Interaction of vinblastine analogues with tubulin[1]

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Summary. Studies of the interaction between vinblastine-like alkaloids and their receptor, i.e. tubulin, are reported. They shed some light on the structure-activity relationships in this medicinally important series: the configurations at $C_{14'}$ and $C_{16'}$ as well as the presence of the methoxycarbonyl group on $C_{16'}$ seem to play an essential role in the determination of biological activity.

The known target for antitumour agents of the so-called 'spindle poisons' group is tubulin[3], an ubiquitous protein of the eukaryotic cell. Tubulin undergoes polymerization to microtubules which constitute the mitotic spindle. It has been shown[4-6] that the alkaloids of the vinblastine group fall into this category of drugs and have a very high affinity for tubulin, preventing its polymerization.

The discovery in our laboratory[7] of an original method for the synthesis of vinblastine-type alkaloids has enabled us to prepare new compounds and to study the structure-activity relationships in this series. Earlier work[8-10] had been restricted only to immediate derivatives of vinblastine and/or vincristine isolated from *Catharanthus roseus.*

Material and methods. 1. Polymerization of tubulin extracted from porcine brain, following the method of Shelanski[11], is followed at 350 nm using a Jobin-Yvon Duospac 203 UV-spectrophotometer and a thermostated cell where the temperature of the sample is raised from 4 to 37 °C. The maximum rate of polymerization (V_{max}) is checked for each sample and I_{50} values (concentration of drug inducing a 50% inhibition of the rate of polymerization) are determined for each analogue.

2. Incubation, for 15 min at 37 °C, of tubulin in the presence of amounts of drugs higher than those necessary for inhibiting polymerization, induces spiralization of tubulin[12] (checked by electron microscopy). The extent of spiralization is determined at 400 nm at 4 °C. It is known that spiralization of tubulin is stopped in the cold but cannot be reversed as in the case of its polymerization[9]. S_{50} (concentration of drug necessary to induce 50% of the maximum spiralization of tubulin) is determined for each analogue.

3. The concentrations of tubulin, determined by the method of Lowry et al.[13], using bovine serum albumine as a standard, are kept in our experiments between 1 and 2 mg/ml. For this range of concentrations, I_{50} and S_{50}

Structure of vinblastine analogues and semi-effect constants for tubulin-alkaloid interaction

Product		R_1	R_2	R_3	R_4	R_5 15′-20′	Double-bond 15′-20′	Configuration 16′	Configuration 14′	I_{50}	$\frac{I_{50}}{I_0}$*	S_{50}	$\frac{S_{50}}{S_0}$*	In vivo assay
Vinblastine	VLB	OH	CO_2CH_3	CH_3	$COCH_3$	CO_2CH_3		N	N	$1.7\ 10^{-6}$	1	$8.9\ 10^{-6}$	1	+
Vincristine	VCR	–	–	CHO	–	–		–	–	$1.4\ 10^{-6}$	0.8	$7.7\ 10^{-6}$	0.9	+
Epi-20′VLB (leurosidine)	VRD	–	–	CH_3	–	–		–	–	$1.8\ 10^{-6}$	1.1	$1.6\ 10^{-5}$	1.8	+
Anhydrovinblastine	AVLB		–	–	–	–	yes	–	–	$3.0\ 10^{-6}$	1.7	$9.3\ 10^{-6}$	1	+
Deoxy-20′ vinblastine	DVLB A	H	–	–	–	–		–	–	$1.8\ 10^{-6}$	1.1	$1.5\ 10^{-5}$	1.7	
Deoxy-20′ leurosidine	DVLB B	H	–	–	–	–		–	–	$2.1\ 10^{-6}$	1.2	$1.1\ 10^{-5}$	1.3	
Deoxy-20′ vincristine	DVCR	H	–	CHO	–	–		–	–	$2.6\ 10^{-6}$	1.6	$9.6\ 10^{-6}$	1.1	+
Epi-16′ anhydrovinblastine	EVLB		–	CH_3	–	–	yes	I	N	$4.3\ 10^{-4}$	250	Inactive		–
Reduced vinblastine	HVLB	OH	CH_2OH	–	H	CH_2OH		N	N	$3.9\ 10^{-4}$	230	–		
BVLB	BVLB		$CO_2CH_2CO_2tBu$	–	$COCH_3$	CO_2CH_3	yes	–	–	$3.1\ 10^{-5}$	18	–		
Vincovaline	VCV	OH	CO_2CH_3	–	–	–		I	I	$8.8\ 10^{-5}$	52	–		
Desmethoxycarbonyl-16′AVLB	DCVLB		H	–	–	–	yes	N	N	Inactive		–		
I	I	H	CO_2CH_3	–	–	–		I	I	$1.8\ 10^{-3}$	1000	–		
II	II	–	–	–	–	–		N	I	Inactive		–		
III	III	–	–	–	–	–		I	N	–		–		
Vincathicine	VTC	OH	–	–	–	–		N	N	$1.3\ 10^{-3}$	750	–		
IV	IV	–	–	–		–		–	–	Inactive		–		
V	V		–	–			yes	–	–	–		–		
Vindoline	VIN									–		–		
Catharanthine	CAT								N	–		–		
Cleavamine	CLI								N	–		–		
VIN + CAT										–		–		
VIN + CLI										–		–		

