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Vinflunine, the latest *Vinca* alkaloid in clinical development A review of its preclinical anticancer properties

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Abstract

Vinflunine is a new *Vinca* alkaloid uniquely fluorinated, by the use of superacid chemistry, in a little exploited region of the catharanthine moiety. In vitro investigations have confirmed the mitotic-arresting and tubulin-interacting properties of vinflunine shared by other *Vinca* alkaloids. However, differences in terms of the inhibitory effects of vinflunine on microtubules dynamics and its tubulin binding affinities have been identified which appear to distinguish it from the other *Vinca* alkaloids. Vinflunine induced smaller spirals with a shorter relaxation time, effects, which might be associated with reduced neurotoxicity. Studies investigating the in vitro cytotoxicity of vinflunine in combination therapy have revealed a high level of synergy when vinflunine was combined with either cisplatin, mitomycin C, doxorubicin or 5-fluorouracil. Furthermore, although vinflunine appears to participate in P-glycoprotein-mediated drug resistance mechanisms, it has proved only a weak substrate for this protein and a far less potent inducer of resistance than vinorelbine. Vinflunine was identified in preclinical studies as having marked antitumour activity in vivo against a large panel of experimental tumour models, with tumour regressions being recorded in human renal and small cell lung cancer tumour xenografts. Overall its level of activity was superior to that of vinorelbine in many of the experimental models used. Interestingly, an in vivo study using a well vascularised adenocarcinoma of the colon has suggested that vinflunine mediates its antitumour activity at least in part via an antivascular mechanism, even at sub-cytotoxic doses. Therefore, these data provide a favourable preclinical profile for vinflunine, supporting its promising candidacy for clinical development. Phase I evaluations of vinflunine have been completed in Europe and phase II clinical trials are now ongoing.

Keywords: *Vinca* alkaloid; Tubulin binding; Microtubule dynamics; Combination chemotherapy; In vivo anticancer activity

1. Introduction

Compounds interfering with microtubule function form an important class of anticancer agents, widely used in combination chemotherapy regimens for treating many solid tumours as well as leukaemias [1]. One of the best-known classes of these agents are the dimeric *Vinca* alkaloids. Their interactions with tubulin, the major component of microtubules in the mitotic spindle, and the subsequent arrest of cells in mitosis are generally accepted as key events in their mechanisms of action [2,3]. Vinblastine, the first natural alkaloid with antiproliferative activity was discovered in extracts of the leaves of the *Vinca rosea* plant, at the University of Western Ontario in 1958 [4] and, independently, at the Lilly Research Laboratories in the USA [5]. This discovery was rapidly followed by that of vincristine [6]. Although these two alkaloids differ structurally only in the functional group on the dihydroindole nitrogen, this minor distinction appears responsible for substantial differences in their activities and toxicities [7,8] and provided the impetus for searching for new analogues with the aim of identifying more active and less toxic compounds exhibiting a broader spectrum of anticancer efficacy. These endeavours, many of which were centred on the use of vinblastine as the starting molecule, since it was the only material readily available in sufficient quantity, resulted in the identification of the third clinically-active *Vinca* alkaloid, vindesine, a desacetyl carboxyamide derivative of vinblastine [9]. In the meantime, new methods of coupling the two precursor alkaloids catharanthine and vindoline enabled chemists to obtain large amounts of the intermediate 3′,4′-anhydrovinblastine, allowing the synthesis of new derivatives, differing from the natural compounds by having an eight—rather than a nine—membered ring in the velbenamine moiety [10,11]. Among these derivatives, vinorelbine was selected for development and this molecule has shown markedly improved clinical efficacy and reduced toxicity [12]. This latest clinically approved *Vinca* alkaloid is now widely used and licensed for the treatment of non-small cell lung cancer, metastatic breast cancer and ovarian cancer [13].

Therefore, despite numerous efforts in the fields of both chemistry and biology, since the early 1970s only two semisynthetic *Vinca* alkaloid derivatives, vindesine and vinorelbine have achieved the rank of approved anticancer drugs. Within this context and encouraged by the clinical utility of vinorelbine in cancer chemotherapy, the French Pierre Fabre Group has continued to search for more active *Vinca* alkaloid-type compounds with a different/wider range of activity against other tumour types and/or with lower/different

spectra of toxic side effects. A joint programme of chemical and pharmacological research has resulted in the identification of vinflunine, a novel semisynthetic *Vinca* alkaloid, which is now in clinical development.

2. Designing a new *Vinca* alkaloid: vinflunine

The major challenge was to find a way of further exploiting the antitumour properties of the *Vinca* alkaloids, knowing that several hundreds of such compounds had already been synthesised and quite a substantial proportion had been evaluated for their potential anticancer activity [14,15]. However, it is noteworthy that most modifications of the *Vinca* alkaloid molecule have been in the reactive parts of the vindoline nucleus and obtained using classic chemistry [16]. Therefore, using an original chemical approach that conceivably could induce dramatic changes in the skeleton of the *Vinca* alkaloid molecule, the reactivity of these highly functionalized compounds was investigated in superacid media [14]. This strategy appeared novel since very few such examples have been described in the literature. Superacid can induce modifications at non-activated bonds [17]. Furthermore, under these unusual conditions, indoles and indolines remain sufficiently stable to react with various electrophiles [18]. This approach resulted in the synthesis of a new family of *Vinca* alkaloids, from which 20′, 20′-difluoro-3′,4′-dihydrovinorelbine or vinflunine has been selected for development on the basis of its initial activity in primary pharmacological screening [19]. The most impressive structural modification of this vinorelbine derivative was the selective introduction of two fluorine atoms at the 20′ position, a part of the molecule previously inaccessible by classic chemistry. During the course of the reaction, the double bond between carbons C3′ and C4′ was also reduced (Fig. 1).

3. Vinflunine interactions with microtubules

It is generally accepted that the antiproliferative properties of *Vinca* alkaloids arise largely from their interactions with tubulin, resulting in disruption of microtubule dynamics [20–23,3]. Microtubules are intrinsically dynamic polymers, which display two types of dynamic behaviour, 'dynamic instability' and 'treadmilling', which appear to be important for cellular function and especially for progression through mitosis [3]. Dynamic instability is characterised by the switching at microtubule ends between phases of slow growth and rapid shortening [24], whereas treadmilling is the net addition of a tubulin subunit to one end of a microtubule (the plus end) and the balanced net loss of tubulin subunits from the opposite (minus) end [25,26]. At the lowest effective concentrations, *Vinca* alkaloids have been shown to suppress both dynamic instability and treadmilling, possibly by binding to microtubule ends, without appreciably reducing the polymer mass [24]. Binding of *Vinca* alkaloids at higher concentrations depolymerizes the microtubules, and at still higher concentrations they induce the formation of large paracrystals made of spiral helices of one or two protofilaments, both in cells and under in vitro test tube conditions [27]. It is believed that the binding of these

