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Interaction of a new bis-indol derivative, KAR-2 with tubulin and its antimitotic activity

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1 KAR-2 (3″-(β-chloroethyl)-2″,4″-dioxo-3,5″-spiro-oxazolidino-4-deacetoxy-vinblastine), is a bis-indol derivative; catharantine is coupled with the vindoline moiety which contains a substituted oxazolidino group. Our binding studies showed that KAR-2 exhibited high affinity for bovine purified brain tubulin ($K_d = 3$ μM) and it inhibited microtubule assembly at a concentration of 10 nM.

2 Anti-microtubular activity of KAR-2 was highly dependent on the ultrastructure of microtubules: while the single tubules were sensitive, the tubules cross-linked by phosphofructokinase (ATP: D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) exhibited significant resistance against KAR-2.

3 The cytoplasmic microtubules of Chinese hamster ovary mammalian and Sf9 insect cells were damaged by 1 μg ml^{-1} KAR-2, as observed by indirect immunofluorescence and transmission electron microscopy. Scanning electron microscopy revealed intensive surface blebbing on both types of cells in the presence of KAR-2.

4 KAR-2 was effective in the mouse leukaemia P338 test *in vivo* without significant toxicity. Studies on a primary cerebro-cortical culture of rat brain and differentiated PC12 cells indicated that the toxicity of KAR-2 was significantly lower than that of vinblastine. The additional property of KAR-2 that distinguishes it from bis-indol derivatives is the lack of anti-calmodulin activity.

Keywords: Vinca alkaloids; bis-indols; vinblastine; vincristine; microtubule

Introduction

Numerous antimitotic drugs developed and used therapeutically for a large number of pathologies belong to different classes based on their chemical structures and other distinctive features. Several synthetic and natural compounds interact specifically with tubulin and/or microtubule (MT), fundamentally destroying its dynamic character, inhibiting tubulin polymerization and leading to cell death. A large number of these agents are plant derived (Lin *et al*., 1988).

These compounds have distinct binding sites on the tubulin molecule and presumably distinct mechanism of action. Two principal binding sites are referred to by their 'classical' binding agents: the colchicine site and the vinca site (Sackett, 1995). Binding of the agents to the distinct sites appears to be independent. On the other hand, several other compounds, e.g. maytansine, rhizoxin that are chemically quite different from Vinca alkaloids, are known to bind at the same (at least overlapping) binding site (for review see Hamel, 1992). Recently, the location of the vinca site on the tubulin dimer as well as structural changes induced in tubulin by binding site occupancy have been established (Sackett, 1995; Rai & Wolff, 1996).

Two Vinca alkaloids from *Catharanthus roseus*, vinblastine (VBL) and vincristine (VCR) are effective antimitotic agents that are used in cancer therapy (Dustin, 1984). Both drugs inhibit the self-assembly of tubulin into MTs at substoichiometric concentrations by the formation of a tubulin-drug complex at the end of a growing MT and consequent blocking of self-assembly (Wilson *et al*., 1976; Margolis *et al*., 1980) and suppression of dynamic instability (Dhamodharan *et al*., 1995). However at higher Vinca alkaloid concentrations, tubulin dimers are self-associated irreversibly into linear polymers (Prakash & Timasheff, 1992) leading to the formation of paracrystals both *in vivo* and *in vitro* (Dustin, 1984; Na & Timasheff, 1986; Prakash & Timasheff, 1992; Takanari *et al*., 1994).

Structurally the dimeric Vinca alkaloid drugs comprise two monomers, catharanthine and vindoline. The monomers are much less effective in bringing about the inhibition of tubulin self-assembly into MTs than VBL and VCR (Prakash & Timasheff, 1991). Catharanthine has no clinically significant antimitotic activity. The vindoline moiety has not been studied in detail. Production of potent semisynthetic antitumour agents is motivated by their extensive need in clinical chemotherapy. Severe, sometimes toxic side effects of the existing drugs make it a necessity to establish new derivatives.

Although the primary target of the bis-indol alkaloids is the microtubular network, they also bind to calmodulin (CaM), a ubiquitous Ca^{2+} receptor protein that is involved in cell proliferation (De Brabander *et al*., 1980) and other physiological processes. Recently, we demonstrated that the bis-indol Vinca alkaloids display significant anti-CaM activity (Molnár *et al*., 1995).

In this paper, we characterize the effects of a new bis-indol derivative, KAR-2 (3″-(β-chloroethyl)-2″,4″-dioxo-3,5″-spiro-oxazolidino-4-deacetoxy-vinblastine) (see Figure 1) in various systems. We have found that this compound is effective in the mouse leukaemia P388 *in vivo* antitumour test and evolutionary different cell lines. Electron microscopic studies have shown that KAR-2 targets the microtubular network causing dramatic morphological alterations. KAR-2 binds to tubulin at the vinca site and extensively inhibits MT assembly *in vitro*. In comparison to vinblastine (VBL) and vincristine (VCR), KAR-2 is less toxic and it does not possess anti-CaM activity.

Methods

Proteins

Phosphofructokinase (PFK) (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), aldolase and glycerol-3-

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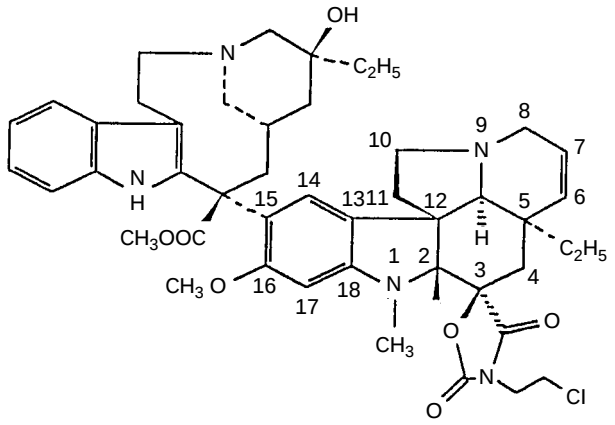

Figure 1 Structural formula of KAR-2.

phosphate dehydrogenase - triosephosphate isomerase purified from rabbit skeletal muscle were purchased from Sigma or Boehringer. The enzyme $(NH_4)_2SO_4$ suspensions were centrifuged at 10,000 *g* for 5 min. The pellets were suspended in standard buffer (50 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulphonic acid (HEPES), pH 7.0, containing 100 mM KCl, 5 mM $MgCl_2$ and 2 mM dithioerythritol) and gelfiltered or dialysed in the same buffer to remove $(NH_4)_2SO_4$.

Tubulin was prepared from calf brain by the Weisenberg method modified by Na & Timasheff (1986). Purified tubulin was virtually free from MT associated protein bands when run on overloaded sodium dodecyl sulphate polyacrylamide gel. Tubulin was stored in 1 M sucrose, 10 mM sodium phosphate buffer, 0.5 mM $MgCl_2$ and 0.1 mM GTP, pH 7.0 at $-80°C$ and dialyzed against the appropriate buffer before use.

