New Anti-Mitotic Drugs with Distinct Anti-Calmodulin Activity

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Abstract: Bisindole *Vinca* alkaloids target microtubule system causing anti-mitotic activity. The problem of their clinical application is the lack of selectivity resulting in toxic side effects. In this paper we review the late history of new bisindole derivatives focusing on KARs recognized as potent anti-cancer drugs with low side effect. KARs, just as other bisindoles, impede microtubule assembly of mitotic spindle, however, they display no anti-calmodulin activity. This new drug family appears to be less potent than vinblastine *in vitro* systems, but it shows high antitumor efficacy with considerably higher doses being well tolerated in the animal tumor models. 3D data of calmodulin complexed with KAR-2 explain the specificity and unique pharmacology of KAR derivatives.

Key Words: Tubulin/microtubule, *vinca* alkaloid, anticancer agent, calmodulin, bisindoles.

ANTICANCER DRUGS TARGETING MICROTUBULAR SYSTEMS

Cancer continues to be one of the major health and social-economic problems despite considerable progress in its early diagnosis and treatment. The search for new anticancer drugs and the development of more effective treatment strategies is a field of utmost importance in current drug discovery and clinical research. Anti-cancer drug discovery has focused extensively on targets related to mitosis, a crucial phase of cell cycle progression, the miscontrol and uncontrol of which can lead to development of human tumors. The major constituent of the highly dynamic mitotic spindle is the microtubule.

Microtubules, key components of cytoskeleton, are crucial in maintenance of cell shape, in several transport processes, in cell signaling and mitosis. Microtubules are highly dynamic polymers and their assemblies are tightly regulated both spatially and temporally. The α/β-tubulin heterodimer, subunit of the polymer, is a very conservative protein, yet its polymers/tube can express multiple functions depending on the cell function. The functional diversity of microtubules is achieved through static and dynamic binding of various regulatory proteins, including microtubule-associated proteins (MAPs) and cytosolic proteins.

The extensive involvement of microtubules in mitosis and cell division makes them an important target for anticancer drugs. Microtubules and their dynamics are targets of chemically diverse groups of anti-mitotic drugs that have been used with great success in the treatment of cancer. In fact, it has been argued that "microtubules represent the vest cancer target to be identified so far, and it seems likely that drugs of this class will continue to be important chemotherapeutic agents" [1].

Microtubule-targeting molecules occur as self-protecting, toxic molecules in plants and animals. Antimitotic agents that act on microtubules can be classified in two categories according to their mechanism of actions. *Vinca* alkaloids and colchicine (and several other molecules) inhibit microtubule polymerization; in contrast, taxanes (paclitaxel, docetaxel) promote polymerization and stabilize microtubules. In this paper we review the history of new potent semi-synthetic *Vinca* alkaloids, KARs, focusing mainly to the most powerful member of KAR derivatives, KAR-2, and compare their properties with that of the well-known therapeutic agents belonging to *Vinca* alkaloids.

Among the natural products, *Vinca* alkaloids, vincristine and vinblastine, were earliest isolated from *Catharanthus roseus* [2] (Fig. (**1**)). 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 polymerization into microtubules than vinblastine and vincristine [3]. Catharanthine has no clinically significant antimitotic activity. The vindoline moiety has no not been studied in detail.

Several hundred vinblastine and vincristine derivatives have been synthesized and evaluated for their pharmacological activities. These semi-synthetic molecules are modified in either the catharanthine or in the vindoline moieties, bearing several reactive centers. These efforts led to the identification of a couple of derivatives, which are potential anticancer agents, however, only a few of them have been used so far in chemotherapy (see recent related reviews: [1, 4-7]).

At the beginning, vinblastine was used as starting molecule since it was the only product readily available in relatively large quantity in the plant extract. The chemical conversion of bisindoles into semi-synthetic anti-tumor agents is motivated, on one hand, by the extensive need for potent anti-tumor agents in clinical chemotherapy and, on the other hand, by the fact that the known drugs have undesired side-effects. The modification of vindoline moiety resulted in the identification of the first semi-synthetic clinically active *Vinca* alkaloid, a desacetyl carboxyamide derivative of vinblastine, vindesine (desacetyl vinblastine amide sulfate) [8] (cf. Fig. (**1**)), registered in Europe in 1980 by Eli Lilly & Co. In meantime, new methods of coupling the two precursor

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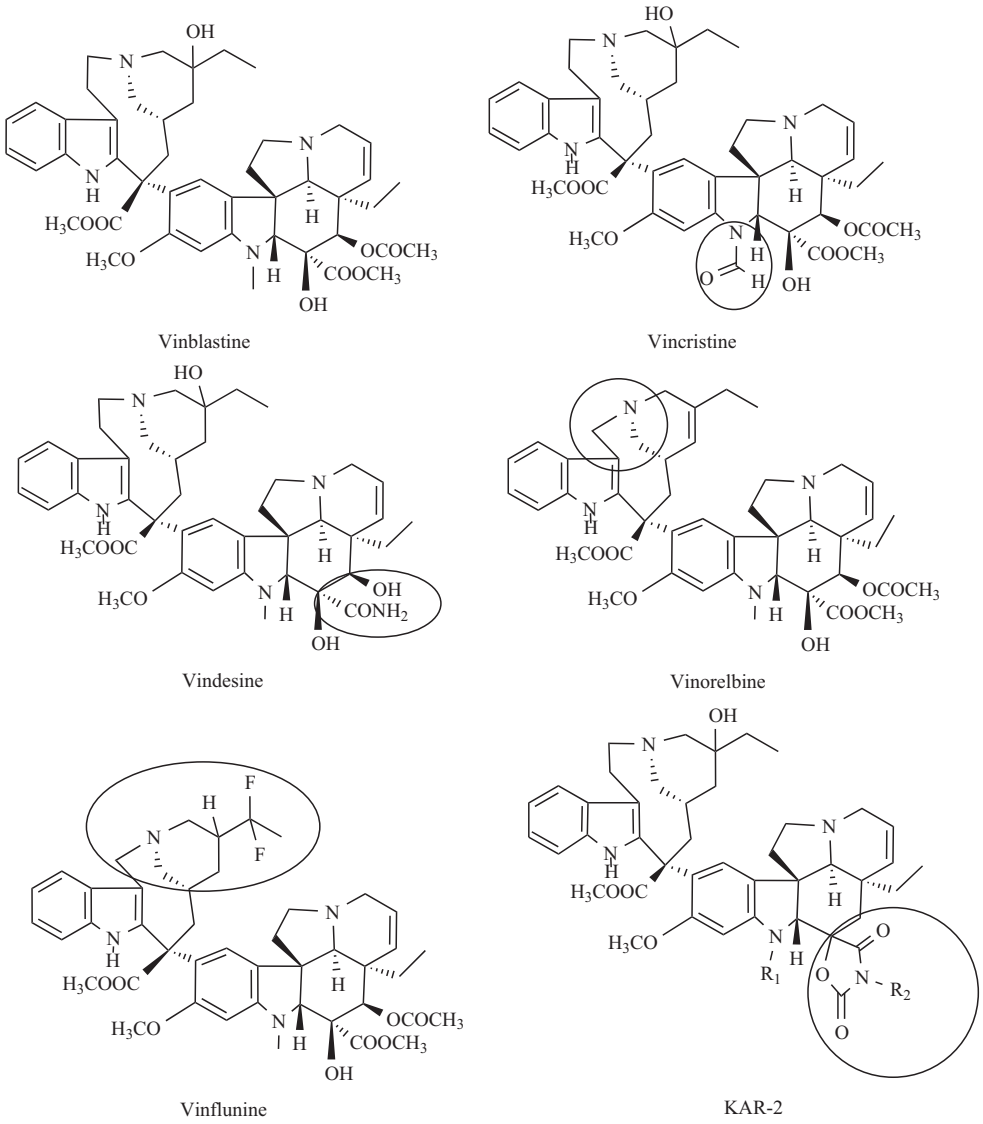

Fig. (1). Chemical structures of selected bisindoles.