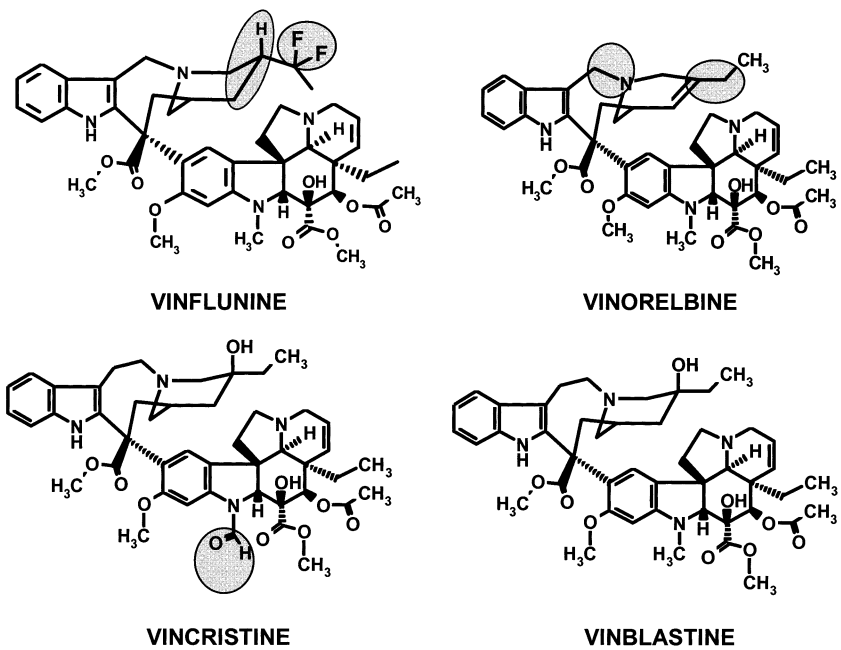

Fig. 1. Chemical structure of vinflunine. The shaded areas indicate the structural differences vis-à-vis vinorelbine structure.

drugs would stabilise microtubule ends through a conformational change that would increase the affinity of the tubulin for neighbouring tubulin molecules [28,29] and, overall, *Vinca* alkaloid binding appears to be linked to tubulin self-association resulting in the formation of spiral aggregates [22].

3.1. Vinflunine exhibits certain characteristics in common with the other Vinca alkaloids

Studies using reconstituted systems have established that vinflunine prevents assembly of microtubules without affecting their disassembly, with an IC_{50} value in the μM range, similar to those obtained with the other *Vinca* alkaloids, namely vincristine, vinblastine or vinorelbine [30]. Ngan et al. [31] confirmed these findings, showing that vinflunine reduced the microtubular mass in a concentration-dependent manner, with an IC_{50} value of 1.2 μM, which was judged comparable to the value of 0.8 μM recorded with vinorelbine under similar experimental conditions. In addition, there is evidence that vinflunine interacts with the *Vinca* alkaloid binding site on tubulin. This was shown first using an assay based on the differential proteolysis of purified tubulin by chymotrypsin or trypsin, and confirmed by the fact that vinflunine, when interacting with tubulin, induced structural changes which favoured an inhibition of GTP hydrolysis [30]. In mammalian tumour cells in vitro, vinflunine caused cell cycle arrest, as judged by flow cytometry, in the $G_2 + M$ phases, which was shown to be associated with an accumulation of cells in mitosis [30]. More specifically, in studying the perturbation of the mitotic spindle by *Vinca* alkaloids, Jean-Decoster et al. [32] have shown that vinflunine (10–100 nM) induced an abnormal location of chromosomes and prevented the formation of chromosomal alignment at metaphase in rat kangaroo PtK2 cells, after a 6-h incubation. These findings are consistent with previous studies demonstrating that at the lowest effective concentration, microtubule poisons, such as vinblastine or vincristine, suppress microtubule dynamics, thereby impairing the intricate movements of the chromosomes and leading to a disappearance of metaphase and post-metaphase figures [27]. Furthermore, it has also been shown, in interphase cells, that low concentrations of microtubule poisons induce separation of the centrosome, reflected by the migration of the two centrioles, which then reach opposite locations separated by a few μm [33]. In this context, Jean-Decoster et al. [32] have shown that vinflunine induced a concentration-dependent separation of the centrosome in interphase PtK2 cells, which was detectable at concentrations as low as 75 nM. Similar effects were observed with vinblastine, vincristine and vindesine, but at 10-fold lower concentrations [32]. At higher concentrations (500 nM) vinflunine also caused a concentration-dependent depolymerisation of the interphasic microtubular network of rat aortic A-10 smooth muscle cells, together with the formation of paracrystalline structures, when still higher concentrations (50 μM) were studied [30]. Overall, in mammalian cells, vinflunine resulted in similar biological effects to those of the other *Vinca* alkaloids. In these respects, therefore, vinflunine appears to function as a specific tubulin-interacting compound, like the classic *Vinca* alkaloids. However, these effects, which all proved to be concentration-dependent, generally required 3- to 17-fold higher concentrations of vinflunine relative to those of the other *Vinca* alkaloids tested.

3.2. Vinflunine also expresses some quite distinctive features relative to the other Vinca alkaloids

Vinflunine has been shown to exhibit different tubulin binding properties relative to the other *Vinca* alkaloids. Using a centrifugal gel filtration method, Kruczynski et al. [30] showed that vinflunine interfered in a different manner with the binding of tritiated *Vinca* alkaloids to tubulin, namely by not inhibiting the binding of [^{3}H]vincristine and only weakly competing with either [^{3}H]vinblastine or [^{3}H]vinorelbine, when used at high concentrations (≤ 100 μM). Furthermore, under these experimental conditions, specific binding of [^{3}H]vinflunine to tubulin was undetectable, whereas [^{3}H]vinorelbine, [^{3}H]vinblastine and [^{3}H]vincristine all showed saturable and specific binding [30]. Overall, in considering the capacity of these molecules to bind to tubulin or to interfere with the binding of the other [^{3}H]*Vinca* alkaloids to tubulin under these experimental conditions, the various *Vinca* alkaloids tested could be classified as follows: vincristine > vinblastine > vinorelbine > vinflunine [30]. The authors put forward the hypothesis that these differences might be due to a higher dissociation constant for vinflunine than for the other *Vinca* alkaloids, thereby permitting the equilibrium between bound and free vinflunine to be easily displaced in favour of free drug during the course of an experiment based on the centrifugal gel filtration technique. Using NMR spectroscopy, Fabre et al. also showed that a higher number of vinorelbine molecules bind to tubulin as compared with vinflunine [34]. No significant binding of vinflunine to tubulin was detected at 30 °C, under their experimental conditions. Furthermore, complementary studies, based on measurements of tubulin self-association, provided direct evidence that vinflunine does bind to tubulin [35]. Indeed, because tubulin self-association is linked to drug binding, it is a measure of the extent of overall drug binding, which can be determined by sedimentation velocity [35]. The results of these studies though demonstrated that vinflunine bound to tubulin with much weaker overall affinity (defined by K_1K_2, according to Lobert et al.