Protein concentrations were determined spectrophotometrically, with molar absorption coefficients of 1.06×10^5 M^{-1} cm^{-1} at 280 nm for tubulin (Na & Timasheff, 1986) and 8.88×10^4 M^{-1} cm^{-1} at 276 nm for phosphofructokinase (PFK; Hesterberg & Lee, 1982). PFK and tubulin concentrations are given in monomers and dimers, respectively. Monomeric molecular weight of PFK is 83 000, that of dimeric tubulin is 100 000 Da. For spectroscopic measurements HP 8451A or JASCO V-550 spectrophotometers were used. Protein purity was determined by discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, as described by Laemmli (1970).

In vivo *mouse leukaemia P338 test*

Mouse leukaemia P388 cells were maintained in DBA/2 inbred mice and transplanted by the intraperitoneal (i.p.) route to groups consisting of six BDF1 hybrid mice each. A dose of 10^6 tumour cells/animals was administered (six mice per group). For antitumour studies KAR-2 and reference drugs (VBL and VCR) were dissolved in physiological saline and were administered i.p. The daily i.p. treatment with the drugs was started 24 h after transplantation. The treatment was continued for 8 days by treating the animals once daily. The body weight and conditions of the animals were recorded daily. Animal mortality was checked daily and necroscopy was performed in order to differentiate death due to tumour from death due to toxicity. Similar tests were repeated with single doses. Antitumour activity was expressed as a percentage of the life span related to the average life span (in days) of the control group (T/C%), where T and C are the median survival time of treated and control mice, respectively.

VCR-resistant leukaemia P388 (P388/VCR) was also used to test antitumour activity of drugs under similar conditions as described for standard P388 tumours.

Cell cultures and treatments

Primary cultures of rat cerebral cortex cells were obtained by trypsinization of the cortex of 16–18 day-old Wistar rat embryos. The cells were plated onto poly-D-lysine-coated 24-well plates (Falcon) at 1.8×10^5 cell/cm^2 density in DMEM (Dulbecco's modified Eagle medium; GIBCO BRT, Paisley, Scotland) supplemented with 10% foetal calf serum (Erdö *et al.*, 1990). Cultures were incubated for 8 days.

PC12 cell lines were purchased from ATCC (Rockville, U.S.A.). Cells were plated onto collagen-coated 24-well plates at 1×10^5 cell/cm^2 density in 85% RPMI1640, 10% horse serum, 5% foetal calf serum medium and incubated at 37°C in a 5% CO_2 atmosphere.

For the treatments of neuronal cells, drugs were dissolved in 0.1 M HCl diluted with N_2 medium (serum free medium supplemented with 100 μg ml^{-1} transferrin, 30 nM sodium selenite, 100 μM putrescine, 5 μg ml^{-1} insulin and 20 nM progesterone) and sterilized. The pH was adjusted to neutral. Cells were exposed to drugs in N_2 medium for 24 h with controls treated with N_2.

The extent of cell death was determined by the measurement of lactate dehydrogenase (LDH) activity released from damaged cells into medium and is expressed as percentage of the total LDH activity (cells + medium) in the same culture as described previously (Erdö *et al.*, 1990).

Chinese hamster ovary (CHO) cells were kindly provided by the National Research Institute of Radiology and Radiohygiene (Budapest, Hungary). The cells were grown on glass cover slips at 37°C in Eagle's Minimum Essential Medium (GIBCO BRT, Paisley, Scotland) supplemented with 10% foetal bovine serum, streptomycin 0.1 mg ml^{-1} and penicillin 100 u ml^{-1}.

Sf9 insect cells, a clonal isolate derived from the immature ovaries of the pupae of the all armyworm (*Spodoptera frugiperda*) (Vaughn *et al.*, 1977) were grown as monolayers in antibiotic free Grace's medium supplemented with 10% foetal calf serum (Serva, Heidelberg, Germany) 0.33% yeast extract and 0.33% lactalbumin hydrolysate (Sigma, St. Louis, MO, U.S.A.) at 26°C (Summers & Smith, 1987).

CHO and Sf9 cells were treated for 2 h with KAR-2 at concentrations of 0.1, 1, 10 and 100 μg ml^{-1} prepared from a stock solution by dilution with the culture medium. For preparation of stock solution 1 mg KAR-2 was dissolved in 250 μl 0.01 N HCl and diluted to 1000 μl with physiological saline.

Immunocytochemistry

Cells were fixed in pure methanol at $-18°C$ for 10 min washed with phosphate buffered saline (PBS) and blocked with 2% (w/v) non-fat dry milk powder in PBS for 30 min. The samples were incubated for 1 h with a mouse monoclonal anti-tubulin IgG solution (generously supplied by Dr Jane Arnold, University of Nottingham) at a dilution of 1:50 in 0.2% (w/v) non-fat dry milk powder in PBS, followed by washing in PBS. Samples were incubated for 1 h in fluorescein isothiocyanate labelled anti-mouse IgG (Vector Laboratories) at a dilution of 1:1000 in PBS. After washing in PBS and mounting in a 1:1 mixture of PBS-glycerol containing 1 mg ml^{-1} para-phenylene diamine, the slides were examined in a Zeiss Axioscope. Photographic images of the cells were recorded on KODAK TMAX-400 film.

Electron microscopy

Samples for routine transmission electron microscopy were prepared by incubating 10 μM tubulin, 20 μM taxol, 4 μM PFK without and with drugs (2 μM) for 60 min at room temperature; then fixed and embedded as described previously (Lehotzky *et al.*, 1994). For negative staining a drop from the unpelleted samples was applied to formvar/carbon coated glow-discharged copper grids for 30 s. The solution was re-

moved and the grid stained with one drop of freshly filtered 1% aqueous uranyl acetate for 30 s. The excess stain was removed by blotting with filter paper.

The specimens were examined and photographed in a JEOL CX 100 electron microscope operated at an accelerating voltage of 80 kV. Magnification was calibrated with a diffraction grating replica (21601/mm, Balzers).

Turbidity and sedimentation measurements

Tubulin polymerization was measured in standard HEPES buffer (Lehotzky *et al*., 1993). The assembly was started by addition of taxol (to a final concentration of 20 μM from a 10 mM stock solution in dimethyl sulphoxide) to the samples. In some cases tubulin polymerization was followed in a medium containing 1.0 M glutamate, pH 6.6, 1 mM Mg-acetate and 1 mM GTP, in the absence of taxol (Hamel & Lin, 1981). Polymerization reaction was started by transferring the reaction mixture from 0°C to 30°C. Tubulin polymerization was monitored in the absence and presence of PFK and drugs by measuring the turbidity at 350 nm in a 1 cm light path cuvette with a HP-8451A spectrophotometer. Drug effect was determined from the initial rates of the polymerization curves measured at different drug concentrations. The IC_{50} values of the drugs were calculated from the concentration-response curves with the method of maximum likelihood probit analysis.