KARs	R_1	R_2
KAR-2	CH_3	CH_2CH_2Cl
KAR-3	CHO	CH_2CH_2Cl
KAR-4	CH_3	$CH_2CH=CH_2$

alkaloids, catharanthine and vindoline, were developed to produce large amounts of the intermediate 3',4'-anhydrovinblastine [9]. The novel chemistry permitted the semi-synthesis of derivatives modified in the catharanthine "upper" part of the molecule, creating a new potential in the *Vinca* alkaloid medicinal chemistry. Vinorelbine or navelbine (5'-nor-anhydro-vinblastine) (cf. Fig. (**1**)) obtained by C' ring contraction of anhydrovinblastine [10] has been marketed worldwide by Pierre Fabre SA. This compound differs from the natural compounds by having an eight-membered rather than nine-membered ring in the catharanthine moiety.

The vindesine and vinorelbine were obtained by modification of reactive parts of the natural bisindole molecules using classic chemistry. Later on an original chemical approach in superacidic media was applied that could cause dramatic changes in the skeleton of these complex molecules. Superacids are able to protect functional groups by protonation and can induce modifications at non-activated bonds [11]. *Via* this approach a new family of fluorinated bisindoles were synthesized [12] from which vinflunine (20', 20'-difluoro-3',4'-dihydrovinorelbine) cf. Fig. (**1**) a bi-fluorinated derivative of vinorelbine, was selected for further stud-

ies on the basis of its activity in initial pharmacological screening. Vinflunine, a product of Pierre Fabre SA, is under Phase III clinical trial.

KARs, have been recently identified as new potential anti-cancer agents [13]. The major structural difference between these products and the mother molecules is the formation of a spiro-ring at the vindoline part of the bisindole [13] (Fig. (**1**)). While KAR-2 [3'-(β-chloroethyl)-2',4'-dioxo-3,5''-spiro-oxazolidino-4-deacetoxyvinblastine], and KAR-4 [3'-allyl-2',4'-dioxo-3,5''-spiro-oxazolidino-4-deacetoxyvinblastine], are derivatives of 4-deacetoxy-vinblastine, KAR-3 [3'-(β-chloroethyl)-2',4'-dioxo-3,5'-spiro-oxazolidino-4-deacetoxyvincristine], is synthesized from 4-deacetoxy-vincristine; both isolated from *Catharanthus roseus* extract according to De Bruyn *et al*. [14]. The crucial step of the synthesis is the ring formation in the 4-deacetoxy-derivatives by addition of chloroethyl-isocyanate in absolute tetrahydrofurane [15]. The KAR compounds synthesized by Drs Keve and Ács, synthetic chemists of Richter G. Chemical Works Ltd., Budapest, were screened in our laboratory for their anti-mitotic activities in various biological systems and KAR-2, KAR-3 and KAR-4 were selected for more detailed analysis. These bisindoles, however, have not undergone clinical trials for cancer therapy yet. The reason of this situation is complex, but one of them is economical, which prevented us as owner of these potent compounds to initiate into clinical trials, thus the results of our extensive research were published in series of scientific papers (cf. References).

For evaluation of antimitotic activities of bisindoles, three of the major pharmacological test procedures were used routinely by pharmaceutical companies for the last 30 years or so. These were centered upon analyses of the molecules' abilities to:

- interfere with the polymerization/depolymerization of isolated microtubules;

- exhibit marked *in vitro* cytotoxicity;

- show impressive *in vivo* antitumor activity against a transplantable murine tumor.

These characteristic features of the most potent bisindoles including KARs are summarized in Table **1**, and used as subtitle for reviewing the novel direction of bisindole research.

INTERFERENCE WITH THE POLYMERIZATION/ DEPOLYMERIZATION OF ISOLATED MICROTUBULES

The inhibition of the polymerization of the tubulin into microtubules (Table **1**) seems to be correlated with the binding affinity of the molecules. The binding affinities to tubulin are vincristine > vinblastine > vinorelbine > vinflunine [19, 20]. It has been shown for years that vinblastine binds to the *Vinca* site on microtubule and stabilizes microtubule plus ends and destabilizes minus ends [21]. More recent data established the effect of vinblastine and derivatives on microtubule dynamics as

- reduce the rate of shortening;

- increase the percentage of time the microtubule spent growing.

It has been suggested that these differences could be associated with differences in the binding of the drugs to the microtubule ends or in the extent of the stabilizing effects of the drugs [22]. Vinorelbine and vinflunine suppress both dynamic instability and treadmilling, but vinflunine inhibited

Table 1. Pharmacological Data of Bisindoles

Test	Inhibition of tubulin assembly[1]	Cytotoxicity *in vitro*[2]	Prolongation of survival of mice grafted with P388 leukemia cells	
Compound	(IC_{50} relative to vinblastine)		Optimal T/C (%)[3]	Optimal dose[4] (mg/kg)
Vinblastine	1^{abe}	1^{abcde}	143^{ab}	$2.5^{f}/5^{b}$
Vincristine	$1^{a}/0.71^{b}$	$0.95^{a}/1^{b}/1.6^{c}/2.6^{e}$	$143^{ab}/147^{e}$	$1.25^{fb}/1^{e}$
Vindesine	0.82^{a}	$1.4^{a}/2.5^{c}$	157^{a}	5^{f}
Vinorelbine	$1^{a}/0.71^{b}$	$1.7^{a}/1.8^{b}/3.2^{c}/6.8^{d}$	157^{ab}	10^{fb}
Vinflunine	$1.8^{a}/1.3^{b}$	$4.9^{a}/6.1^{b}/18^{c}/25^{d}$	200^{ab}	40^{fb}
KAR-2	0.81^{e}	62^{e}	207^{e}	60^{e}
KAR-3	0.55^{e}	62^{e}	187^{e}	20^{e}
KAR-4	0.75^{e}	70^{e}	214^{e}	40^{e}

[1] IC_{50} values represent the concentrations of bisindoles required to inhibit tubulin polymerization by 50%. Tubulin polymerization was followed by turbidimetry at 350 nm. IC_{50}s for vinblastine are: 1.7 μM (a), 2.4 μM (b), 0.25 μM (e).

[2] IC_{50} values represent the concentrations of bisindoles required to inhibit cell growth by 50%. L1210 murine leukemia (a,b), PtK2 (c) SK-N-SH (d) or SH-SY5Y (e) neuroblastoma. IC_{50}s for vinblastine are 16.3 nM (a), 16 nM (b), 4.8 nM (c), 2 nM (d), 5 nM (e).

[3] T/C (%) = surviving factor of drug-treated animals obtained by (median day of survival of treated animals / median day of survival of control animals) x 100. Drugs were given i.p. as a single dose.

[4] Dose used in single administration of animals which induced the highest T/C ratio.

Data were obtained from [a][23] [b][4] [c][17] [d][18] [e][13] [f][19].

the rate of treadmilling 4-fold less strongly than vinorelbine and 7-fold less strongly than vinblastine [23]. It is already known that small changes in the dissociation rate constant at microtubule minus ends can result in greatly modified treadmilling rates [24]. It is, however, unclear yet, whether these differences are related to antitumor activity or to the toxic side effect. Recently, it has been recognized that the most potent action of these drugs is the suppression of microtubule dynamics, rather than increasing or decreasing microtubule-polymer mass [7].