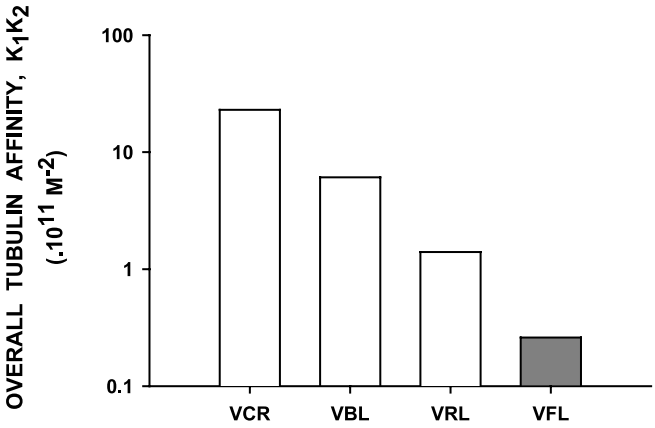

Fig. 2. Vinflunine exhibits lower overall affinity (K_1K_2) for tubulin relative to the other *Vinca* alkaloids. Illustrative representation of the data originating from Lobert et al. [35]. VCR, vincristine; VBL, vinblastine; VRL, vinorelbine and VFL, vinflunine.

[35]) than vinorelbine, which itself had been shown previously to exhibit a lower overall affinity for tubulin than either vinblastine or vincristine (Fig. 2) [36,35]. More specifically, the order observed in overall affinity of the various *Vinca* alkaloids for tubulin (K_1K_2) was vincristine > vinblastine > vinorelbine > vinflunine [35], in agreement with the conclusions from the studies of Kruczynski et al. [30]. The weaker binding of vinflunine to tubulin has been shown not to be reflected in the drug binding to tubulin heterodimers (K_1 according to Lobert et al. [35]) but rather in the affinity of liganded heterodimers for spiral polymers (K_2) and in the binding of the drug to polymers (K_3). This results in the formation of fewer spirals of a smaller size to those induced by other *Vinca* alkaloids, as judged by sedimentation velocity. According to Lobert et al. [35], this means that overall less vinflunine binds to tubulin than is the case for the other *Vinca* alkaloids. The vinflunine-induced smaller spirals exchanged tubulin heterodimers more readily, reflected by a short relaxation time, as measured by stopped-flow light-scattering drug dilution experiments aimed at investigating kinetics of the reequilibration of drug-induced spirals [35,22]. As argued by Lobert et al. [35], these kinetic data contribute to an understanding of the previously published results of iodoacetamide alkylating experiments [30]. Monitoring of the alkylation of the sulfhydryl groups of tubulin by iodo[^{14}C]acetamide in the presence of *Vinca* alkaloids had shown that vinflunine or vinorelbine only inhibited tubulin alkylation in a transitory manner, since their inhibition was reversed after a 2 h incubation, whereas in contrast, vinblastine and vincristine induced persistent inhibition throughout the experimental period. Since relaxation times for vinflunine- and vinorelbine-induced spirals are shorter than those for vinblastine- and vincristine-induced spirals, Lobert et al. [35] proposed that in the presence of vinflunine or vinorelbine, tubulin heterodimers are more readily reexposed to alkylation than is the case when either vinblastine or vincristine is present. Interestingly, Jean-Decoster et al. [32] showed that the separation of centrosome units induced by vinflunine was readily reversed after drug 'wash-out', while this process was slower in cells treated with the three other *Vinca* alkaloids, with the effects of vincristine proving the least reversible. Therefore, vinflunine, which exhibits the weakest overall affinity for tubulin, appears to result in the most readily reversible interaction with tubulin.

Furthermore, the action of vinflunine on microtubules dynamics appears to differ significantly from that of vinblastine [31]. Microtubule dynamics were analysed by quantitative video microscopy [31]. Microtubule images were captured in real time on videotape allowing measurement of microtubule length over time and therefore calculation of various kinetic parameters (microtubule growth or shortening rates or pause time) illustrated diagrammatically in Fig. 3. Vinflunine suppresses microtubule dynamic instability and its most prominent effects are to slow the microtubule growth rate, to increase the duration of growth events, and to reduce the duration of shortening events. In marked contrast, to the action of vinblastine, vinflunine does not reduce the rate of microtubule shortening, nor does it increase the percentage of time the microtubules spend in an attenuated or paused state. Vinflunine also suppresses microtubule treadmilling, but with a weaker potency than vinorelbine or vinblastine. Ngan et al. [31] suggest that these various differences may indicate that vinflunine affects the rate constants for tubulin association and dissociation at opposite microtubule ends differently than vinorelbine and vinblastine. Since microtubules treadmilling or flux is necessary for normal chromosome progression through mitosis, these data suggest overall that vinflunine might have different subtle effects on certain mitotic events.

In summary, vinflunine binds to tubulin and induces tubulin self-association, like the other *Vinca* alkaloids. Overall, the addition of drug, liganded dimer, or spiral at the ends of microtubules is presumed to be sufficient to disrupt microtubule dynamics. However, vinflunine exhibits distinctive tubulin binding properties from the other *Vinca* alkaloids, which may account for its differential action(s) on microtubule dynamics.

4. In vitro cytotoxicity

4.1. Vinflunine as a single agent

Vinflunine exerts in vitro cytotoxic activity against a wide spectrum of tumour cell lines with IC_{50} values ranging from 10^{-8} to 10^{-7} M, when tested against two murine leukaemias as well as seven human cell lines

derived from lung, colon, prostate, breast, ovarian and bladder tumours [30]. Experiments conducted concurrently with vincristine, vinblastine and vinorelbine revealed that these IC_{50} values obtained with vinflunine proved generally higher by factors of 2- to 40-fold [30]. However, the high order of correlations between $\log IC_{50}$ values, noted when evaluating cytotoxicity against this panel of unselected human tumour cell lines, for vinflunine, vinorelbine or vincristine relative to vinblastine were consistent with all these compounds having a similar intracellular target [30]. Furthermore, Jean-Decoster et al. [32] set out to determine whether vinflunine interactions with microtubules could account for its cytotoxicity using PtK2 (rat kangaroo) cells. They demonstrated that the cell growth inhibitory concentrations (50–75 nM) indeed led to impairment of the formation of the chromosomal alignment as well as to the first signs of separation of the centrosome units, reflecting a mild destabilisation of the mitotic and interphasic microtubules, respectively. They concluded therefore that vinflunine cytotoxicity is compatible with its action(s) on the most dynamic microtubules. It is also interesting to note that, intracellular accumulation of vinflunine appeared to be higher (9-fold) than that of vinorelbine, vinblastine and vincristine, as shown by Jean-Decoster et al. [32] using PtK2 cells.

4.2. Vinflunine in combination therapy

The potential for including vinflunine in combination chemotherapy regimens was investigated by evaluating the cumulative in vitro cytotoxicity of vinflunine when co-incubated simultaneously with a series of standard anticancer agents with differing modes of action [37]. Synergy was identified when vinflunine was combined with the DNA-damaging agents cisplatin and mitomycin C, the DNA-intercalator doxorubicin, the antimetabolite 5-fluorouracil, and the topoisomerase I inhibitor, camptothecin. However, only additivity was noted with combinations of vinflunine and the topoisomerase II poison etoposide, the antimetabolite gemcitabine, or two tubulin-interacting agents, paclitaxel and vinorelbine. It is interesting to note that no antagonism was observed when vinflunine was combined with any of these nine antitumour agents tested (Fig. 4). Therefore, these findings suggest a wide range of possibilities for including vinflunine in combination chemotherapy.