*I_0 and S_0 are the values of I_{50} and S_{50} for vinblastine.

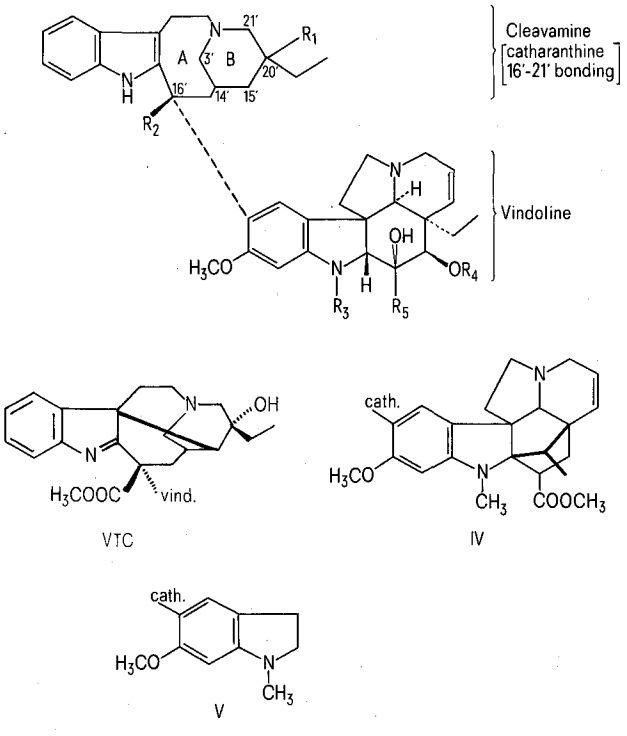
values are proportional to the tubulin concentration (unpublished observations).

4. The buffer used is RB (0.1 M 2-(N-morpholino)ethane sulfonic acid, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP from Sigma, pH 6.6).

5. The various analogues of vinblastine are prepared either by extraction from natural sources (*Catharanthus roseus* stems and leaves) or by synthesis[14]; they are used in the form of their hydrochloride or tartrate salts.

6. In vivo tests have been carried out at the Laboratoire de Pharmacologie et Toxicologie Fondamentales du CNRS in Toulouse (Prof. C. Paoletti and Mme C. Cros) using L 1210 and P 388 leukaemias. These results will be reported in full detail elsewhere.

Results and discussion. The various substances tested are represented in the figure. Their concentrations are raised to 5×10^{-4} M for the analogues of low or zero activity, higher concentrations inducing nonspecific aggregation of the tubulin. To allow comparison between analogues, every semi-effect is related to the I_{50} or S_{50} of vinblastine used as a reference for both polymerization inhibition and spiralization of the same tubulin.



The table reflects that the inhibitory activity of these drugs seems to be correlated to spiralization induction: a substance of comparable activity with VLB for the polymerization inhibition will also be comparable for the spiralization; for the less active substances, the second effect is not observable.