Fluorescence measurements

Fluorescence studies were conducted on a JASCO Model FP-777 spectrofluorometer, with excitation and emission slits of 3 nm. Fluorescence excitation and emission spectra of tubulin (1 μM) were measured in the absence and presence of Vinca alkaloids at 25°C, in 20 mM potassium phosphate buffer, pH 6.9, containing 1 mM ethylene glycol *bis*(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and 0.5 mM $MgCl_2$. The excitation wavelength was 295 nm. The fluorescence intensities of VBL and KAR-2 are much less (with 2 and 3 orders of magnitude) than that of tubulin, in this way the drugs did not disturb directly the fluorescent spectra of tubulin. Spectra were corrected for inner filter effects according to

$$F_{corr} = F_{obs}[10^{(OD_{ex}+OD_{em})/2}]$$

Where OD_{ex} and OD_{em} refer to the optical density of the sample (Lakowicz, 1983).

Circular dichroism measurements

Circular dichroism spectra were recorded with a JASCO J-720 spectropolarimeter. Measurements were performed at 25°C in thermostated cuvettes, in 20 mM potassium phosphate pH 6.9 buffer, containing 1 mM EGTA and 0.5 mM $MgCl_2$. For measuring drug-protein mixtures, drug was added to the protein solution, mixed and the circular dichroism spectrum recorded in the 250–360 nm wavelength range. Scanning was repeated twice and the spectra were averaged. Spectra measured immediately after mixing were stable for at least 20 min, i.e. complex formation was complete in 2–5 min at the concentrations used in our experiments.

u.v. absorbance spectra and difference spectra

The direct and difference spectra were recorded on a Hewlett Packard 8451A spectrophotometer at 25°C in a standard buffer. Difference spectra were obtained by using 1 cm tandem cells (Hellma). A tubulin concentration of 5 μM was used in all experiments.

PFK activity measurements

Drugs at 20 μM were preincubated with 3 μM calmodulin (CaM) in the presence of 100 μM $CaCl_2$ for 60 min at 25°C in standard buffer. PFK (2 μM) was added to the CaM-drug mixture and incubated for an additional 20 min. The enzyme activity was assayed in 50 mM Tris, pH 8.0 containing 2 mM fructose-6-phosphate, 1 mM ATP, 3 mM $MgCl_2$, 0.2 mM NADH, 3 mM dithioerythritol, 0.1 mM EGTA, 2 u aldolase, 12 u triosephosphate isomerase, and 2 u glycerol-3-phosphate dehydrogenase at 30°C (Mayr, 1987; Lehotzky *et al*., 1993). The specific activity of PFK was 185 u mg^{-1} measured at 0.8 μg ml^{-1} final concentration after dilution from a 1 mg ml^{-1} stock solution into activity assay.

Chemicals

VBL, VCR, catharantine, vindoline and KAR-2 were kindly provided by Chemical Works of Gedeon Richter Ltd. (Budapest). The structure of KAR-2 was supported by its ^{13}C-n.m.r., IR and mass spectra (Hungarian Patent Description: 978/95). Taxol and GTP were purchased from Sigma and Aldrich, respectively. All other chemicals were reagent-grade commercial preparations.

Results

Anti-tumour potency on mouse leukaemia P388

Mouse leukaemia P388, an *in vivo* tumour model was used to test the antitumour activity of KAR-2 in comparison to effective Vinca alkaloids. Table 1 shows that KAR-2 possesses an antitumour effect after an 8 day treatment with 0.4 mg kg^{-1}, its most effective doses lie in the higher dose range where VBL and VCR are toxic; in addition, toxic mortality occurs in the presence of these latter two drugs.

Antitumour activity of KAR-2 could not be demonstrated on VCR resistant mouse ascites lymphoma (Table 1). As expected, KAR-2 was inactive on VCR-resistant tumours in doses which were effective on standard P388 tumours; the slight effect seen with higher doses cannot be considered to be significant.

Based on the possibility of administering high doses as observed during the experiments, the tests were repeated with

Table 1 *In vivo* antitumour effects on P388 and P388/vincristine (VCR) leukaemias

Daily dose (mg kg^{-1}) *for 8 days*	*T/C%* *P388 VBL*	*P388 VCR*	*P388 KAR-2*	*P388/VCR KAR-2*
8×0.1	165 ± 28	193 ± 22	110 ± 6	
8×0.2	199 ± 22	209 ± 24		
8×0.4	170 ± 20	187 ± 16	146 ± 19	
8×0.7	toxic	toxic		
8×1	toxic	toxic	174 ± 17	
8×8	toxic	toxic	171 ± 3	124 ± 24
8×12	toxic	toxic	170 ± 14	125 ± 7
8×16	toxic	toxic	162 ± 16	
8×20	toxic	toxic	145 ± 24[a]	
Single dose				
1×1		147 ± 36		
1×2		138 ± 8		
1×20	toxic	toxic	144 ± 13	
1×40	toxic	toxic	173 ± 9[b]	
1×60	toxic	toxic	207 ± 24[b]	107 ± 14
1×80	toxic	toxic	181 ± 19[a]	

Efficacy of the compounds was tested on P388 and P388/VCR (resistant to VCR) mouse ascites tumours, after single or repeated intraperitoneal doses. The antitumour activity (T/C%) was evaluated according to the formula T/C $\times100$, where T = median day of survival of treated animals and C = median day of survival of control animals. [a]1/5 toxic mortality occurred; [b]1/5 tumour-free survivor.

single doses (Table 1). KAR-2 was effective as a single i.p. dose, the threshold dose being 20 mg kg^{-1}. In groups treated with 40 and 60 mg kg^{-1} KAR-2 tumour-free survivors were found. No toxic mortality, no paralysis of bladder or lower extremities, indicating neurotoxic effects, were observed after KAR-2 administration even during and after treatments with effective doses for several days, whereas these adverse effects are known for VCR. No single dose, including that in the toxic range, was found to be effective for VCR.

Toxicity on neuronal cell lines

The cytotoxicity of KAR-2 was investigated on primary embryonic brain cells as well as on nerve growth factor (NGF) differentiated PC12 cell line and compared with that of VBL. Figure 2a shows the LDH leakage of the cells, characteristic of cell damage, as a function of drug concentration. The toxicity of VBL manifested itself at a much lower concentration (approximately three orders of magnitude) than that of KAR-2; IC_{50} values of KAR-2 and VBL were 166 nM and <1 nM, respectively. On the other hand, the saturating values of the LDH leakage of the KAR-2- or VBL-treated cells were only partial, 20–25%.