5. Resistance mechanisms

The *Vinca* alkaloids as a class participate in so-called 'classical' multidrug resistance (MDR), a phenomenon whereby overexpression of a plasma membrane efflux pump termed P-glycoprotein (Pgp) results in lower intracellular drug levels and hence, in reduced cytotoxicity [38]. Amongst the tubulin-interacting agents, *Vinca* alkaloids and their derivatives have generally shown common cross-resistance patterns and this is especially true of Pgp-mediated resistance [39]. It was important to ascertain whether the unique fluorination within the vinflunine molecule, in the little exploited region of the catharantine moiety, might have influenced interactions of vinflunine with Pgp. As assessed by several criteria using both in vivo and in vitro models, the studies reported have indicated that, like the other *Vinca* alkaloids, vinflunine appears to belong to the family of compounds inducing Pgp-dependent MDR [40]. Indeed, tumour cells selected for resistance, either in vivo (murine P388 leukaemia) or in vitro (human A549 lung carcinoma), to vinflunine revealed an increase in Pgp

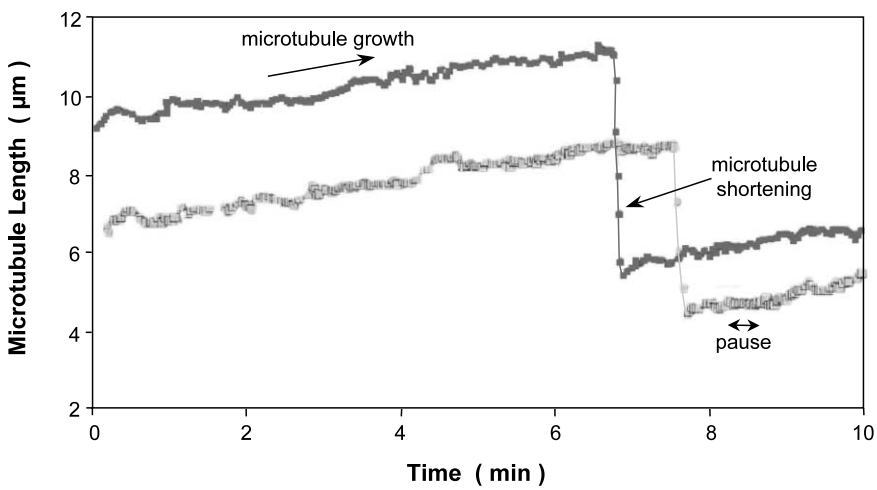

Fig. 3. Diagrammatic illustration of microtubule instability.

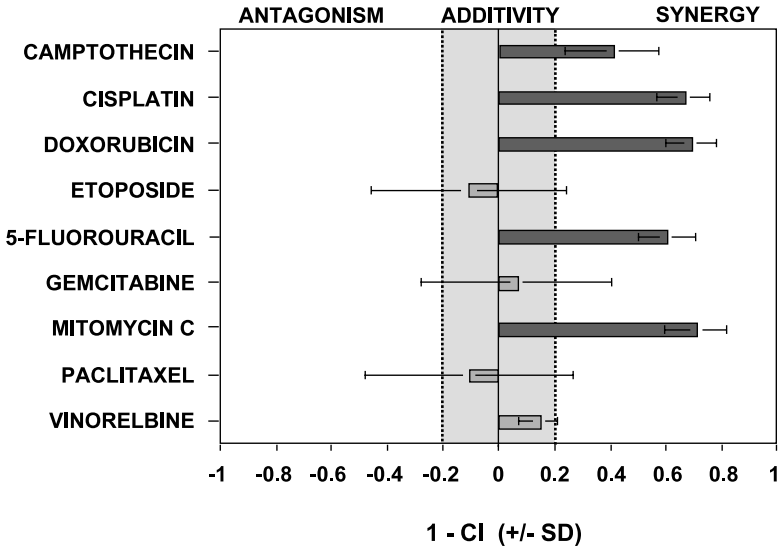

Fig. 4. Synergy or additivity was identified with combinations of vinflunine with a series of anticancer agents. Data extracted from Barret et al. [37] and reproduced with permission.

expression and proved cross resistant, to other *Vinca* alkaloids, to doxorubicin and to etoposide, but not to drugs not implicated in the MDR phenotype, such as cisplatin and camptothecin [40,41]. However, although in various in vitro models of human MDR tumour cells, resistance to vinflunine was identified in cells overexpressing Pgp, these MDR cells exhibited a rather different profile since they proved generally less cross resistant to vinflunine relative to the other *Vinca* alkaloids [40]. More specifically, the Pgp-associated MDR sublines, CEM/VBL1000 (human leukaemia), MCF7/200R (human breast carcinoma), T24M (human bladder carcinoma) and P388/ADR (murine leukaemia) were less cross resistant to vinflunine relative to vincristine and vinorelbine by factors of 2.5- to 13-fold [40]. Full sensitivity to vinflunine was also retained in cells expressing alternative non-Pgp-mediated MDR mechanisms, such as GLC4/ADR (human small-cell lung cancer) and CEM/VM1 (human lymphoblastoid T-cell leukaemia) cells [40]. Other mechanisms of resistance described for microtubule-interacting agents have involved tubulin modifications. A decrease in the level of β4 tubulin isoforms has been reported recently in a human lung cancer subline selected for resistance to vinflunine [41].

Although vinflunine appears to induce Pgp-mediated resistance, there could be positive clinical implications if this resistance would not develop readily. Indeed, in a study aimed at establishing whether there were differences in the rate and extent of development of resistance both in vivo and in vitro to either vinflunine or vinorelbine under identical selection conditions, Etiévant et al. [41] demonstrated that resistance to vinflunine was generated far less readily than to vinorelbine. The in vivo studies, carried out using the murine P388 leukaemia cells, showed that resistance to vinflunine and vinorelbine developed gradually over time in P388 cells, as shown in Fig. 5 [41]. However, complete resistance to vinflunine was only obtained after 36 weekly treatments, whereas complete resistance to vinorelbine was already reached after 11 such treatments weekly with comparable therapeutic doses. A similar approach applied in vitro supported these findings since a period of 8 months was needed to induce resistance in A549 (human lung carcinoma) cells so that