In fact, the effect of bisindoles on microtubule dynamics is concentration-dependent: they produce a remarkable range of effects that depend upon the drug concentration used [7, 25, 26]. High concentrations of *Vinca* alkaloids cause extensive depolymerization of microtubules and the rounding of the cells and formation of tubulin paracrystals. Lower concentrations induce depolymerization of microtubule network, and the lowest effective concentrations suppress microtubule dynamics [18] and references therein).

KARs show properties comparable to vinblastine concerning its binding affinity to the tubulin, and its inhibitory potency on microtubule assembly is even higher *in vitro* assays [13, 27] (cf. Table **2**). The dissociation constants of KARs and other bisindoles are in the micromolar range, which is much higher than the IC_{50} values of the inhibition of the tubulin polymerization (Table **2**).

The effects of KARs on microtubule assemblies were investigated *in vitro* under conditions when microtubule bundling occurred as well [13]. This was done by *in vitro* cross-linked microtubules to mimic *in vivo* situation. Fig. (**2**) shows that the formation of bundled microtubules (cross-linked by phosphofructokinase) is not affected by KAR-2, while the polymerization of tubulin into single microtubules is virtually arrested [13]. This simple experiment reveals that the anti-microtubular activity of KAR-2 highly depends on the organization state of microtubules. This phenomenon could have pharmacological relevance since the relative amounts of tubulin dimers, microtubules and bundled microtubules vary in a wide range depending on the cell type and/or on the intracellular conditions. Thus the efficacy of the drugs may vary due to factors influencing the organization of microtubules. Microtubules bundled by MAPs and other proteins largely occur in axons of neuronal cells which might be less sensitive to KARs as compared to mitotic spindle microtubule [33, 34]. This issue was investigated at cell level.

EXHIBITION OF *IN VITRO* CYTOTOXICITY

While vincristine, vindesine or vinorelbine, as active chemotherapeutic agents, show a couple of similarities to vinblastine at cell level, the cytotoxicity of vinflunine and especially KARs is significantly lower as compared to other bisindoles. Concentration-dependent effects of vinblastine,

Table 2. Anti-Tubulin and Anti-Calmodulin Effects of *Vinca* Alkaloids

Compound	Anti-tubulin effect	Binding to		Anti-calmodulin effect, %		
	Inhibition of tubulin polymerization, (IC_{50} relative to vinblastine)[1]	tubulin[2]	calmodulin[3]	Binding[4]		Activity[5]
		K_d, μM		ELISA	Phosphofructokinase	
Momomers						
Catharanthine	>1300[b]	>100[a]	n.m.	5[b]	n.m.	0[b]
Vindoline	>400[b]	30[a]	n.m.	0[b]	n.m.	0[b]
Bisindoles						
Vinblastine	1[be]	4.5[a]	3.0[b]	34[d]	39[d]	61[b]
Vincristine	1.5[b]/1.2[e]	4.0[a]	n.m.	43[d]	40[b]	62[b]
Vinorelbine	0.53[b]	5.0[a]	0.5[b]	68[d]	48[d]	83[b]
KAR-2	0.26[b]/0.81[e]	3.0[a]	4.6[c]/5.2[d]	18[d]	3[d]	7[d]
KAR-3	0.55[e]	n.m.	n.m.	n.m.	n.m.	45[e]
KAR-4	0.75[e]	n.m.	n.m.	n.m.	n.m.	5[e]
Phenothiazine (TFP)	n.m.	n.m.	1.6[c]	94[d]	87[d]	69[b]

[1] IC_{50} values were obtained and defined as described in the legend to Table 1.

[2] K_d values were determined by ELISA measurements.

[3] K_d values were determined by fluorescence (vinblastine, KAR-2) and circular dichroism (vinblastine, vinorelbine, TFP) spectroscopy.

[4] Inhibitory effects were determined by ELISA and fluorescence anisotropy measurements, at 250 μM and at 20 μM drug concentrations, respectively.

[5] Effect of drugs on calmodulin-modulated phosphofructokinase activity measured as described in [28].

Data are from: [a][29]; [b][30]; [c][31]; [d][32]; [e][13].

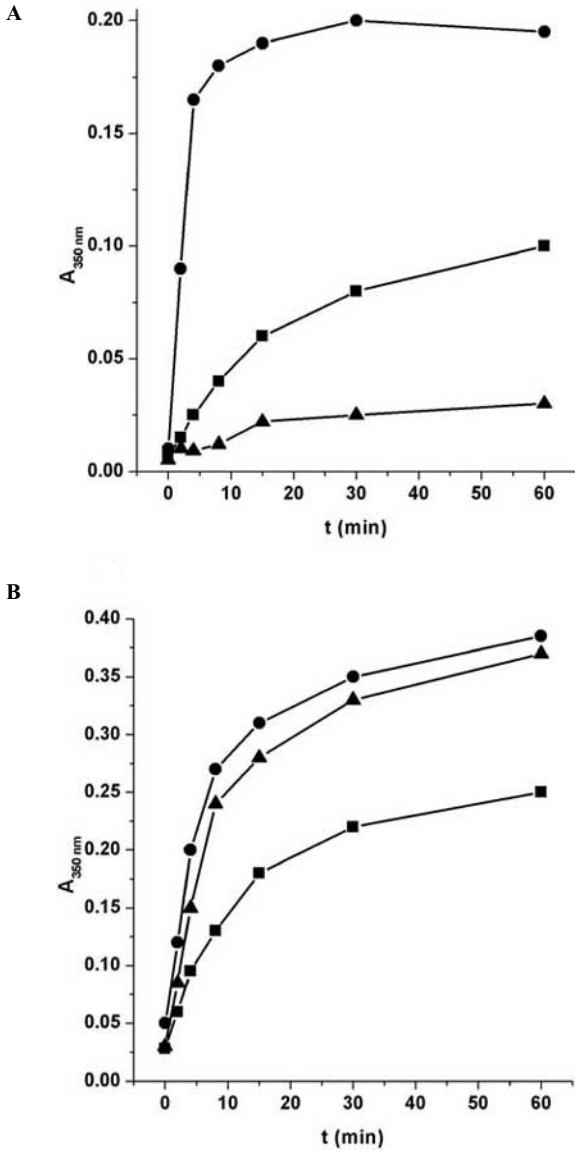

Fig. (2). Taxol-induced polymerization of 10 μM tubulin obtained by measuring turbidity at 350 nm in 50 mM 2-morpholinoethanesulphonic acid (MES) buffer, at pH 6.8 containing 100 mM KCl, 5 mM $MgCl_2$ at 37 C^o in the absence (A) and in the presence (B) of 2 μM rabbit muscle phosphofructokinase. (●) control (■) 2 μM vinblastine (▲) 2 μM KAR-2. Data published in [13].

vinorelbine and vinflunine on cell growth were investigated on various cell lines, for example, on SK-N-SH neuroblastoma or on HeLa cells, where IC_{50} values were 2, 13.5 and 50 nM or 0.45, 1.25 and 18 nM, respectively, as demonstrated by MTT assay [18, 26]. Similar data were obtained on various tumor cell lines as well. Table **1** shows the order of cytotoxicity of the active chemotherapeutic bisindoles, and new derivatives. The relative IC_{50} values show that vinblastine and vincristine are the most cytotoxic drugs in the *in vitro* assay. Vinflunine is less cytotoxic than its mother molecule, at least respect to their inhibitory effects *in vitro* on the cell proliferation.