Similar experiments were carried out with PC12 cells, a cloned rat phaeochromocytoma cell line which expresses many of the characteristics of neuronal cells. Exposure to nerve growth factor (NGF) causes differentiation in cells similar to sympathetic neurones with extensive neurites. Figure 2b shows concentration - response curves for both KAR-2 and VBL on PC12 cells differentiated by NGF. These neurone-like cells remained partly resistant to KAR-2 even at 100 μM, while VBL caused almost complete cell death under the same conditions.

Immunofluorescence and electron microscopic studies

Morphological studies with KAR-2 and VBL were performed on two evolutionary highly distant cells, on mammalian CHO, widely used for testing antimitotic drugs (Bai *et al*., 1993) and Sf9 insect cells.

Figure 3 shows the tubulin immunofluorescence pattern for control and KAR-2-treated CHO cells. The untreated cells displayed well developed microtubular network radiating from the centrosomes to the periphery. In cells treated with 1 μg ml^{-1} KAR-2 for 2 h, the network had collapsed and needle-like bodies appeared in the cytoplasm which were identified as tubulin paracrystals by transmission electron microscopy (data not shown). Despite the disappearance of cytoplasmic MTs, the structure of centrioles apparently remained intact. In addition to the disorganization of MTs, several other effects of KAR-2 were observed in CHO cells. These included accumulation of small vesicles around the Golgi stacks, formation of bundles of intermediate filaments in the cytoplasm, appearance of long paired cisterns originating from the rough endoplasmic reticulum (data not shown) and intensive surface blebbing, indicating the collapse of the cytoskeleton. All these effects of KAR-2 treatment were found to be concentration-dependent, i.e., they were conspicuous in dose ranges of 10 to 100 μg ml^{-1}, absent at 0.1 μg ml^{-1} and are moderate at 1 μg ml^{-1} KAR-2.

The effects of KAR-2 on Sf9 insect cells were similar to those observed with CHO cells. The microtubular network disappeared from the cytoplasm and the cell surface became covered with blebs and folds instead of the fine, long filopodia characteristic of the control, untreated cells (Figure 4). However, no needle-like inclusions, characteristic of paracrystal formation, were seen in this cell line even after application of high doses of KAR-2.

Anti-tubulin activities

Recently, we showed that KAR-2 is a powerful ligand in inhibiting immunocomplex formation (Liliom *et al*., 1995).

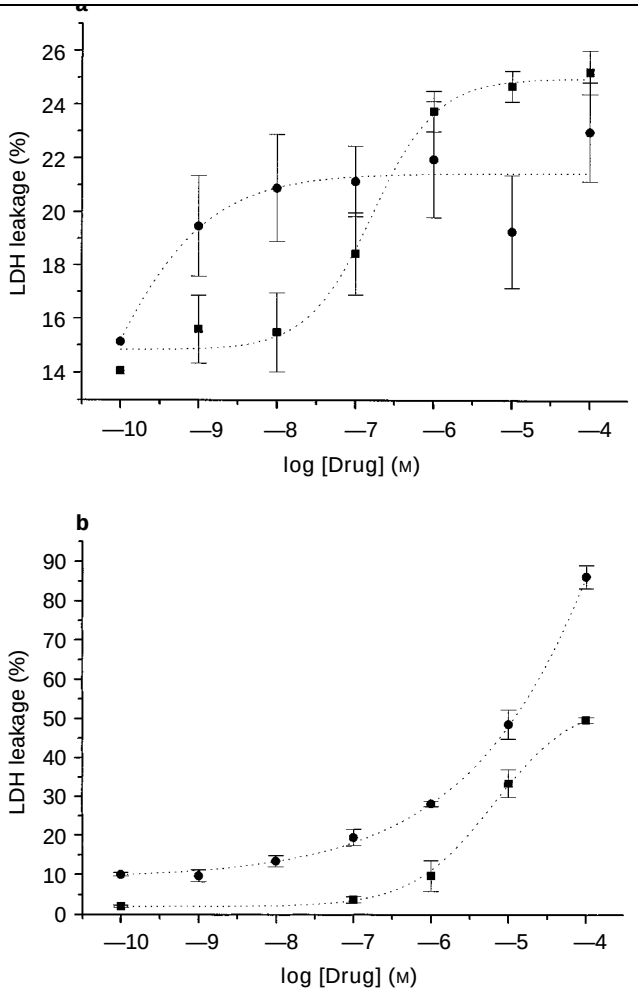

Figure 2 Effect of KAR-2 (■) and vinblastine (VBL; ●) on differentiated PC12 cells in the presence of nerve growth factor (NGF) (a) and on the viability of the cells of primary cerebro-cortical culture (b). Details are described in Methods section. Points show means and vertical lines indicate s.d.; $n=3$.

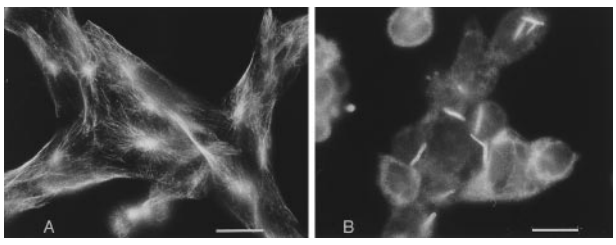

Figure 3 Effect of KAR-2 on the organization of microtubules (MTs) in CHO cells. The cells were incubated in the absence (a) and presence (b) of 10 μg ml^{-1} KAR-2 for 2 h and fixed and stained with anti-tubulin antibody followed by fluorescein isothiocyanate labelled secondary antibody as described in Methods. The extensive network of MTs characteristic of control cells is absent in the KAR-2 treated ones and needle-like inclusions appear in their cytoplasm. Bars: 5 μm.

Displacement indirect ELISA experiments showed that the inhibitory potency of KAR-2 was slightly higher than that of VBL or VCR (cf. Table 2). The effect was due to the binding of these bis-indol derivatives to tubulin dimers which perturbed the binding of polyclonal antibodies to the immobilized tubulin. The effects of drugs on tubulin polymerization were investigated. The IC_{50} values for KAR-2 and some standard molecules are presented in Table 2. The IC_{50} values for KAR-2

Table 2 Anti-tubulin and anti-calmodulin (CaM) effect of Vinca alkaloids

	Anti-tubulin activity				*Anti-CaM activity*	
	Turbidity[a]		*ELISA*[b]		*Effect on CaM-induced PKF inactivation*[c]	
Drug	*HEPES* IC_{50} (nM)	*Glutamate* IC_{50} (nM)	IC_{50} (μM)	K_d (μM)	*Activity* (umg^{-1})	*Efficiency %*
KAR−2	10±1	380±19	24±2	3.0±0.3	18.1±0.6	7
VBL	38±2	420±15	37±4	4.5±0.4	39.0±1.5	61
VCR	58±3	480±21	33±3	4.0±0.4	39.6±1.1	62
Vindoline	>300	$>3\times10^5$	240±25	30±5	15.5±0.8	0
Catharantine	>300	$>1\times10^6$	>500	–	15.4±0.8	0
TFP	–	–	–	–	42.4±2.0	69