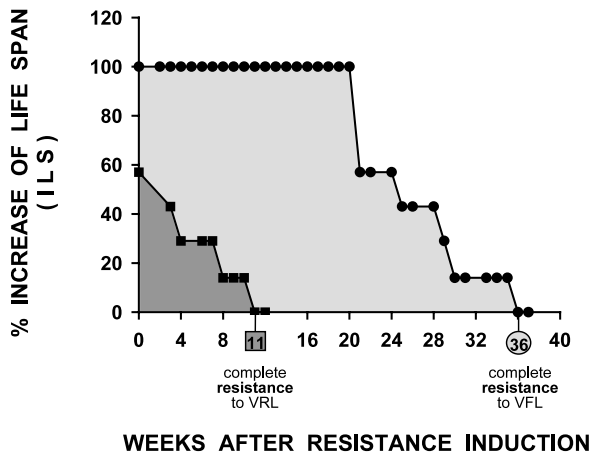

Fig. 5. Resistance of P388 leukaemia in vivo to vinflunine was generated far less readily than to vinorelbine. P388 parental sensitive cells were exposed in vivo weekly to equivalent sub-therapeutic doses of either vinflunine (2.5 mg/kg) or vinorelbine (0.63 mg/kg). Then therapy experiments, aimed at measuring the level of sensitivity of these P388-treated cells to the resistance-inducing compound, were conducted at various passages in vivo (or weeks after resistance induction) to monitor the development of resistance, as assessed by percent increase of life span (%ILS). Illustrative representation of the data originating from Etiévant et al. [41].

they survived continuous exposure to a $2 \times IC_{50}$ concentration of vinflunine, whereas under similar experimental conditions, resistance to vinorelbine was readily obtained (within 2 months) [41]. Therefore, in summary, although vinflunine appears to participate in Pgp-mediated multidrug resistance, it is a less potent inducer of such resistance than vinorelbine and seems to be a weaker substrate for Pgp than the other *Vinca* alkaloids, especially vincristine and vinorelbine, an observation which has positive implications for its clinical usage.

6. Cell death mechanisms

A study, aimed at defining the molecular mechanisms of cell killing by vinflunine in murine P388 cells, showed that vinflunine initiated a series of events leading to apoptotic cell death [42]. Cellular morphological changes specific for apoptosis, as well as DNA fragmentation, were observed after vinflunine treatment of these tumour cells. Apoptosis-inducing concentrations of vinflunine caused caspase-3 and/or -7 activation and the cleavage of one of its specific substrates, the poly-(ADP-ribose) polymerase (PARP). Furthermore, Ac-DEVD-CHO, a caspase 3/7 inhibitor, was shown to inhibit vinflunine-induced caspase activation as well as vinflunine-induced apoptosis, suggesting therefore that vinflunine-induced apoptotic cell death in P388 cells is mediated through caspase-3 and/or -7. Vinflunine also stimulated a stress-activated protein kinase, JNK1 [42], whose activation has been shown to occur in response to diverse stress stimuli, including cellular treatment with microtubule inhibitors [43]. These authors have suggested that this may represent a general stress response to microtubule dysfunction. Furthermore, several studies have proposed a role for Bcl-2 phosphorylation in the apoptotic response of tumour cells to microtubule damaging agents [44]. Interestingly, however, the apoptotic signal triggered by vinflunine in P388 cells was not mediated through Bcl-2 phosphorylation, whereas this is the case when another tumour cell line was used, namely the CEM (human leukaemia) line [42]. Therefore, these findings led to the hypothesis that the capacity of vinflunine to cause Bcl-2 phosphorylation might depend on the cellular type. In addition, in order to assess whether apoptosis contributed to the cellular sensitivity to vinflunine, the capacity of vinflunine to induce apoptosis in P388 parental and vinflunine-resistant (P388/VFL) cells was measured [42]. This study revealed that resistance to vinflunine cytotoxicity developed in P388/VFL cells was associated with resistance to vinflunine-induced apoptosis, as reflected by a loss of the capacity of vinflunine to induce DNA fragmentation, PARP degradation and specific cellular morphological changes in these resistant P388/VFL cells. These vinflunine-resistant cells were also characterised by relatively higher level of expression of the anti-apoptotic proteins, Bcl-2 and Bfl-1/A1 [42]. In summary, this study has indicated that vinflunine is able to induce apoptotic cell death in P388 leukaemic cells, involving a series of cellular events such as caspase 3/7 activation and the participation of certain Bcl-2 family members that may play a role in the overall cellular response to vinflunine treatment.

7. In vivo antitumour activity

7.1. Marked antitumour activity of vinflunine in a panel of experimental tumour models

Vinflunine has demonstrated a broad spectrum of antitumour activity against a panel of experimental tumours with different biological properties and chemosensitivities [45–48]. Marked activity was recorded against the murine P388 leukaemia implanted i.v., with vinflunine given i.p. as a single or as multiple doses according to various schedules or as single i.v. or p.o. doses. This activity was not associated with any major toxicities, as judged by a lack of any major body weight loss and early deaths [46]. More specifically, a single i.p. injection of vinflunine at the highest non-toxic dose of 40 mg/kg resulted in a major increase of life span (ILS) of 100% (T/C of 200%), which was judged as being of high level of activity (T/C > 175%), according to the National Cancer Institute (NCI) USA criteria. Furthermore, this value of ILS proved markedly superior to those of 43–57% (T/C of 143–157%) obtained with the other *Vinca* alkaloids tested concurrently (Fig. 6). It is noteworthy that this superior antitumour activity of vinflunine relative to the other *Vinca* alkaloids was reached at 4- to 16-fold higher doses. A comparison of the various schedules of administration of vinflunine in this P388 model revealed that although multiple daily injections ('daily $\times 4$' on days 1–4) and intermittent treatments over 2 weeks ('every other week day for 2 weeks' on days 3, 5, 7, 10, 12 and 14) did not markedly increase either the resultant antitumour activity or the total dose that could be given, the schedule of longest duration, i.e. weekly treatments over 4 weeks ('once a week for 4 weeks', days 1, 8, 15 and 22) achieved the greatest antitumour effect (T/C = 457%) and permitted administration of the highest total tolerated dose of 160 mg/kg (Fig. 7). Therefore dosing at weekly intervals proved the most effective schedule in this P388 model. Furthermore, significant survival prolongation (log rank $P < 0.001$) and tumour growth inhibition (optimal inhibition of 76%) were also shown by treating the relatively refractory s.c.-implanted B16 melanoma with multiple doses of vinflunine [46]. The extent of this activity was again superior to that noted

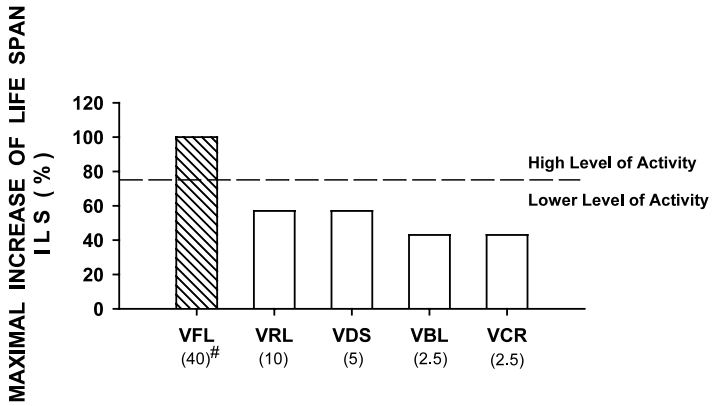

Fig. 6. Vinflunine induced marked in vivo antitumour activity against the P388 leukaemia, which is superior to that of the other *Vinca* alkaloids. Illustrative representation of the data originating from Kruczynski et al. [46]. ILS (%) = Increase of Life Span = T/C(%) − 100, with T/C = (median survival of treated mice/median survival of control mice) × 100. VFL, vinflunine; VRL, vinorelbine; VDS, vindesine; VBL, vinblastine and VCR, vincristine. # dose (mg/kg) resulted in the maximal increase of life span without significant toxicity.

