleas et al., 1984), the progression into and through S phase (Sasaki and Hidaka, 1982; Chafouleas et al., 1982; López-Girona et al., 1992), and the initiation and the exit of mitosis (Chafouleas et al., 1982). Accordingly, we suggest that these differences with KARs could be explained, taking into account that vinblastine and vincristine present anticalmodulin activity and that KAR-2 binds to a different site on calmodulin than vinblastine (Vértessy et al., 1998) and thus does not display anticalmodulin activity (Orosz et al., 1997a). Moreover, cell cycle perturbations observed when we treated neuroblastoma with a mixture of KAR-2 and W13 (a calmodulin inhibitor) were similar to those obtained with vinblastine alone. Although these results agree with our hypothesis that the effect of vinblastine on the cell cycle is a result of the combination of its antimicrotubular activity and its anticalmodulin activity, other possibilities could be considered to explain differences between vinblastine and KAR-2.

Because exposure of cells to KARs leads to higher levels of cyclin A and B1 than exposure of cells to vinblastine and vincristine, we concluded that KARs caused stronger mitotic arrest and seem to be more specific antimitotic agents than vinblastine or vincristine. This is corroborated by the fact that Cdc2 activity doubled in KAR-2–treated cells with respect to vinblastine-treated cells. KARs do not display polyploidy at 72 h, which is a desirable characteristic for a drug to be used in antitumor chemotherapy and also indicates that neuroblastoma cells activate the mitotic spindle checkpoint in response to KARs treatment. The cell cycle phase arrest specificity of antitumor drugs is important in oncology in developing clinical treatment protocols and designing antitumor strategies involving specific drug combinations. For instance, Stone et al. (1996) demonstrated that drugs that induce overexpression of p16 make normal cells more resistant than cancer cells to antimitotic drugs, because normal cells respond to p16 overexpression reversibly by arresting at G_1, whereas in many tumors, the p16 regulatory pathway is inactivated and thus cells progress to mitosis, where they become susceptible to antimitotic drugs. Testing several anticancer agents, they concluded that the most dramatic effect was observed with vinblastine and suggested that other agents more specifically directed against the G_2 or M phases than vinblastine might be more effective (Stone et al., 1996). From this point of view, the new KAR family of Vinca-alkaloids could be an excellent candidate for this therapeutic approach; this will be one of the objectives of our future studies with these drugs. The ability of KAR-2 to induce the formation of aberrant mitotic cells without any apparent destruction of the microtubular network of interphasic cells at their IC_{50} level is in marked contrast to its mother compound vinblastine. These differences can be very important in a possible selective effect of KAR-2 against dividing cells, which is a desirable characteristic in any antitumoral drug. These differences can also be related to the lack of anticalmodulin activity of KAR-2; it has been reported that some of the microtubule-associated proteins are calmodulin-binding proteins (Ortega-Perez et al., 1994; Gonzalez et al., 1995).

We found that KARs, like vinblastine and vincristine, cause apoptosis rather than necrosis in human neuroblastoma cell line SH-SY5Y. However, the percentage of apoptotic cells was always low, which is consistent with the percentages of apoptosis after vincristine treatments reported in the literature for other cell lines (Tsurusawa et al., 1997). This finding is consistent with the higher percentages of mitotic cells after treatment of these compounds and with the evidence that cells may undergo apoptosis at any phase except mitosis (Pittman et al., 1994). In addition, it has been suggested that tubulin reorganization may play a role in the apoptotic process (Cotter et al., 1992).

Thus, the combination of the three differential characteristics of KAR-2 with regard its parental compounds vinblastine and vincristine, the fact that it completely arrests cell cycle at mitosis, and its capacity to generate aberrant mitosis without affecting interphase microtubules makes this compound particularly interesting from a pharmaceutical point of view.

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