[a] Tubulin (20 μM) polymerization was measured in HEPES or in glutamate at 30°C and IC_{50} values were calculated as described in Methods. The data are the average of three independent measurements. [b] From Liliom *et al.* (1995). [c] Concentrations of PFK (in protomers), CaM (for activity measurements) and drugs were 2 μM, 3 μM, and 20 μM, respectively. Residual activity of phosphofructokinase after 20 min incubation in the absence and presence of CaM was 54.3 umg^{-1} (100%) and 15.4 umg^{-1} (0%), respectively. Efficiency was calculated from the activity data by the following formula:

$$\text{Efficiency} = \frac{\text{activity}_{\text{PFK-CaM-drug}} - \text{activity}_{\text{PFK-CaM}}}{\text{activity}_{\text{PFK}} - \text{activity}_{\text{PFK-CaM}}}$$

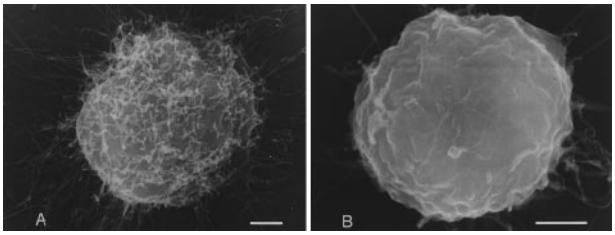

Figure 4 Scanning micrographs of Sf9 cells incubated in the absence (a) and presence of (b) 10 μg ml^{-1} KAR-2 for 2 h. Note the disappearance of fine filopodia from the surface of drug-treated cells. Bars: 2 μm.

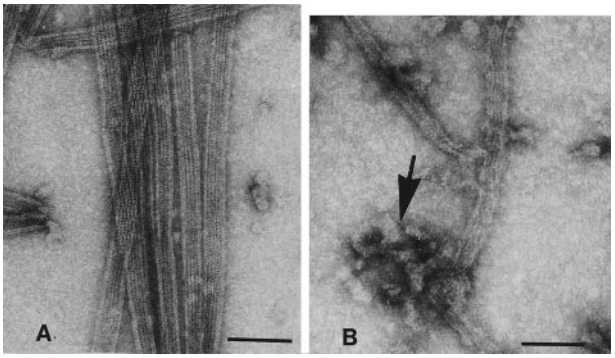

Figure 5 Negatively stained samples of microtubules (MTs) assembled from tubulin before (a) and after (b) the addition of phosphofructokinase (PFK) and KAR-2. The samples were prepared as described in Methods. Note the ring-like aggregate (arrow) in (b). Bar: 100 nm.

were lower than those obtained with VBL and VCR. The latter values are in good agreement with published data (Potier, 1980; Timasheff *et al.*, 1991). It is of interest that the IC_{50} of KAR-2 is lower than the binding constant, suggesting that the inhibition of polymerization is substoichiometric, similar to other Vinca alkaloids.

Previously, we demonstrated that PFK can bind to and cross-link the MTs as visualized by electron microscopy. The bundling process can be monitored by turbidimetry: a relatively rapid increase of turbidity at 350 nm due to the tubulin assembly is followed by a slow additional increase caused by bundling of MTs (Lehotzky *et al.*, 1993; 1994). In order to investigate the sensitivity of tubulin assembly and PFK-induced bundling to the drugs, the turbidity was measured in the presence of VBL or KAR-2, in the absence and presence of PFK. We have found that 2 μM VBL or KAR-2 significantly inhibited the tubulin polymerization (cf Table 2). However, the presence of 4 μM PFK resulted in an extensive increase in the turbidity, characteristic of both the assembly and bundling processes. This effect was more pronounced in the case of KAR-2, in the presence of which the turbidity curve was virtually the same as observed in control samples measured with PFK in the absence of the drug (data not shown).

To examine the structure of the tubulin polymer formed in the presence of KAR-2 and PFK, the samples were negatively stained for electron microscopic visualization. The polymerization of tubulin in the presence of PFK plus KAR-2 resulted in tubules which were similar to the native ones. Tubules of similar morphology were observed in samples assembled from tubulin within the absence and presence of PFK and KAR-2. Nevertheless, the sample with KAR-2 also contained ring-like aggregates and C-shaped profiles that probably represent tubulin oligomers (Figure 5). These data strengthen the conclusion that PFK exerts a protective effect on MT bundling against the anti-tubular action of KAR-2.

Anti-CaM activities

CaM can modulate the activity of numerous enzymes, which can be abolished by CaM antagonists. CaM can stimulate (e.g. phosphodiesterase) or inhibit (e.g. PFK) the activity of enzymes (Orosz *et al.*, 1988 and references therein). We found that the inhibitory potency of drugs at suppressing the modulating effect of CaM manifested itself in both phosphodiesterase and PFK systems in a similar manner (Orosz *et al.*, 1990).

The anti-CaM activity of KAR-2 was tested in the well-established PFK-CaM system, since the antagonist effects of trifluoperazine (TFP), a 'classical CaM antagonist' as well as VBL have been demonstrated in this and other CaM-modulated systems (Molnár *et al.*, 1995). Table 2 shows that the inhibitory effect of 3 μM CaM on PFK activity was significantly attenuated by 20 μM VBL and TFP, but not by KAR-2, under similar experimental conditions. Control experiments indicated that the drugs themselves had no effect on PFK activity at concentrations up to 20 μM. This result demonstrates the unique character of KAR-2 in comparison to other bisindol derivatives.

Spectroscopic characterization of the drug binding to tubulin

Binding of vinca site agents alters the structure of tubulin that induces the quenching of tubulin fluorescence at 330 nm excited at 295 nm (Sackett, 1995). We have found that both VBL

and KAR-2 caused concentration-dependent quenching, resulting in about a 20% decrease in fluorescent intensity at saturation (approx. 30 μM) (data not shown). The emission difference spectra of the tubulin-VBL and tubulin-KAR-2 complexes have a similar shape. These structural changes probably occur due to the perturbation of tryptophan and other fluorophore residues involved in drug-tubulin interactions.