with vinorelbine under similar experimental conditions [46]. Interestingly, Cros et al. [49] had reported earlier that activity for vinorelbine in this B16 model was superior to that obtained with vinblastine and vincristine. Vinflunine, administered as a single i.p. dose, also reduced by 75% the number of lung metastatic foci developed in the i.v.-implanted B16F10 melanoma bearing mice [47]. To examine in more depth the overall spectrum of in vivo antitumour activity of vinflunine, a panel of 11 human solid tumours xenografted onto nude mice, including various histological types of breast, lung, bladder, pancreas, kidney, colon, central nervous system (CNS) and prostate cancers, were studied. These evaluations were carried out concurrently in four independent research centres using standardised procedures and evaluation criteria in accordance with NCI criteria (USA) and European Organisation for Research and Treatment of Cancer (EORTC) guidelines [45,46,48]. Vinflunine, given as weekly i.p. injections over 4 successive weeks, demonstrated antitumour activity against seven of the 11 xenograft models, with high activity against RXF944 (renal) and NCI-H69 (small cell lung cancer) and moderate activity against MX-1 (breast), LX-1 (small cell lung cancer), TC37 (colon), PAXF549 (pancreas) and PC-3 (prostate) (Fig. 8). Of particular note, this activity was generally sustained and was not associated with excessive toxicity, as judged by monitoring for early lethality and body weight loss. More specifically, the high levels of activity of vinflunine against the RXF944 (renal) and NCI-H69 (small cell lung cancer) tumour models, recorded at the highest non-toxic dose of 40 mg/kg per injection, were associated with optimal tumour growth inhibition exceeding 90%, as well as with partial or complete tumour regressions. It is noteworthy that tumour regressions in animal experimental tumour models are considered an important end point of clinical relevance [50]. Furthermore, the definite activity obtained in mice bearing the LX-1 tumours, with i.p. administrations of vinflunine was reproduced, with i.v. administrations according to the same schedule, as reflected by an optimal tumour growth inhibition of 63% (T/C = 37%).

Overall, therefore, a consistently high level of antitumour activity for vinflunine has been documented in these preclinical studies, with evidence of activity against a broad spectrum of different histologic human types. Concurrent experiments with vinorelbine [45], have served to emphasise the superiority of vinflunine, similar to historical comparisons with published data on vinblastine and vincristine [51,52].

In conclusion, the extent of activity of vinflunine against this panel of experimental human tumours encouraged its consideration for clinical development.

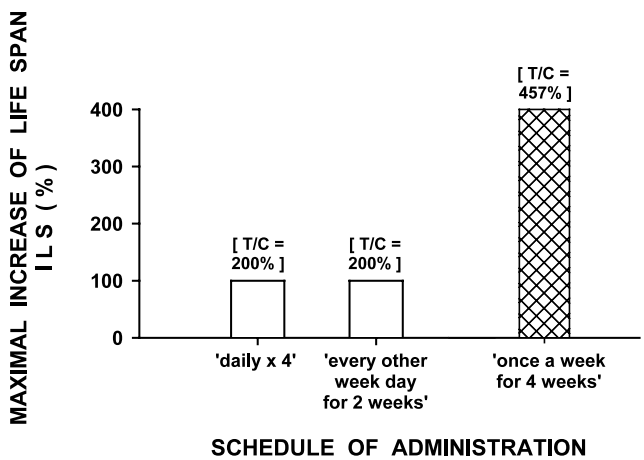

Fig. 7. Vinflunine dosing at weekly intervals ('once a week for 4 weeks') provided the most effective schedule in the P388 leukaemia model in vivo. Illustrative representation of the data originating from Kruczynski et al. [46]. ILS (%) = Increase of Life Span = T/C(%) − 100, with T/C = (median survival of treated mice/median survival of control mice) × 100. # Corresponding T/C values; 'daily ×': treatments on days 1, 2, 3 and 4; 'every other week day for 2 weeks': treatments on days 3, 5, 7, 10, 12 and 14; 'once a week for 4 weeks': treatments on days 1, 8, 15 and 22.

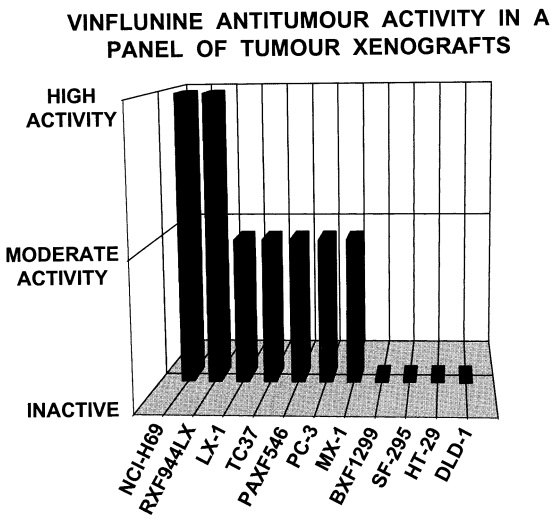

Fig. 8. Vinflunine demonstrate definite in vivo antitumour activity in 7/11 human xenografts. Illustrative representation of the data originating from [45,46]. Levels of antitumour activity were recorded according to following criteria: High level of activity: T/C < 10%; moderate activity: T/C < 50% and > 25%, with T/C, % = (median tumour volume of drug-treated group on day X/median tumour volume of control group on day X) × 100. The tumour types of the various xenograft models were as follows: NCI-H446, lung; RXF944LX, renal; LX-1, lung; TC37, colon; PAXF546, pancreas; PC-3, prostate; MX-1, breast; BXF1299, bladder; SF-295, central nervous system; HT-29, colon; DLD-1, colon.

7.2. Antivascular activity of vinflunine

The contribution of potential antivascular effects of vinflunine to its antitumour efficacy was investigated using a well vascularised tumour model, namely the murine MAC15A colon adenocarcinoma [53]. A single i.p. administration of the maximum tolerated dose (MTD) of vinflunine in MAC15A-tumour bearing mice resulted in tumour growth delay, associated with the appearance of tumour haemorrhagic necrosis. This antivascular effect was confirmed by an Hoechst perfusion study that showed vascular shutdown over a minimum of 24 h, which was achieved even at doses below the MTD. Whilst such effects have also been described with other tubulin-interacting agents like vincristine, vinblastine [53,55] or colchicine [56], they have only been obtained at doses approaching the MTD. Therefore, these newer data suggest that vinflunine mediates its antitumour activity at least in part, via an antivascular mechanism. This may have implications for the inclusion of vinflunine in combination therapies since the combining of drugs with different mechanisms of action at doses below their MTD may result in a synergistic antitumour activity with minimal toxicity.