The two dynamic features of microtubules, dynamic instability and treadmilling are important for cell cycle progress [35, 36]. Nowadays it is accepted that *Vinca* alkaloids block cell cycle in G2/M phase and inhibit the dynamic features of microtubules. The comparison of the effects of vinblastine, vinorelbine and vinflunine showed that the extent of inhibition was significantly different from each other, however, these differences did not result in morphologically detectable differences in spindle effects. This finding indicates that the mitotic block induced by the three drugs is the major contributor to their anti-proliferative action [26].

The quantitative differences in the effects of drugs on cell proliferation and mitotic block were not originated from differences in the accumulation of drugs at cellular level. The time courses of uptake of the three drugs revealed that all drugs entered HeLa cells gradually, reaching maximal levels within 4h, and these intracellular concentrations were significantly higher than the extracellular levels for vinflunine, vinorelbine and vinblastine, respectively. The accumulation of vinblastine and vinflunine was similar. Therefore, the increased potency of vinblastine cannot be accounted for its preferential cellular accumulation [26].

The effect of KAR concentrations on the proliferation of SH-SY5Y neuroblastoma cell was also determined using MTT assay [37]. As can be seen from the IC_{50} values presented in Table **1**, there is negligible difference in cytotoxicity of the KAR derivatives. KAR-3 and KAR-4 exhibit similar or even slightly lower cytotoxicity than KAR-2 does. The IC_{50} values of KARs are much higher, with more than one order of magnitude, than those of the other *Vinca* alkaloids, except vinflunine. The dose-response curves of these bisindoles on neuroblastoma cell lines shown in Fig. (**3**) reveal that KAR-2 inhibited the cell growth at significantly higher concentration than vinblastine, vinorelbine or vinflunine.

The effects of vinflunine and KAR-2 on cell cycle distribution at their IC_{50} values as compared to that of vinblastine are shown in the case of neuroblastoma cells (Table **3**). In the case of the control (no drug) most of the cells were in G1 or S phase and about 10% were in G2/M phase. The treatment of the cells with vinblastine resulted in increase of cell number occurring in G2/M phase due to the inhibition of cell cycle specifically at G2 phase indicating that the cell cycle is arrested at mitosis. The treatment of the cells with KAR-2 led to an entirely different pattern of cell cycle distribution of neuroblastoma cells: 68% of the SH-SY5Y cells were in G2/M phase, most of them in M, and only 7% remained in G1 (cf. Table **3**).

Low concentration of vinflunine (using SK-N-SH or SH-SY5Y neuroblastoma cells) slows down mitotic progression but fails to block cells in G2/M (22% - Table **3**) as quantified by mitotic index as well [18]. A slight increase in mitotic index corresponds to a slowing down of the progression into mitosis suggesting that the cells at the IC_{50} value are succeeded in exiting from a slower mitosis, and undergo postmitotic diploid G1 arrest. However, high concentration of vinflunine such as 500 nM induces G2/M block with high mi-

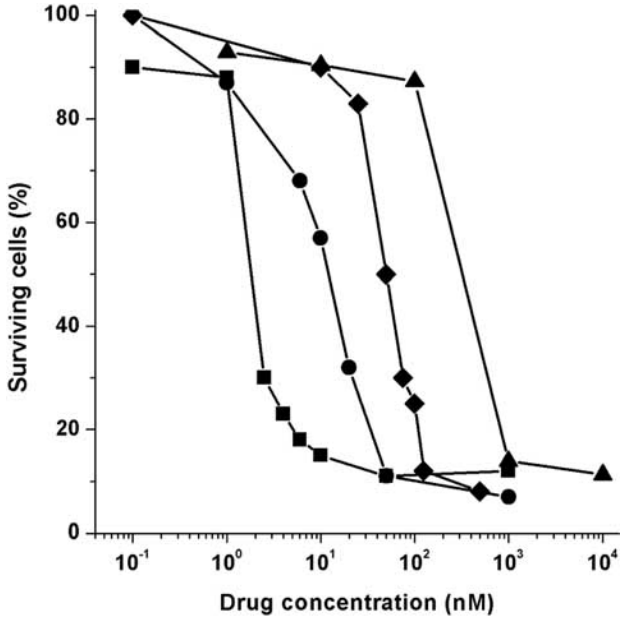

Fig. (3). Concentration dependent inhibition of cell growth by KAR-2 (▲), vinflunine (◆), vinorelbine (●) and vinblastine (■). Exponentially growing SH-SY5Y [37] or SK-N-SH [18] neuroblastoma cells were incubated with the drugs for 72 h, and using the MTT reagent assessed cell proliferation. Data are taken from [37] (KAR-2) and [18] (vinflunine, vinorelbine and vinblastine).

totic index (cf. Table **3**). The concentration and time-dependent mitotic arresting properties of vinflunine at G2/M phase was confirmed in a series of cell culture systems [16].

There are additional extensive cellular studies concerning the effects of the two new bisindoles, vinflunine and KAR-2, and their mother molecules, vinblastine on the spindle organization and other molecular events of the cell cycle process which might contribute to our understanding of their low but still distinct cytotoxicities. The arrangement of microtubules and chromosomes induced by these drugs at their IC_{50} concentrations were investigated by immunofluorescent microscopy using anti-tubulin antibody and 4'-6-diamidino-2-phenylindole (DAPI) staining. The experiments were performed with two different human cell lines (HeLa and SH-SY5Y, respectively) by different research groups, however, in both sets of experiments vinblastine was used as reference drug.

As shown in Fig. (**4**) the control cells in metaphase contain well-organized bipolar spindles, and all of chromosomes are organized in compact equatorial plane. After incubation of the cells with vinblastine at its IC_{50} concentrations

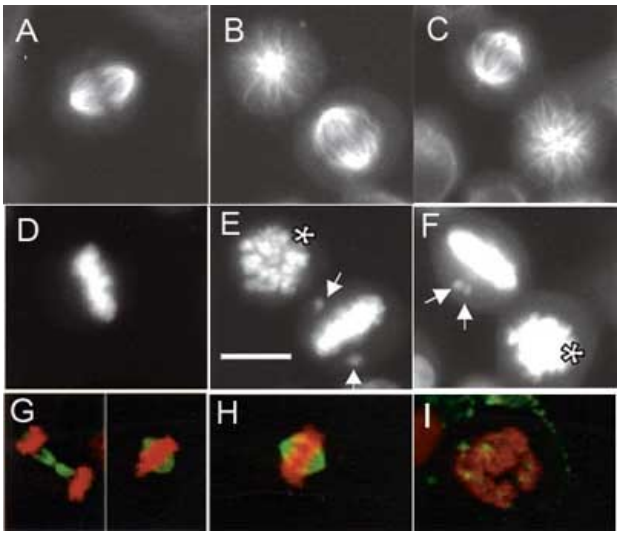

Fig. (4). Effect of bisindoles on the mitotic microtubule network and chromosomes detected by immunofluorescence microscopy. Images for HeLa (A-F) and SH-SY5Y (G-I) are taken from figures published in [26], and [37], respectively. IC_{50} concentrations of the vinblastine (B,E,H), vinflunine (C, F) and KAR-2 (I) were used for treatments of cells. Cells were immunostained for tubulin (A-C, G-I) and for DNA (D-I). Arrows and asterisk show chromosomes uncongressed and arranged in a ball, respectively. Bar: 10 μm.

