Cancer Chemother Pharmacol (2000) 45: 471–476

ORIGINAL ARTICLE

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In vitro synergistic effects of vinflunine, a novel fluorinated vinca alkaloid, in combination with other anticancer drugs

Received: 6 September 1999 / Accepted: 1 January 2000

Abstract *Purpose*: Vinflunine (20′-20′-difluoro-3′,4′-dihydrovinorelbine), a novel derivative of vinorelbine characterized by marked antitumour activity in vivo in a series of experimental murine and human tumours is currently undergoing phase I evaluation. To investigate its potential for inclusion in combination chemotherapy regimens, this preclinical study was undertaken. The in vitro cytotoxicity of vinflunine incubated simultaneously with one of the following drugs was investigated: camptothecin, cisplatin, doxorubicin, etoposide, 5-fluorouracil, gemcitabine, mitomycin C, paclitaxel or vinorelbine. *Methods*: The combinations were first evaluated in vitro against the A549 human non-small-cell lung cancer cell line using median-effect analyses. *Results*: The results revealed synergistic cytotoxicity when vinflunine was combined with cisplatin, mitomycin C, doxorubicin or 5-fluorouracil. Synergy was also observed when testing similar combinations against CCRF-CEM human leukaemia cells. Finally, these findings were comparable with those resulting from such combinations involving vinorelbine instead of vinflunine. *Conclusion*: Vinflunine appears a promising candidate for combining with other anticancer drugs.

Key words Vinflunine · Combination index · Synergy · Vinorelbine · Cultured cells

Introduction

Vinflunine (20′-20′-difluoro-3′,4′-dihydrovinorelbine) is a novel derivative of vinorelbine, synthesized using superacidic media [8], that has shown markedly superior antitumour activity in vivo in a series of experimental tumour models [11, 17]. In vitro studies have confirmed the mitosis-arresting and tubulin-interacting properties of vinflunine, but have identified certain quantitative differences relative to other vinca alkaloids [16] suggesting that vinflunine may be less neurotoxic than vinorelbine [20]. Recent studies have also indicated that vinflunine may be only a weak substrate for Pgp, a membrane glycoprotein involved in the classic multidrug-resistance phenotype [7]. Vinflunine has now entered phase I clinical trials.

The vinca alkaloids are one of the oldest identified classes of cytotoxic agents used in humans [23] and they are a group of agents with a broad spectrum of antitumour activity [2, 27]. The initial drugs used were vincristine and vinblastine, but newer analogues have since been prepared with the intention of enhancing therapeutic efficacy by either increasing cytotoxicity or reducing side effects. However, relatively few of these agents have reached the clinic, with only vindesine and vinorelbine becoming commercially available [2]. Clinical trials have demonstrated the major value of vinorelbine as a single agent for antitumour therapy against breast and non-small-cell lung cancer and more recently have served to emphasize the particular value of vinorelbine when combined with another anticancer drug, such as 5-fluorouracil (5-FU), doxorubicin, mitoxantrone or, particularly, cisplatin [3, 28]. In this context, it is reasonable to envisage in the future combining vinflunine with other anticancer drugs.

This preclinical study was therefore undertaken to investigate the in vitro cytotoxicity of vinflunine in combination with a series of other anticancer drugs. Those tested, selected for their wide usage in cancer chemotherapy and for their differing modes of action, were camptothecin and etoposide (two inhibitors of, respectively, topoisomerase I and II [6]), 5-FU and gemcitabine (antimetabolites from two different chemical families [15]), paclitaxel and vinorelbine (two agents which, respectively, stabilize and destabilize tubulin polymerization [27]), cisplatin and mitomycin C (two different DNA crosslinking agents [26]), and doxorubicin (a DNA-intercalating agent [1]). Combinations were

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evaluated against the A549 human non-small-cell lung cancer cell line using median-effect analyses and calculating the combination index (CI) according to the method described by Chou and Talalay [5]. The results were then compared with those from similar combinations tested against CCRF-CEM human leukaemia cells and from combinations involving vinorelbine instead of vinflunine.