It has been shown previously (Lee *et al*., 1975) that the binding of VBL to tubulin induces small changes of the circular dichroism spectrum in the near ultraviolet wavelength range. We have found that the circular dichroism spectrum of KAR-2 differs from that of VBL around 258 nm where VBL has a well-defined peak while KAR-2 does not (cf. Figure 6a). This difference is probably due to the derivation of the vindoline moiety of VBL. Both drugs show a smooth positive circular dichroism signal in the 300–330 nm wavelength range. Figure 6b shows the difference circular dichroism spectra of both tubulin, VBL plus tubulin and KAR-2 mixtures at stoichiometric drug to tubulin concentrations between 250 and 360 nm. At 258 nm, the altered maximum measured in the presence of KAR-2 was significantly larger than that measured with VBL, but the characteristics of the difference spectra for the molecular conformation of tubulin-drug complexes were similar.

The effect of the binding of KAR-2 on the perturbation of aromatic residues of tubulin was also investigated by differential spectroscopy and compared with that of VBL. The ultraviolet spectra of VBL and KAR-2 were identical in the range 240 to 360 nm, although there were significant differences in the chemical structure of the vindoline part of the bis-indol molecule (data not shown). Figure 7 shows the difference spectra of tubulin, VBL and tubulin-KAR-2 complexes between 240 and 360 nm at equimolar concentrations. The extent of both positive and negative peaks were sensitive to alterations of drug concentration.

In the case of the tubulin-VBL complex the differential peaks at 292 nm and 300 nm are consistent with the perturbation of tryptophans by transfer to a less polar environment (Lee *et al*., 1975). These peaks are represented also in the case of KAR-2 but to a lesser extent. Another maximum around 240 nm and also minima between 260 nm and 280 nm are present in both cases. Therefore, the qualitative characteristics of the difference spectra of tubulin-drug complexes suggest the involvement of similar chromophores in the formation of tubulin-drug complexes.

Discussion

Bis-indol derivatives are among the most potent MT inhibitors. One of the strategies to produce new potent molecules is the synthetic conversion of natural molecules. KAR-2, a new bis-indol, was synthesized from deacetoxy-VBL occurring in the *Catharanthus roseus* extract (60–200 mg kg^{-1}) (Hungarian Patent Description: 978/95.).

KAR-2 differs chemically from VBL by the substitution of 3 and 4 carbon atoms of the vindoline moiety: the acetoxy group on the C4 atom is replaced by H atom and it contains a substituted oxazolidine group on the C3 atom. Oxazolidine derivatives of bis-indol alkaloids have been synthesized by Eli Lilly (Miller & Antowsk, 1984). However, those molecules were substituted by acetoxy and acetyl groups at position C4. These derivations of VBL result in compounds structurally diverse in the vindoline moiety, but the same in the catharanthine moiety that is responsible for the interaction with tubulin (Wilson *et al*., 1974). In this way, transformations carried out on the catharantine moiety have usually led to compounds devoid of antitumour activity.

Although true competitive binding of the two drugs has not been ascertained, our present spectroscopic studies suggest that KAR-2 acts in a manner analogous to VBL with regard to its interaction with tubulin, suggesting that it binds to tubulin at or near the VBL binding site. Independent evidence supporting this possibility comes from our previous displacement

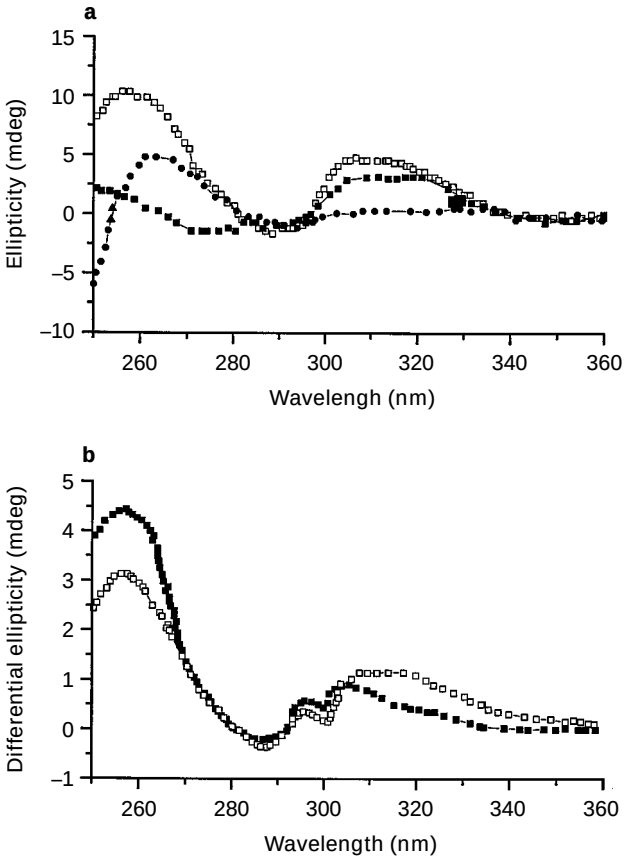

Figure 6 Near u.v. circular dichroism spectra of tubulin, vinblastine (VBL) and KAR-2. The concentrations were 10 μM for tubulin, and 20 μM for drugs. (a) Spectra for tubulin (●), VBL (□) and KAR-2 (■). (b) Difference spectra for tubulin-VBL (□) and tubulin-KAR-2 (■). CD spectra of the components were recorded alone and in the mixture, and the difference spectra were computed.

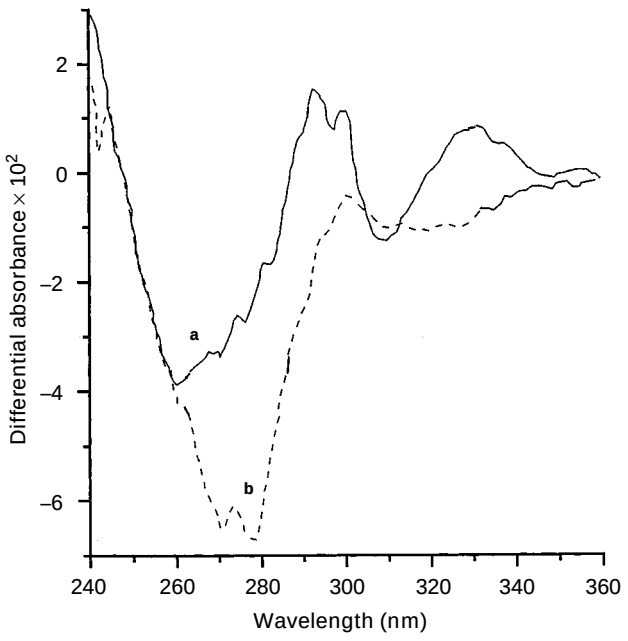

Figure 7 Differential u.v. spectra of tubulin-VBL (a) and tubulin-KAR-2 (b). Difference spectra were recorded at equimolar concentrations (5 μM) of tubulin and drugs as described in Methods.