Table 3. Effect of Bisindoles on the Cell Cycle Distribution

bisindole	IC_{50} nM	G1	S	G2/M	M	mitotic index
control		62[a]	27[a]	11[a]/16[b]	3.4[a]	4.3[b]
vinblastine	2[b]/5[a]	38[a]	16[a]	46[a]	18[a]	
vinflunine	50[b]	high[b]		22[b]		6.2[b]
				59 (at 500nM)[b]		41(at 500nM)[b]
KAR-2	316[a]	7[a]	25[a]	68[a]	41[a]	
W13	30[a]	partial stop in G0/G1, S and G2/M phase[a]				
KAR-2+W13		similar to vinblastine[a]				

SH-SY5Y (a) or SK-N-SH (b) neuroblastoma cells were treated for 24h at the IC_{50} values of the drugs. Data are taken from [37] and [18] for SH-SY5Y and SK-N-SH cells, respectively.

(2-5 nM) the bipolar spindles apparently do not damaged, however, the appearance of aberrant chromosome structures could be visualized cf. Fig. (**4**). At higher concentrations of vinblastine and other *Vinca* alkaloids, vincristine and vindesine, the abnormality mostly fell into various types of abnormal spindles [26, 38]. In SH-SY5Y cells treated with vinblastine the number of cells with condensed chromosomes was higher than in untreated cells, and some of them had mitotic spindle with normal appearance (around 30% of mitosis were normal) [37]. In contrast, KAR-2-treated cells had a high number of aberrant mitotic spindle cf. Fig. (**4**), less than 10% of mitosis were normal. In fact, vinblastine and vinflunine showed the same sequence of concentration-dependent changes in spindle morphology, albeit over different concentration ranges. Interestingly, the microtubule network of interphasic cells was more damaged in the vinblastine-treated cells than in the KAR-2-treated cells (data not shown). The unique feature of KAR-2 to cause selective arrest of cell cycle may have high impact in drug development.

The fact that KAR-2 causes stronger and selective mitotic arrest as antimitotic agent than vinblastine, was further corroborated by the following observations: i) levels of cyclin A and B1 were lower; ii) Cdc2 activity doubled in KAR-2-treated cell with respect to vinblastine-treated cells [37]. The specificity of anti-tumor drugs in arresting cell cycle is of importance in oncology in developing clinical treatment protocols and designing antitumor strategies involving specific drug combinations. For instance, Stone *et al*. [39] demonstrated that normal cells overexpressing p16 induced by specific drugs were more resistant to anti-mitotic drugs as compared to cancer cells. This is due to the fact that normal cells respond to p16 overexpression reversibly by arresting cell cycle at G1, while in many tumors the p16 regulatory pathway is inactivated, and thus cells progress to mitosis, where they become susceptible to anti-mitotic drugs. Testing several anticancer agents they concluded that the most dramatic effect was observed with vinblastine. However, it was suggested that other agents, more specifically directed against the G2 or M phases than vinblastine, might be more effective [39]. From this point of view the new KAR family of Vinca-alkaloids could be an excellent candidate to test this hypothesis.

Bisindoles, like other microtubule damaging agents are able to induce apoptosis exhibiting typical morphological changes and DNA fragmentation in the treated cells. The mechanism of this complex process is unclear yet. The vinflunine-induced apoptosis in SK-N-SH neuroblastoma cells is highly concentration-dependent: low concentrations induce apoptosis through post-mitotic G1 arrest and a mitochondrial pathway [18]. At the IC_{50} concentration vinflunine induced 64% apoptosis and blocked cells in G2/M. KARs caused apoptosis rather than necrosis in human neuroblastoma cell line SH-SY5Y. Both the KARs and vinblastine at their IC_{50} slightly increased the number of apoptotic cells (about 10%), whereas the proportion of necrotic cells, including late apoptotic cells was very low (about 3%)[37]. An increase in the number of apoptotic and necrotic cells was evident in cells exposed to high doses of vinflunine or KAR-2. Therefore, it can be concluded that vinflunine and KAR-2 behaves similarly to each other on neuroblastoma cell lines.

Therefore, there seems to be consensus that the *Vinca* alkaloids block mitosis at the metaphase/anaphase transition (G2/M phase) by depolymerizing microtubules and /or inhibiting their dynamics leading to apoptosis [7], however, this mechanism highly depends on the drug, on its concentration, and on cell type [18]. Among these drugs, the effect of KAR-2 on cell cycle inhibition appears to be the most specific but there is the less knowledge how the inhibition of microtubule dynamics-involved in this process.

IN VIVO ANTITUMOR ACTIVITY AGAINST A TRANSPLANTABLE TUMOR

The main pharmacological test to show impressive *in vivo* antitumor activity of drugs is the P388 murine leukemia grafted to mice. The quantitative measure of this activity is the T/C value of life span, a surviving factor of the drug-treated animal as compared to the controls. The superiority of vinflunine as well as KARs relative to the mother molecules was demonstrated in P388 assays; the higher T/C % values were achieved in the cases of the two new bisindoles at their optimal dose (cf. Table **1**). Although both vinflunine and KARs are least potent in the cytotoxicity assays (cell level experiments), only these derivatives exhibited "high level of antitumor activity" (T/C ratio of > 175%) according to the NCI standard [40]. At single administration of vinflunine and KAR-2, the T/C values were around or even higher than 200 (cf. Table **1**). Increases in life span achieved with vinflunine in multiple doses, as assessed by T/C ratios, ranged from 200% to 457% and thus vinflunine proved marked superiority comparing to the T/C values of 129%-186% obtained with the other *Vinca* alkaloids. Therefore, *in vivo* vinflunine and KAR-2 are the most effective in terms of antitumor efficacy although considerably higher doses relative to vinblastine, vincristine or vinorelbine (8- to 48-fold) are required to express their effect; these doses are however, are well tolerated in the mouse [13, 23].

In another *in vivo* tumor models, B16 melanoma or Ehrlich ascites carcinoma were grafted to mice, and treated with vinflunine and KAR-2, respectively. In these cases the decrease of the tumor volumes were measured, and significant tumor growth inhibition (T/C value of tumor volume <42%) was observed at optimal doses higher than in the case of the mother compounds [13, 16]. Against s.c.-implanted B16 melanoma, multiple i.p. administration of vinflunine proved active in terms of both survival prolongation and tumor growth inhibition, with optimal T/C values. The extent of this activity was superior to that noted for vinorelbine under the same conditions [19]. At relatively high dose (20-60 mg/kg), using single administration, KAR-2 slowed the growth of tumors, yet exhibited lower toxicity to the host animal than the parent compound did.

Since tubulin is considered as the primary target of *Vinca* alkaloids, it can be expected that differential interactions with tubulin should correlate to some extent with differences in their clinical utility. Lobert *et al.* [20] first pointed out that the order of the tubulin binding affinities, namely: vincristine

> vinblastine > vinorelbine, correlated well with the weekly intravenous doses used clinically, where vincristine is used at the lowest dosage (0.4 - 1.4 mg/m^2) and vinorelbine at the highest (25-35 mg/m^2). Newest studies showed that this tendency is valid for vinflunine as well (350 mg/m^2) [23]. In fact, the cytotoxicity data followed the same order [17, 19] (cf. Table **1**).

The strength of binding of the bisindoles to tubulin, however, is not necessarily related to antitumor efficacy. There are *Vinca* derivatives which interact with tubulin with a high affinity have negligible antiproliferative effects, as well as others identified as having a weaker affinity more effectively prevent tumor production in mice. Vinflunine can be categorized into the latter group of bisindoles. However, there is a new group represented by KARs which interact with tubulin with a high affinity, however, display low cytotoxicity and high efficacy to inhibit tumor production in mice (cf. Table **4**). A good measure of the therapeutic index is the AAI value (antitumor activity index) as suggested by Duflos and his coworkers [4], which shows the concentration range of the antitumor activities. They assumed that this value might be discriminatory in selecting the most efficacious drugs, with the highest T/C values over the widest range of therapeutically active doses. Vinflunine displayed significantly higher AAI than the other *Vinca* derivatives; however, as can be seen in Fig. (**5**), KAR-2 showed similarly high therapeutic index as vinflunine.