In summary, these preclinical in vivo studies have shown that vinflunine has a broad spectrum of activity against a panel of experimental tumours. This activity with vinflunine was consistently superior to that of vinorelbine and, although it was achieved at higher overall dose levels, their administration was not associated with any identified increased toxicity.

8. Discussion: potential implications of vinflunine preclinical data

Vinca alkaloids have been widely used in many cancer chemotherapy protocols since the discovery of vinblastine and vincristine more than 30 years ago, and the more recent availability of vinorelbine has certainly resulted in renewed interest in this group of compounds [57,12,13]. However, as yet any clear understanding of their differential reported antitumour activities and toxic side effects remains elusive. Although, it has been argued that, as antimitotic drugs, their mechanisms of action on tubulin are likely to have a major impact on their activities and toxicities [32,22,31].

8.1. Comparison of vinflunine pharmacological properties with those of vinorelbine, vinblastine and vincristine

Data previously published and reviewed here show that vinflunine, like the other *Vinca* alkaloids, binds to tubulin and induces an accumulation of cells in mitosis. However, vinflunine exhibits different pharmacological properties relative to those of the other *Vinca* alkaloids. These differences are presented in a summary table (Table 1). More specifically, vinflunine binds to tubulin with much weaker (+, in Table 1) overall affinity (K_1K_2) than vinorelbine, vinblastine and vincristine, with the relative order of overall affinity for tubulin being vincristine (+ + + +) > vinblastine (+ + +) > vinorelbine (+ +) > vinflunine (+). Therefore, vinflunine appears to extend further a classification of *Vinca* alkaloids based on overall tubulin affinities. It is also apparent that strong binding to tubulin is not necessarily required for antitumour efficacy. For example, Singer and Himes [58] reported that the relative binding affinities to bovine brain tubulin of vinepidine, vincristine, vindesine and vinblastine were inversely correlated with their effects on B16 tumour cell proliferation, with vinepidine interacting with tubulin with the highest affinity, yet being the least efficient inhibitor of proliferation. It is interesting to note that, in the various preclinical studies reported here, the overall binding affinities of vincristine, vinblastine, vinorelbine and vinflunine inversely correlate with the capacity of the drug to inhibit microtubule treadmilling and cell proliferation (Table 1). They also inversely correlate with the amount of drug required to disassemble the interphasic microtubular network, to induce paracrystal formation

in A-10 cells, and to inhibit in vivo tumour growth, as well as to increase the survival of tumour-bearing animals (Table 1). In these cases vinflunine with the weakest overall tubulin affinity is used at the highest concentration and vincristine with the strongest overall affinity is used at the lowest concentration. These findings are also consistent with the relatively higher concentrations of vinflunine needed to impair the formation of the chromosomal alignment and to result in the separation of centrosome pairs in PtK2 cells, relative to the three other *Vinca* alkaloids (Table 1). Furthermore, the reversibility of the action of these four *Vinca* alkaloids appears inversely proportional to their overall tubulin binding affinities, with vinflunine with the weakest overall affinity inducing the most reversible effect on the centrosome.

8.2. Potential relationship with clinical dosage

Lobert and Correia [22] have suggested that the relative affinity for tubulin may also be related to the clinical doses used since the most potent tubulin binder vincristine is used at the lowest dosage and vinorelbine at the highest [57,12]. This, of course, does not exclude the importance of other factors such as pharmacokinetic parameters. Therefore, based on preclinical data, it can be postulated that vinflunine will be used in the clinic at still higher doses, a finding confirmed by recently completed Phase I clinical trial studies [59,60]. Furthermore, the clinical dosage is determined not only by the potency of the drug but also by its dose-limiting toxicities, which also vary among the *Vinca* alkaloids.

8.3. Potential relationship with neurotoxicity

More specifically, the main clinical toxicity of vincristine, which exhibits the strongest overall affinity for tubulin, is neurotoxicity, whereas vinorelbine, with weaker overall affinity, is the least neurotoxic *Vinca* alkaloid [57,12]. Recently presented clinical data on vinflunine identified neutropenia and febrile neutropenia as dose-limiting toxicities using either a weekly or a three-weekly schedule of administration [59,60]. In this context, Lobert et al. [35] put forward the hypothesis that the overall drug affinity for tubulin may contribute to the severity of the neuropathies observed clinically and therefore suggested that vinflunine is likely to result in reduced clinical neurotoxicity relative to vinorelbine, to vinblastine and to vincristine. Indeed, differences in tubulin affinities between vinflunine and the other *Vinca* alkaloids have been found in terms of the affinity of liganded tubulin heterodimers for spiral polymers (K_2 value), and the data indicate that vinflunine, with the lowest overall affinity for tubulin (K_1K_2 value), induces fewer and smaller spiral polymers than vinorelbine, which in turn induces a lower extent and smaller spiral polymers than vinblastine and vincristine. It is noteworthy that these biophysical data support other published studies reporting that vinorelbine is a poor inducer of spiral aggregates relative to vinblastine and vincristine [61]. The smaller spiral polymers induced by vinflunine have a more rapid relaxation time and thus a potential for faster clearance from cells, whereas vincristine, which makes the largest spirals, exhibits the longest relaxation time [22]. It is postulated that the

Table 1
Quantitative differences between the pharmacological properties of vinflunine and those of vinorelbine, vinblastine and vincristine

	Vinflunine	Vinorelbine	Vinblastine	Vincristine
Tubulin binding				
Overall tubulin binding affinity	+	++	+++	++++
Microtubule dynamics inhibition				
Treadmilling inhibition	+	++	+++	nd
Cytotoxicity				
Cell proliferation inhibition in: murine L1210 cells	+	++	+++	+++
human DLD-1 cells	+	++	+++	++++
Concentration required to reach the respective cellular effects				
Interphasic microtubule network depolymerisation	++++	+++	++	nd
Tubulin paracrystal formation	++++	+++	++	nd
Impairment of the chromosomal plaque formation at metaphase	++++	++	+	++
Maximal centrosome separation in interphasic cells	++++	++	+	++
Reversibility of cellular effects				
Reversibility of drug-induced centrosome separation	++++	+++	+++	+
In vivo antitumour activity				
Drug-induced max increase of life span in P388-bearing mice	++++	+++	++	++
Dose required to induce the maximal increase of life span	++++	+++	++	++

L1210: murine leukaemia cells; DLD-1: human colon adenocarcinoma; nd: not determined under the experimental conditions used with vinflunine; max: maximal; + to ++++ = weak effect/activity/concentration to strong.

formation of larger drug-induced spiral polymers might result in relatively longer drug retention by tubulin in cells and tissues and therefore in the least reversible effects, which could lead to a greater potential for toxicity. This of course does not exclude the importance of other parameters that contribute to their pharmacokinetics profiles [35].