However, the different active substances that we have tested are not classified in the same order for I_{50} and for S_{50} (for instance, VRD is more efficient than AVLB in the inhibition of polymerization, whereas AVLB is more efficient than VRD in inducing spiralization). These results are consistent with the hypothesis of 2 vinblastine-binding sites, with different affinities, as suggested by some authors[15] to explain the 2 observed effects on tubulin.

The importance of spiralization is not known in vivo; it can be related to the already known paracrystallization of tubulin obtained in cells[12, 15, 16].

Concerning the inhibition of polymerization, 3 categories of analogues are discernable: the very active compounds, the compounds having moderate activity and the inactive ones, having respectively good, little and no efficacy in vivo, as tested on mice. Considering the products we have tested, there is a good agreement between the activity on tubulin and antitumour activity. The inhibition of the polymerization of tubulin may be considered as a necessary condition for the antimitotic activity of these compounds, if no metabolisation occurs in vivo[17].

Our results disclose some interesting features: all the active compounds have the same 'natural' (N) 16′ and 14′ configurations as VLB; however, compounds having both 14′ and 16′ inversed configurations (I) exhibit a moderate activity (VCV). This can be explained after inspection of the molecular models of these compounds: a) either, each moiety of the molecule, particularly at the level of the 2 aromatic rings, has a conformation similar to VLB; b) or, the methoxycarbonyl group has the same spatial arrangement as in VLB.

As the conformation of the 9-membered ring of the catharanthine moiety (A, figure) is not exactly known in these compounds, it is difficult to choose between the 2 hypotheses. The methoxycarbonyl group in 16′ is essential for activity (no inhibition of DCVLB); this is not only due to the concomitant steric hindrance (a more bulky group as in BVLB reduces the activity), the ester function itself seems necessary (HVLB, the reduced product from VLB, has very poor activity). This is consistent with a polar type interaction or hydrogen bonding with an amino acid of the tubulin.

The other compounds tested provide evidence for the requirement of the integrity of both moieties of the molecule (**IV** and **V**) and of their presence in the dimeric form (VIN, CAT, CLI, VIN+CAT, VIN+CLI); the modifications of the ring B in the catharanthine moiety (figure) - hydroxyl in 20′*a* or *β*, double-bond, hydroxyl elimination - are not preponderant for the activity (these observations are in disagreement with ambiguous results of Boder et al.[18] who reported the influence of $C_{20'}$ configuration).

In conclusion, it is clear that one of the key positions in vinblastine type molecules is the 16′ carbon; by the configuration of its substituents on the one hand, and by the presence of the methoxycarbonyl group on the other hand. The interaction of these products with tubulin is a good test for a first screening of their pharmacological activity. Regarding the precise mechanism of inhibition, still not known, the importance of the substoechiometry of vinblastine compared with tubulin must be pointed out. Other analogues currently under study in our laboratory should allow us to make some progress in this field.

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Wound healing properties of naphthaquinone pigments from *Alkanna tinctoria*

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Summary. From the roots of *Alkanna tinctoria* Tausch, the alkannin esters of the following acids were isolated and identified: β,β-dimethylacrylic acid, β-acetoxy-isovaleric acid, isovaleric acid, and angelic acid. These esteric pigments showed excellent wound healing properties in a clinical study on 72 patients with ulcus cruris.

The healing of a wound, although among the oldest of surgical problems, retains many of its secrets. However, some wounds become contaminated or contain foreign material that cannot be removed during the acute inflammatory reaction. A condition of chronic or atonic ulcer then appears, for whose treatment there is a lack of effective drugs. Conventional treatment usually entails the prevention of infection and any further aggravation of the wound. If a risk of septicemia exists, amputation may be advised.

As stated in the 'Greek Herbal of Dioscorides'[2] compiled in the 1st century, the roots of the plant *Anchusa tinctoria* or *Alkanna tinctoria*[4] were used for the healing of wounds: 'The root is of a binding nature, being good for burnings and old ulcer...'. But since that time, the medicinal value of this plant has either been forgotten or assumed to be nothing but folklore. This paper describes the rediscovery and verification of this medicinal property, as well as the determination of the active components of the root.