While vincristine, vindesine or vinorelbine, as active chemotherapeutic agents, show a couple of similarities to vinblastine at cell level, the cytotoxicity of vinflunine and KARs are significantly lower as compared to vinblastine (Table **1**). In the case of vinflunine, it is not surprising, it corresponds to its low binding affinity to tubulin and low inhibitory activity on microtubule assembly. However, KARs bind to tubulin with high affinity, and are very effective in both *in vitro* and *in vivo* tests, yet exhibit low cytotoxicity, in contrast to other bisindoles (Table **1** and **4**).

The lack of any marked correlation between the effect of KARs as compared to other *Vinca* alkaloids at molecular, cellular and animal levels suggests that the extensive cytotoxicities of the latter ones may not be exclusively related to their antimicrotubular activities, and it suggests the involvement of additional factors/processes in the bisindole-mediated pathological processes.

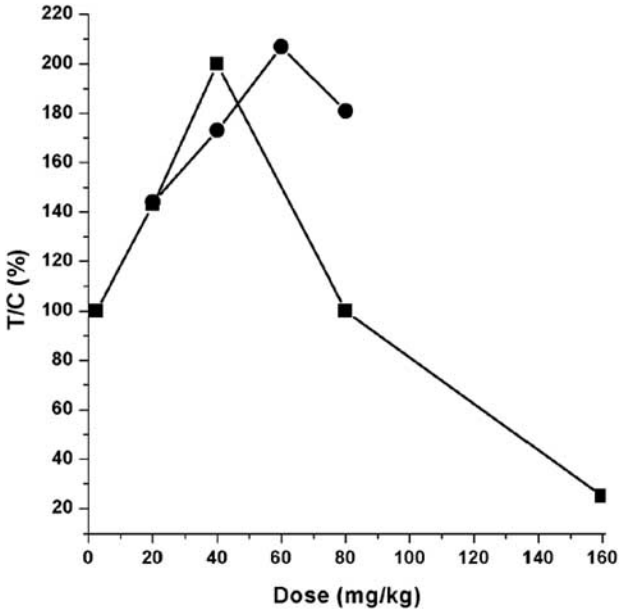

Fig. (5). Graphic representation of the % T/C, determined as the survival of treated relative to control mice, for vinflunine (■) and KAR-2 (●). Data are taken from [27] for KAR-2 and from [4] for vinflunine.

CALMODULIN IS A POTENT TARGET OF BISINDOLES

Nowadays more and more proteins have been discovered which are involved in the complex machinery of the mitosis as well as in the pathomechanism of the cell proliferation. A potential player is certainly a key Ca^{2+} receptor molecule, calmodulin. As an essential element of mitotic apparatus, it is involved in the attachment of kinetochore microtubules to the centrosome [41]. Microtubule perturbation by antimitotic drugs causes redistribution of calmodulin in mammalian cells. In addition, calmodulin has also role in the regulation of cell cycle *via* the actions of protein kinases and phosphatases, for example, the G1/S and G2/M transition are modulated by calmodulin-dependent protein kinase II and calcineurin [42].

Calmodulin, a ubiquitous, multifunctional Ca receptor protein, regulates the function of at least 100 different proteins and enzymes. When Ca^{2+} ions bind to calmodulin, extensive hydrophobic surfaces become exposed to the solvent,

Table 4. Relationship Between *In Vitro* and *In Vivo* Effects of Bisindoles

	tubulin binding	inhibition of MT[1] assembly	cytotoxicity	anti-CaM[2] potency	therapeutic index (AAI)[3]	toxic side effect
vinblastine	high	high	high	high	low	high
vinflunine	low	low	low		high	low
KAR-2	high	high	low	low	high	low

[1]MT - microtubule; [2]CaM - calmodulin; [3]AAI / antitumor activity index as defined in [4]

forming hydrophobic pockets which promote the interactions of calmodulin with target proteins. The calmodulin-binding domains of the target proteins are highly diverse in their amino acid sequences. One of the few unifying principles is the ability of all of these peptides to form basic, amphiphilic helices when complexed with Ca^{2+}-calmodulin [43]. Three-dimensional structures of Ca^{2+}-calmodulin complexed with a peptide from a target enzyme have been solved. In most of them, the two domains of calmodulin wraps around the peptide, contacting hydrophobic residues in the pocket between them [44-46].

CALMODULIN-DRUG BINDING

Antagonists of calmodulin differ both in their chemical structures and in their mechanisms of action [44-50]. Phenothiazines, arylalkylamines, naphtalenesulfonamides, calmidazolium and felodipine are widely used in studying calmodulin functions. All these drugs express their anti-calmodulin activity by competing with the target protein for calmodulin binding. In fact, triflouperazine (TFP) from the phenothiazines has been extensively used as classic calmodulin antagonist, the binding sites on calmodulin as well as their binding affinities have been identified. With a few exceptions (e.g. W7, W13 (naphtalenesulfonamide derivatives) and AAA, an arylalkylamine), the antagonists bind to calmodulin with much lower affinity than do peptides [28, 47-50]. K_d is in the micromolar range for drugs and in nanomolar range for target peptides [43] and references therein.

The direct interactions of calmodulin with bisindoles: vinblastine, vincristine, vinorelbine or KARs, were detected by using different spectroscopic techniques [30-32]. The K_d values of calmodulin - drug complexes are summarized in Table **2**. Extensive studies concerning the potential role of anti-calmodulin activity of bisindoles have been carried out only with KARs as compared to vinblastine. It has to be noted, nevertheless, that the affinity of bisindoles to calmodulin is in the same order of magnitude as to tubulin/microtubules (cf. Table **2**). Thus one can address the question why the microtubule system is exclusively considered as the target of bisindoles?

The binding affinity of vinblastine and KAR-2 to calmodulin is similar in contrast to that they have distinct binding sites on calmodulin as suggested by indirect circular dichroism measurements in Fig. (**6**). Binding of two TFP molecules to calmodulin induces two distinct peaks, negative and positive ones which are formed consecutively. These peaks could be assigned on the basis of the crystallographic data of calmodulin-1TFP and calmodulin-2TFP. Accordingly, the first TFP molecule binds to the hydrophobic pocket of the C-terminal domain, the second TFP molecule interacts with an inter-domain site. As shown in Fig. (**6**) while vinblastine diminishes the peak corresponding to the C-terminal bound TFP, KAR-2 eliminates the signal of the binding of TFP to the second, interdomain site [31]. Therefore, KAR-2 and vinblastine presumably bind to different sites on calmodulin. This indirect result now has been supported by recent x-ray data ([51] and Harmat, V. Budapest, unpublished data).

3D STRUCTURE OF CALMODULIN-KAR-2 COMPLEX

X-ray crystallography provided the 3D structures of calmodulin complexed with competitive antagonists, such as TFP [52-54] or an arylalkylamine derivative, AAA [55]. These data supported the previous indirect results that the hydrophobic pocket of the C-terminal domain of calmodulin is the primary binding site for the antagonists. This finding explains the competition of target peptide/enzyme and antagonists for calmodulin binding, in addition, provides explanation why there is no specific antagonist so far.