8.4. Potential implication of vinflunine interactions with microtubule dynamics

The effects of vinflunine on microtubule dynamics may also play a role in its antitumour activity. Indeed, dynamic instability of microtubules and treadmilling are crucial mechanisms for normal processing of mitosis, especially in the equi-partitioning of chromosomes to the two daughter cells by the mitotic spindle [62,25,63]. Vinflunine, like the other *Vinca* alkaloids, suppresses both dynamic instability and treadmilling, however differences in its effects have been shown relative to those of vinblastine and vinorelbine [31]. Vinflunine inhibits treadmilling less powerfully than vinblastine and vinorelbine (Table 1), and does not suppress the rate of microtubule shortening, whereas vinblastine does. These authors suggested that these different actions are likely to have varied effects during mitosis which may lead to differential effects on cell cycle and therefore on cell killing. However, in a recent publication [64] the importance of the overall suppression of microtubule dynamics in blocking mitosis is stressed.

8.5. Superiority of vinflunine antitumour activity in vivo

It is though noteworthy that in preclinical in vivo studies, vinflunine exhibited marked antitumour activity against a large and varied panel of experimental tumours [45,46,48]. Of particular interest, vinflunine induced tumour regressions in small cell lung and renal cancer xenografts. Furthermore, these preclinical data provided evidence of the superiority of vinflunine over vinorelbine in many of the experimental models tested. Therefore, since vinorelbine as the latest new semisynthetic *Vinca* alkaloid has shown improved efficacy and reduced toxicity both experimentally and in the clinical situation relative to first generation of *Vinca* alkaloids, results are eagerly awaited to see if the enhanced experimental efficacy of vinflunine translates into a wider spectrum of clinical activity.

9. Perspectives

Recent evidence has highlighted the fact that one of the key features associated with tumour progression is an activation of the 'angiogenic switch'. Interestingly, it has been shown that various conventional chemotherapeutic drugs can block angiogenesis or even kill activated, dividing endothelial cells [65]. Therefore, it has been proposed that combinatorial effects of continuous low-dose therapy of conventional cytotoxic agents with newer more specific angiogenesis inhibitors could improve antitumour efficacy in vivo [66]. Since the in vivo antitumour effects of vinflunine have been associated with antivascular properties, it appears interesting to determine whether sub-therapeutic doses of vinflunine could potentate the in vivo activity of standard cytotoxic compounds or whether vinflunine could potentate certain specific angiogenesis inhibitors. In vitro combinations of vinflunine with a series of classic cytotoxic agents have already provided evidence of synergic activity, and in vivo combination therapy experiments, which are currently being performed, seem promising. Furthermore, it appears that the novel generation of anticancer agents, such as for example, the inhibitors of prenyltransferase, of signal transduction or of cell cycle regulators, are more likely to be used in combination therapies since most of these molecules show cytostatic rather than cytotoxic properties. In that context, could vinflunine enhance the antitumour activity of these novel agents? It is clear that all these different types of combination therapies with vinflunine have to be tried. Indeed, if vinflunine could increase the antitumour activity of standard cytotoxic agents as well as that of angiogenesis inhibitors or of the novel generation of anticancer agents, that would greatly extend the potential of clinical usage of this novel *Vinca* alkaloid.

Furthermore, since vinflunine has shown antivascular properties, it appears sensible to wonder whether it might also affect tumour metastasis formation, a process requiring neovascularisation. Several experimental metastasis models, involving orthotopic implantation of tumours, have proved their utility in preclinical studies, and could be used to try to answer that question in relation to vinflunine.

In terms of the mechanism of action of vinflunine, recent advances in our understanding of the process intimately associated with mitosis, have led to our consideration that vinflunine, and probably also the other *Vinca* alkaloids, might well have more subtle effects on certain of these essential processes than initially thought [31]. Although these *Vinca* alkaloids all suppress microtubule dynamics, yet they do so in distinctive ways. Furthermore, since microtubules are coordinated by an undefined number of molecular motors to bring about the equipartioning of chromosomes to the two daughter cells by the mitotic spindle, it is tempting to wonder whether a mitotic blocker, such as vinflunine could also target certain of these motor proteins. Could vinflunine also interfere with certain of

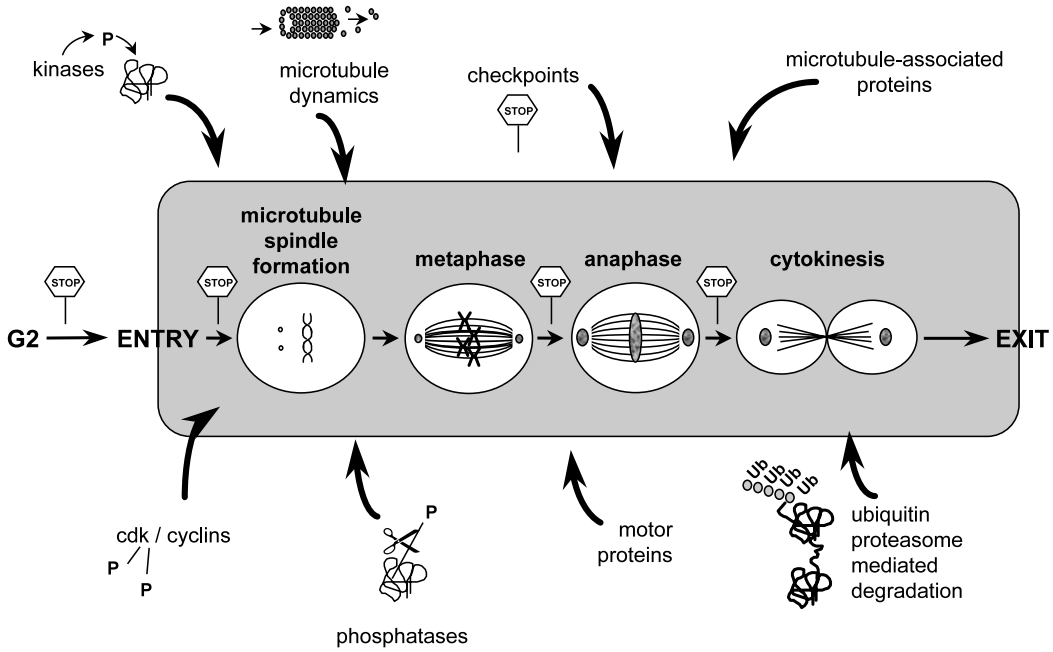

Fig. 9. A large number of 'actors' form the cast involved in regulating mitosis.

the regulators of mitosis? In fact, a large number of 'actors' now appear to form the cast involved in mitosis regulation, as illustrated diagrammatically in Fig. 9. As, so elegantly suggested by Cortez and Elledge [67], mitosis is like a symphony in which many instruments, working individually, are co-ordinated to produce a collective piece of perfection. In cancer cells, this symphony is often modified and offers targets for anticancer therapy. How many 'instruments' does vinflunine target and how does it target them? In answering these questions it may be possible to more fully understand the outstanding preclinical in vivo antitumour activity of vinflunine and even direct approaches to identifying another generation of *Vinca* alkaloids or other effective novel mitotic-interfering agents, as discussed recently [68].

Reviewers

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