ELISA experiments with polyclonal anti-tubulin antibody (Liliom *et al*., 1995). ELISA studies provide quantitative data for tubulin-drug interactions under conditions when no drug-induced tubulin aggregations occur. This is important when obtaining quantitative data, since antimitotic agent-tubulin interactions are frequently coupled to tubulin polymerization (Correia, 1991) which may result in alteration in the affinity of drugs. We found that while the antibodies were completely displaced from immobilized tubulin by the vinca site agents VBL and VCR, neither the colchicine site agent colchicine nor taxol showed any activity in displacement experiments (Liliom *et al*., 1995). KAR-2 appeared to behave as a typical vinca site agent: it inhibited the immunocomplex formation with an IC_{50} value lower than those for VBL and VCR (cf Table 2 and Liliom *et al*., 1995). These ELISA results, in accordance with the present spectroscopic investigations, suggest that the KAR-2 binding site on tubulin is distinct from the colchicine site and might be identical to or at least overlap with the vinca site.

As turbidity measurements showed, KAR-2 exhibits significant anti-tubular activity, which manifests itself at lower concentrations than in the case of the clinically active bis-indol derivatives. In addition, KAR-2 is more potent in inhibiting MT assembly while less potent in inhibiting PFK-induced MT bundling, i.e. more specific as compared to VBL (Vértessy *et al*., 1997).

The results of the turbidity measurements suggest that KAR-2 depolymerizes the single tubules but not the bundled (stabilized) ones. Since ATP prevents the cross-bridges of MTs by PFK (Lehotzky *et al*., 1994; Vértessy *et al*., 1997) and other glycolytic enzymes (Durrieu *et al*, 1987) involved in energy production, the organization state of MTs may counteract the ATP level in cells. The level of ATP is significantly higher in many tumour cells compared to normal cells due to intensive glycolysis, consequently, the organization state of MTs may be changed during neoplastic transformation. This hypothesis raises the intriguing possibility that the MTs of neoplastic cells may be more sensitive to the action of KAR-2 than normal cells.

Our morphological observations showed that in the $\mu g\ ml^{-1}$ dose range KAR-2 disrupts the MT network of cells, probably by blocking the polymerization of tubulins resulting eventually in the disappearance of MTs. At higher concentrations (above 10 $\mu g\ ml^{-1}$) KAR-2 induced formation of tubulin paracrystals in CHO cells. These effects were qualitatively similar to those exerted by VBL (Dustin, 1984; Takanari *et al*., 1994; Dhamodharan *et al*., 1995). Therefore, we think that the mechanisms by which these drugs influence tubulin assembly/disassembly are not significantly different.

On the other hand, there are differences in their action at the cellular level. The mouse leukaemia P388, forms a panel of tumours sensitive and insensitive to Vinca alkaloids (Cros *et al*., 1989 and references therein). It has been suggested as a reasonable model for prescreening because it is sensitive to most classes of clinically effective drugs, but is sufficiently restrictive to avoid overloading the panel. Experiments on leukaemia P388 showed that KAR-2 was less toxic than VBL or VCR. In addition, the administration of KAR-2 did not induce neurotoxic side effects as observed in the case of VCR. The administration schedule revealed an additional advantage property of KAR-2, i.e., it was active even in a single high dose; while single doses of VCR were less effective or caused death of the animals.

Because of the extreme complexity of the nervous system, cell cultures are frequently used for neuropharmacological studies. In many cases the biochemical behaviour of these cells can mimic that found in the animal. However, availability of these cell types for study as clonal populations is very limited since the normal differentiated nerve cell may not divide (Schubert & Carlisle, 1979). The cytotoxicity of KAR-2 was investigated on primary embryonic brain cells as well as on the NGF differentiated PC12 cell line and compared with that of VBL. In both cases the leakage of LDH as a characteristic parameter for the cell death was more extensive with VBL than with KAR-2 over a wide range of drug concentrations. Therefore, KAR-2 is significantly less toxic than VBL.

A crucial problem in cancer chemotherapy is the neurotoxic side effects of drugs. Neurotoxicity means a temporary or permanent loss of neuronal function, which can appear as morphological changes, disturbed synthesis of transmitters, or the inhibition of their uptake and release. The role of CaM seems to be important in these processes (Asano *et al*., 1982). Simple enzyme kinetic measurements rendered it possible to evaluate a difference in the anti-CaM activity of KAR-2 and other biologically active bis-indols. Whereas VBL, VCR and TFP, a 'classic CaM antagonist' could suspend the modulating effect of CaM on PFK activity, KAR-2 was virtually as ineffective as the biologically inactive monomers (catharanthine and vindoline). This result suggests that the different action of VBL and KAR-2 *in vivo* must be due to some biological processes other than a direct interaction with tubulin or MT. Additional data concerning the character of KAR-2 in various CaM systems is published in the subsequent paper.

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References

ASANO, M., SUZUKI, Y. & HIDAKA, J. (1982). Effects of various calmodulin antagonists on contraction of rabbit aortic strips. *J. Pharmacol. Exp. Ther*., **220,** 191 – 196.

BAI, R., CICHACZ, Z.A., HERALD, C.L., PETTITE, G.R. & HAMEL, E. (1993). Spongistatin 1, a highly cytotoxic sponge-derived, marine natural product that inhibits mitosis, microtubule assembly, and the binding of vinblastine to tubulin. *Mol. Pharmacol*., **44,** 757 – 766.

CORREIA, J.J. (1991). Effects of antimitotic agents on tubulin-nucleotide interactions. *Pharmacol. Ther*., **52,** 127 – 147.

DE BRABANDER, M. NUYDENS, R., GEUENS, G., MOEREMANS, M., DE MEY, J. & HOPKINS, C. (1980). Nanovid ultramicroscopy: a new non-destructive approach providing new insights in subcellular motility. In *Microtubules and Microtubule Inhibitors*. ed., De Brabander, M. & De Mey, J. pp. 187 – 196. Amsterdam, New York: Elsevier.

CROS, S., WRIGHT, M., MORIMOTO, M., LATASTE, H., COUZINIER, J.P. & KRIKORIAN, A. (1989). Experimental antitumor activity of navelbine. *Semin. Oncol*., **16,** (suppl. 4), 15 – 20.

DHAMODHARAN, R., JORDAN, M.A., THROWER, D., WILSON, L. & WADSWORTH, P. (1995). Vinblastine suppresses dynamics of individual microtubules in living interphase cells. *Mol. Biol. Cell*., **6,** 1215 – 1229.

DURRIEU, C., BERNIER-VALENTIN, F. & ROUSSET, B. (1987). Microtubules bind glyceraldehyde 3-phosphate dehydrogenase and modulate its enzyme activity and quaternary structure. *Arch. Biochem. Biophys*., **252,** 32 – 40.

DUSTIN, P. (1984). *Microtubules*. Berlin, New York: Springer-Verlag.

ERDÖ, S.L., MICHLER, A., WOLFF, J.R. & TYTKO, H. (1990). Lack of excitotoxic cell death in serum free cultures of the rat cerebral cortex. *Brain Res*., **526,** 328.