Recently, the 3D structure of calmodulin-KAR-2 complex was solved at atomic level in crystal and the binding

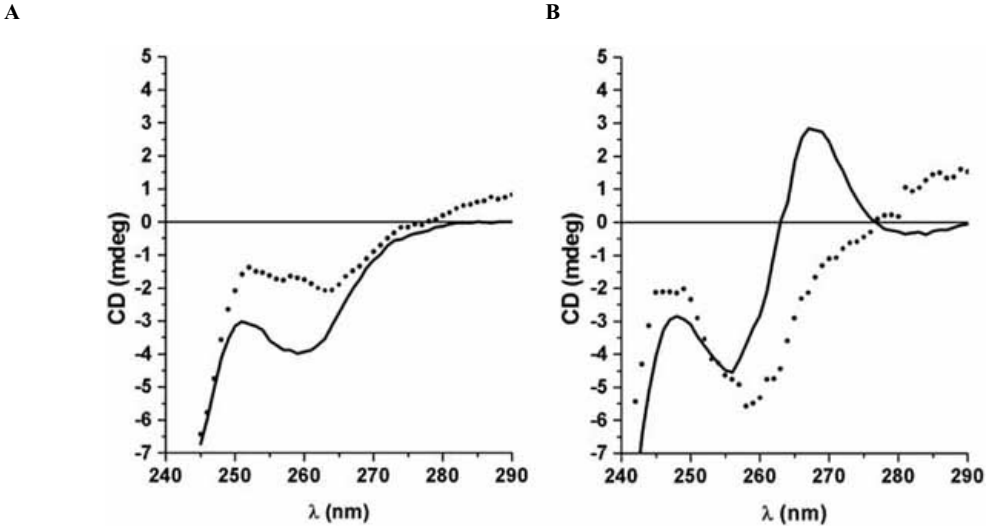

Fig. (6). Distinct effects of KAR-2 and vinblastine on the formation of calmodulin-TFP complexes. (A) difference spectra of 10 μM calmodulin and 10 μM TFP without (*solid line*) and with 10 μM vinblastine (*dotted line*). (B), difference spectra of 10 μM calmodulin with 30 μM TFP without (*solid line*) and with 30 μM KAR-2 (*dotted line*). Data are from [31].

site was also determined in solution with NMR [51]. These data showed that KAR-2 was not accommodated into the hydrophobic pocket, but - in contrast to the antagonists - it bound to a distinct domain of calmodulin. NMR data revealed that the binding of KAR-2 to calmodulin causes global conformational changes in the protein structure resulting in a closed compact conformation. The structure of the calmodulin-KAR-2 complex in solution is consistent with that determined by X-ray crystallography.

As illustrated in Fig. (**7**), the bound KAR-2 molecule is situated in an interdomain position between the N- and C-terminal lobes of calmodulin. The compact conformation of calmodulin induced upon binding KAR-2 resembles that assumed by calmodulin when it is bound by TFP molecule or by most cognate peptides [44-46, 52-54]. Overlaying the structures of calmodulin-KAR-2 and calmodulin-TFP1 complex it clearly demonstrates that KAR-2 and the C-terminal-bound TFP are accommodated in distinct sites on calmodulin. Indeed, most of the amino acid residues contacted by the KAR-2 are different. The catharanthine region of KAR-2 principally contacts hydrophobic residues (Phe-19, Leu-39, Phe-68, Met-71, Met-72) of the N-terminal domain of the protein while most of the contacts of the vindoline moiety (which contains the oxazolidino ring characteristic of this bisindole derivative) are formed with residues in the C-terminal lobe of calmodulin. In contrast to the catharanthine region, however, this part of the drug does not exclusively contact hydrophobic side chains (Ile-85, Met-109, Met-124, Met-144, Met-145), but also makes several interactions with polar residues (Glu-84, Glu-114, Lys-148). A surprising feature of the interface between KAR-2 and calmodulin is that the two hydrophobic pockets are abolished by the intradomain rearrangement, induced by the drug binding. In fact, this conformational change by the accommodation of KAR-2 to a new binding domain can cause limited, if at all, inhibitory effect on calmodulin mediated events.

Fig. (7). Superposition of the structures of calmodulin complexed with KAR-2 (from blue to red) (PDB id: 1XA5) and with TFP (grey) (PDB id:1CTR). KAR-2 (yellow) and TFP (pink) bind to different sites on calmodulin. Although the global conformation is similar, TFP binds to the hydrophobic pocket of the C-terminal domain, while in the accommodation of KAR-2 both the N- and the C-terminal domains are involved [51].

EFFECT OF BISINDOLES ON THE CALMODULIN-MEDIATED FUNCTIONS

The distinct properties of KAR-2 in relation to other calmodulin-binding drugs, vinblastine and TFP, are manifested in their calmodulin-mediated functions as shown by competitive binding and activity assays. The indirect binding assays such as ELISA, fluorescence anisotropy and surface plasmon resonance were used to monitor the modulating effect of drugs on the calmodulin-target protein complex [30, 32, 51] (cf. Table **2**). Different target proteins, aldolase, phosphofructokinase and phosphodiesterase, were used in the binding and activity assays, [30, 32, 51]: while the two glycolytic enzymes are inhibited, phosphodiesterase is activated by calmodulin. The use of these enzymes in these assays was substantiated by two factors; they exhibit very different biological functions, and ii) calmodulin interacts with comparable affinity with the two glycolytic enzymes and the bisindoles [28, 56], thus relatively low drug concentrations can be used and no direct binding of the bisindoles to the target enzymes could occur.

Comparative data for the inhibitory potency of KAR-2 and vinblastine and/or TFP shown in Fig. (**8**) were obtained by surface plasmon resonance measurements and in the classic phosphodiesterase assay. While TFP prevented the binding of aldolase to immobilized calmodulin, KAR-2 only partially inhibited the heteroassociation. The phosphodiesterase assay had the same outcome: vinblastine exhibited competitive antagonist activity even if in less extent that that of TFP; KAR-2, in contrast, did not decrease calmodulin-dependent phosphodiesterase activity below about 60% of the control (no drug). Similar qualitative picture was obtained in the phosphofructokinase assay concerning the inhibitory potency of bisindoles (Table **2**). As shown in Table **2** all of the tested bisindoles (vinflunine was not available) displayed anti-calmodulin activity but KAR-2 was the least potent bisindole in this respect; it was able to abolish neither the binding of calmodulin to aldolase nor the activation of phosphodiesterase by calmodulin [32] (Table **2** and Fig. (**8**)).

The structural and functional studies revealed that calmodulin could simultaneously accommodate two drugs with different chemical structures. The fact that TFP and KAR-2, as well as vinblastine and KAR-2 can form ternary complex with calmodulin, but not TFP and vinblastine [31, 51] suggests that KAR-2 interacts with distinct binding domain of calmodulin. This feature of KAR-2 was supported by functional test.