A series of trials with different extracts of the roots of *Alkanna tinctoria,* on skin ulcers experimentally induced on lab animals (rats, cats, dogs), indicated an excellent healing effect[4]. Subsequent to these findings, studies were carried out to determine the active principles in these extracts and also to formulate a suitable pharmaceutical preparation for clinical studies. For this purpose, a large number of root samples of *Alkanna tinctoria,* obtained from different locations, were phytochemically analyzed[5]. The initial hexane extract, after further extraction and chromatographic separation, was divided into 4 fractions: waxes[6], fluorescent compounds, natural polymers[5] and pigments[7]. Of these fractions, only that containing the pigments showed a healing effect when tested experimentally on skin ulcers. Following these preliminary results, the investigation was focused on the pigment-containing fractions, from which I isolated and identified the following esters of alkannin (**1a**): β,β-dimethyl acrylate, **1b**[5], angelate, **1c**[8], isovalerate, **1d**[8], and the novel β-acetoxy isovalerate, **1e**[9]. Brockmann has reported that the structure of the pigment found in *Alkanna tinctoria* was the levorotatory form of 5,8-dihydroxy-2-(1-hydroxy-4-methylpent-3-enyl)-1.4-naphthaquinone (alkannin), **1a**[10]. Contrary to Brockmann's findings, however, I was unable to isolate free alkannin from any of the samples analyzed, irrespective of their origin. I believe that Brockmann's alkannin was an artifact, caused by the caustic alkali which he used for the isolation of the pigments.

Following the chemical and animal studies described above, a pharmaceutical ointment was formulated[11]. Since this ointment is to be used for topical application to human skin, it was necessary first to test it, as well as its active ingredients, for mutagenicity in Ame's Test[12]. The ointment

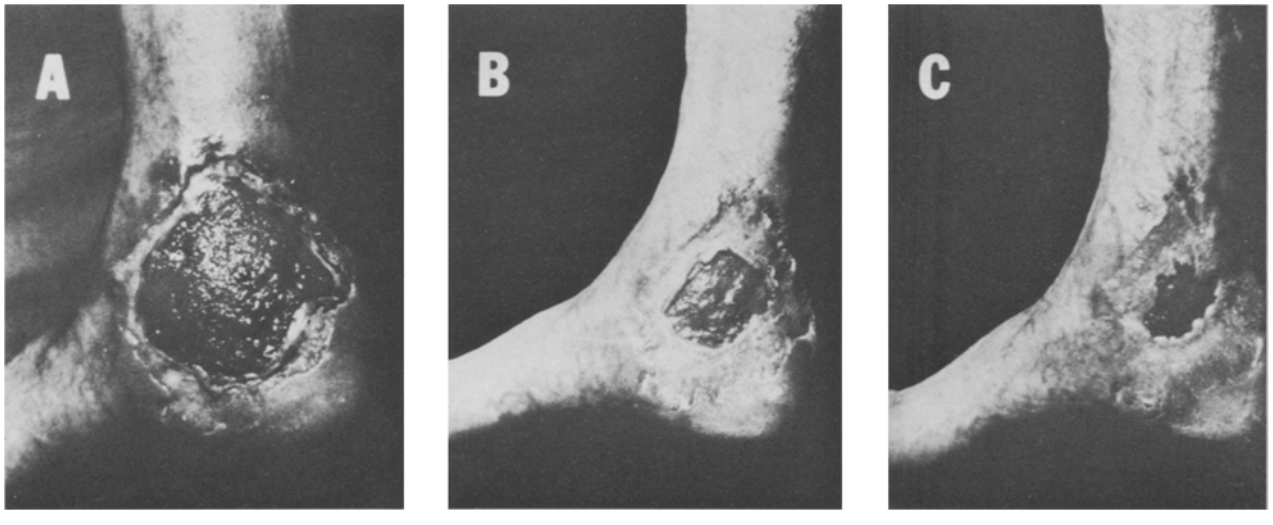

Fig. 1. Showing the response to alkannin esters of a severe case of ulcus cruris: *A* before treatment, *B* after 2 weeks, and *C* after 18 weeks of treatment.