HAMEL, E. (1992). Natural products which interact with tubulin in the vinca domain - maytansine, rhizoxin, phomopsin-A, dolastin-10 and dolastin-15 and halichondrin-B. *Pharmacol. Ther.*, **55,** 31 – 51.

HAMEL, E. & LIN, C.M. (1981). Glutamate-induced polymerization of tubulin: Characteristics of the reaction and application to the large-scale purification of tubulin. *Arch. Biochem. Biophys.*, **209,** 29 – 40.

HESTERBERG, L.K. & LEE, J.C. (1982). Self-association of rabbit muscle phosphofructokinase: effects of ligands. *Biochemistry*, **21,** 216 – 222.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, **227,** 680 – 688.

LAKOWICZ, J.R. (1983). *Principles of Fluorescence Spectroscopy*. New York: Plenum Press.

LEE, J.C., HARRISON, D. & TIMASHEFF, S.N. (1975). Interaction of vinblastine with calf brain microtubule protein. *J. Biol. Chem.*, **24,** 9276 – 9282.

LEHOTZKY, A., PÁLFIA, Z., KOVÁCS, J., MOLNÁR, A. & OVÁDI, J. (1994). Ligand-modulated cross-bridging of microtubules by phosphofructokinase. *Biochem. Biophys. Res. Commun.*, **204,** 585 – 591.

LEHOTZKY, A., TELEGDI, M., LILIOM, K. & OVÁDI, J. (1993). Interaction of phosphofructokinase with tubulin and microtubule: quantitative evaluation of the mutual effects. *J. Biol. Chem.*, **268,** 10888 – 10894.

LILIOM, K., LEHOTZKY, A., MOLNÁR, A. & OVÁDI, J. (1995). Characterization of tubulin-drug interactions by ELISA. *Anal. Biochem.*, **228,** 18 – 27.

LIN, C.M., SINGH, S.B., CHU, P.S., DEMPCY, R.O., SCHMIDT, J.M., PETTIT, G.R. & HAMEL, E. (1988). Interactions of tubulin with potent natural and synthetic analogs of the antimitotic agent combretastin: a structure-activity study. *Mol. Pharmacol.*, **34,** 200 – 208.

MARGOLIS, R.L., RAUCH, C.T. & WILSON, L. (1980). Mechanism of colchicine-dimer addition to microtubule end: implication for the microtubule polimerization mechanism. *Biochemistry*, **19,** 5550 – 5557.

MAYR, G.W. (1987). Interaction of calmodulin with phosphofructokinase: binding studies and evaluation of enzymatic and physicochemical changes. *Methods Enzymol.*, **139,** 745 – 763.

MILLER, J.C. & ANTOWSK, G.E. (1984). BE 861 417. *Drugs Future*, **9,** 913.

MOLNÁR, A., LILIOM, K., OROSZ, F., VÉRTESSY, B.G. & OVÁDI, J. (1995). Anti-calmodulin potency of indol alkaloids in *in vitro* systems. *Eur. J. Pharmacol.*, **291,** 73 – 82.

NA, C.N. & TIMASHEFF, S.N. (1986). Interaction of vinblastine with calf brain tubulin: multiple equilibria. *Biochemistry*, **25,** 6214 – 6222.

OROSZ, F., CHRISTOVA, T.Y. & OVÁDI, J. (1988). Modulation of phosphofructokinase action by macromolecular interactions. Quantitative analysis of phosphofructokinase-aldolase-calmodulin interacting system. *Biochim. Biophys. Acta*, **957,** 293 – 300.

OROSZ, F., TELEGDI, M., LILIOM, K., SOLTI, M., KORBONITS, D. & OVÁDI, J. (1990). Dissimilar mechanisms of action of anti-calmodulin drugs: quantitative analysis. *Mol. Pharmacol.*, **38,** 910 – 916.

POTIER, P. (1980). Synthesis of the antitumor dimeric indole alkaloids from *Catharantus* species (vinblastine group). *J. Natl. Prod.*, **43,** 72 – 86.

PRAKASH, V. & TIMASHEFF, S.N. (1991). Mechanism of interaction of vinca alkaloids with tubulin: catharantine and vindoline. *Biochemistry*, **30,** 873 – 880.

PRAKASH, V. & TIMASHEFF, S.N. (1992). Aging of tubulin at neutral pH: the destabilizing effect of vinca alkaloids. *Arch. Biochem. Biophys.*, **295,** 137 – 145.

RAI, S.S. & WOLFF, J. (1996). Localization of the vinblastine-binding site on β-tubulin. *J. Biol. Chem.*, **271,** 14707 – 14711.

SACKETT, D.L. (1995). Vinca site agents induce structural changes in tubulin different from and antagonistic to changes induced by colchicine site agents. *Biochemistry*, **34,** 7010 – 7019.

SCHUBERT, D. & CARLISLE, W. (1979). Neuronal cells from rodent neoplasms. *Methods Enzymol.*, **58,** 584 – 591.

SUMMERS, M.D. & SMITH, G.E. (1987). A manual of methods for Baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulettin*, **1555,** 1 – 57.

TAKANARI, H., MORITA, J., YAMANAKA, H., YADA, K., TAKAHASHI, A. & IZUTSU, K. (1994). Effects of acidic pH on the formation of vinblastine-induced paracrystals in Chinese hamster ovary cells. *Biol. Cell.*, **82,** 51 – 57.

TIMASHEFF, S.N., ANDREU, J.M. & NA, G.C. (1991). Physical and spectroscopic methods for the evaluation of the interactions of antimitotic agents with tubulin. *Pharmacol. Ther.*, **52,** 191 – 210.

VAUGHN, J.P., GOODWIN, R.H., TOMPKINS, G.J. & MCCAWLEY, P. (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *In vitro*, **13,** 213 – 217.

VÉRTESSY, B.G., KOVÁCS, J., LÖW, P., LEHOTZKY, A., MOLNÁR, A., OROSZ, F. & OVÁDI, J. (1997). Characterization of microtubule-phosphofructokinase complex: specific effects of MgATP and vinblastine. *Biochemistry*, (in press).

WILSON, L., ANDERSON, K. & CHIN, D. (1976). Nonstoichiometric poisoning of microtubule polymerization: A model for the mechanism of action of the vinca alkaloids, podophyllotoxin and colchicine. In *Cell Motility*, ed., Goldman, R., Pollard, T. & Rosenbaum, J.L. pp. 1051 – 1064. New York: Cold Spring Harbor Laboratory.

WILSON, L., BAMBURG, J.R., MIZEL, S.B., GRISHAM, L.M. & CRESWELL, K.M. (1974). Interaction of drugs with microtubule protein. *Fedn. Proc.*, **33,** 158 – 166.

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