The presence of KAR-2 significantly reduced the inhibitory effect of TFP in calmodulin-activated phosphodiesterase assay which in the absence of KAR-2 resulted in complete inhibition. However, this effect was not seen with the mixture of vinblastine and TFP [51]. These data together with the 3D data of calmodulin-KAR-2 complex render it possible to interpret the situation in this complex system: KAR-2 is accommodated by a novel conformation of calmodulin which does not allow binding of TFP to the hydrophobic pocket of the C-terminal domain of calmodulin which otherwise occurs. Therefore, KAR-2 functions as a “liberator molecule” which counteracts with the antagonist effect of TFP [15]. In the light of these observations one can hypothesize that in the combination of vinblastine or vincristine with

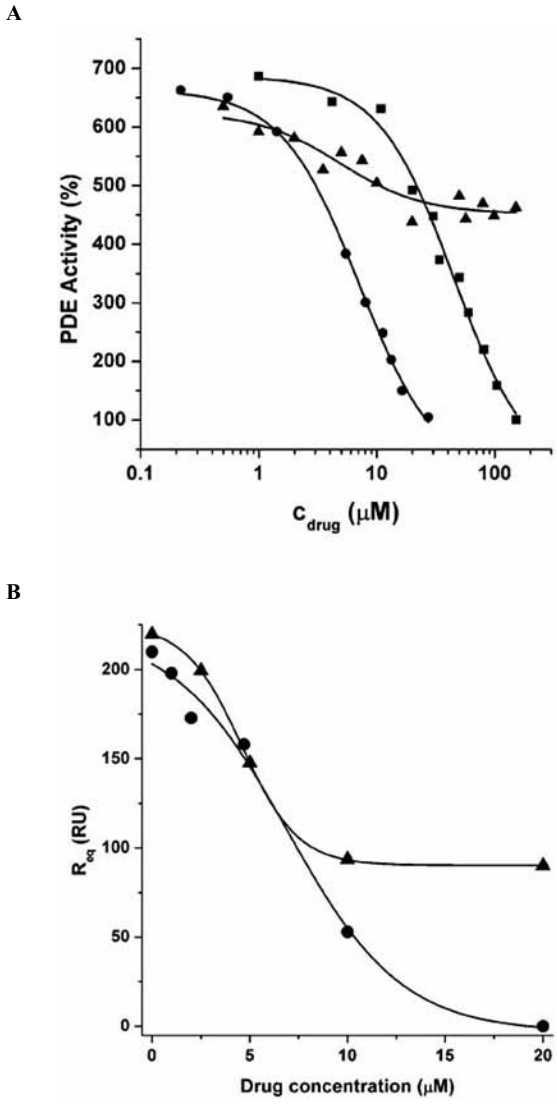

Fig. (8). The anticalmodulin potency of KAR-2, vinblastine, and TFP tested by calmodulin-activated phosphodiesterase (PDE) assay (A) and surface plasmon resonance measurements (B).

(A): Displacement curves for TFP (●), vinblastine (■), and KAR-2 (▲). 100% phosphodiesterase activity corresponds to the reaction rate measured without calmodulin (B): effect of KAR-2 on aldolase binding to immobilized calmodulin. Calmodulin was immobilized on the chip, and 5 μM aldolase was injected together with various concentrations of TFP (●) and KAR-2 (▲). The RUeq is proportional to the amount of surface bound aldolase at equilibrium. Data published in [51].

KAR-2 in chemotherapy, KAR-2 would reduce the undesirable toxic effect of the former bisindoles.

UNDESIRED SIDE-EFFECT OF BISINDOLES

Vinblastine and vincristine are extensively used in chemotherapy, they successfully arrest mitosis, cell division, however, there is definitive necessity to overcome their undesired neurotoxic side effect by increasing their "selectivity and specificity". Although the mechanism responsible for the neurotoxic effect of bisindoles is poorly understood, it "undoubtedly involves the effects of the drugs on microtubules, which are key components of neurons" [1]. There are suggestions for interpretation of the neurotoxicity e.g., disruption of axonal flow and neuronal retraction [57], steric hindrance of motor protein binding to microtubules, altered microtubule dynamics in axonal processes [1]; or demyelinization [58].

Ngan and coworkers [26] have concluded that the diverse actions of these drugs on microtubules are likely to produce different effects on mitotic spindle function, leading to different effects on cell cycle progression and cell killing. Non-tumor cells with "normal" checkpoint proteins may tolerate the relatively less powerful inhibitory effects of vinflunine and vinorelbine on microtubule dynamics rather than the more powerful effects of vinblastine. The situation might be similar with KAR-2. Furthermore, since checkpoint mechanisms in tumor cells are frequently faulty, the tumor cells may be more susceptible than normal cells to bisindoles. Thus the unique constellation of effects of vinflunine and vinorelbine on dynamic instability and treadmilling may play an important role in their superior experimental antitumor efficacies. It is an important question, however, whether the anti-calmodulin activity of these drugs has any impact on the "checkpoint mechanism" of tumor cells. Calmodulin participates in the regulation of the G0/G1 transition [59], in the progression into and through S phase [60-62], and in the initiation as well as in the exit of mitosis [60]. Anti-calmodulin drugs inhibit the re-entry of growth-arrested cells into the cell cycle (G0/G1) [63], the progression into and through S phase [64], the initiation of mitosis (G2/M) [65] and mitosis exit [66]. Arrest on G1 and G2/M was detected in SH-SY5Y cells after treatment with vinblastine or with a combination of W13 (a selective calmodulin antagonist) and KAR-2, at concentration of these compounds equivalent in causing 50% of inhibition of cell growth [37]. In contrast, KAR-2 alone arrested the cell cycle mainly in G2/M phase, whereas W13 alone caused a typical anticalmodulin effect, a partial stop in G0/G1, S and G2/M phase (cf. Table **3**). The fact that the combination of KAR-2 and W13 arrests cell cycle similarly as vinblastine alone may suggest that the effect of vinblastine on cell cycle is a result of the combination of its antimicrotubular activity and its anticalmodulin activity. Therefore, the anti-calmodulin activity of vinblastine probably plays significant role in its high toxic side effect. In fact, the perturbation of the distribution of calmodulin in mammalian cells in the presence of antimitotic drugs was detected by fluorescence microscopy using calmodulin-EGFP fusion protein. This effect could be the consequence of the perturbation of microtubule system [41], however, it is unclear yet whether the anti-calmodulin activity of drugs contributed to the redistribution of calmodulin. It is possible that the calmodulin-containing substructures of centrosomal matrix stabilized by microtubules could be damaged by vinblastine but in less extent or not at all by KAR-2. It still remains to be established whether vinflunine, which shares some characteristic features with KAR-2 (cf. Table **4**), exhibits any anti-calmodulin activity.

CONCLUSION

Cytotoxic agents have very little or no specificity, although the cancer chemotherapy requires the preferential killing of rapidly proliferating cancer cells. The lack of specificity leads to systemic toxicity causing undesirable side effects [67]. In order to increase the selectivity of anticancer drugs and to minimize the undesirable side effects, various strategies have been developed. For example, derivatives and conjugates have been synthesized mainly by large pharmaceutical companies, such as Lilly or Pierre Fabre, which influence the polymerization and dynamics of the microtubule system more effectively and/or by different mechanisms. However, searching bisindole derivatives with low toxic side effects we have followed a different strategy. (Table **4**)

We objected to elaborate why the therapeutic bisindoles (e.i., vinblastine, vincristine) are so toxic when their antimicrotubular potencies can not be responsible for it alone: vinblastine and KAR-2 have similarly high affinity to tubulin and exhibit similar *in vitro* antimicrotubular activity, but different (desired + undesired) cytotoxic effect at cellular level (Table **4**). Therefore, one can propose that these bisindoles may target proteins other than microtubule, the identification of which is crucial for design and development of specific therapeutic agents.

We have identified calmodulin as a new potent target, which is involved in regulation of many metabolic and signaling processes including cell cycle, which is crucial in the biology of tumor development. Further studies are necessary to find one or few calmodulin-modulated processes inhibited by vinblastine but not by KAR-2, and to elaborate how these processes are affected by bisindoles already used in chemotherapy. Although there are several black spots concerning the mechanism of action of KARs, they remain a drug family with a continuing interest for future anticancer therapy.

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ABBREVIATIONS

MAP = Microtubule associated protein

TFP = Trifluoperazine

MTT = [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

AAI = Antitumor activity index

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