Materials and methods

Cell lines and cell culture

The A549 human non-small-cell lung tumour cell line was obtained from the American Type Culture Collection (ATCC, Rockville, Md.). Cells were cultured at 37 °C as monolayers in an incubator containing a humidified atmosphere of 5% CO_2 in air, and were split twice a week in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells of the CCRF-CEM lymphoblastoid T-cell leukemia cell line, originally obtained from Dr. V. Ling (Ontario Cancer Institute, Toronto, Canada), were cultured in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. All cell culture media were complemented with fungizone (final concentration 1.25 μg/ml), penicillin-streptomycin (final concentration 100 μg/ml, 100 IU) and glutamine (final concentration 4 m*M*). MEM, FCS, fungizone and penicillin-streptomycin were purchased from Gibco (Cergy-Pontoise, France). RPMI-1640 medium, L-glutamine and trypsin-EDTA were purchased from Seromed (Polylabo, Strasbourg, France).

Drugs

Vinflunine ditartrate (20′,20′-difluoro-3′,4′-dihydrovinorelbine, Fig. 1) was synthesized at the Centre de Recherche Pierre Fabre (Castres, France) as described elsewhere [8]. Vinorelbine ditartrate and etoposide were supplied by Pierre Fabre Medicament (Gaillac, France). Camptothecin was purchased from CIPLA (Bombay, India) and gemcitabine from Léderlé (Puteaux, France). All other compounds tested were purchased from Sigma (Saint-Quentin Fallavier, France). Vinflunine, vinorelbine, 5-FU, mitomycin C, gemcitabine and doxorubicin hydrochloride were solubilized in water (final concentration 1%), cisplatin was solubilized in 0.9% sodium chloride solution (final concentration 1%), while camptothecin, etoposide and paclitaxel were solubilized in DMSO (final concentration 0.1%).

Cell growth inhibition

For adherent cultures of A549 cells the drug-induced cytotoxic effects were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [7]. Briefly, cells were inoculated into 96-well microtitre plates at 2.5×10^4 cells/ml to ensure their logarithmic growth throughout the experiments. After allowing 24 h for cell attachment, fresh medium containing test compound alone or in combination, or solvent at the required concentration was added to each well. Cells were placed in an incubator containing an atmosphere of 5% CO_2 in air at 37 °C for 48 h. Supernatants were then discarded and replaced by 0.1 ml 1 mg/ml MTT solution in RPMI-1640 medium without phenol red and incubated for 3 h with MTT. Finally, 0.1 ml DMSO was added to each well to dissolve the formed formazan crystals, and then the plates were read using a spectrophotometer (MR7000; Dynex Technologies, Issy les Moulineaux, France) at a test wavelength of 570 nm and a reference wavelength of 630 nm. For the CEM cells, 5 ml cell suspension was seeded at 2.0×10^4 cells/ml into a series of 15-ml cell culture tubes containing solvent or the test compound(s) and then the drug-induced cytotoxic effects were determined by growth inhibition assays after 48 h, based on the counting of cells using an automated Coulter counter (Beckman Coulter, Villepinte, France). Irrespective of the cell line used, each condition was evaluated at least three times in three independent experiments in sextuplet.

Combination index method

The combination effects of two drugs in terms of synergy, additivity or antagonism were analysed by the median-effect plot [5]. This method was selected since it takes account both of the potencies of each drug and their combinations and the shapes of their dose-effect curves. However, such a method is specifically valid for analysing simultaneous coincubations of drugs. This method involves plotting dose-effect curves for each drug (Fig. 2A) and

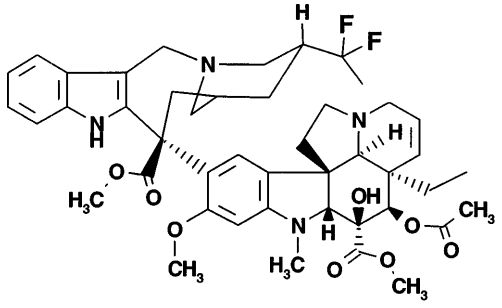

Fig. 1 Structure of vinflunine

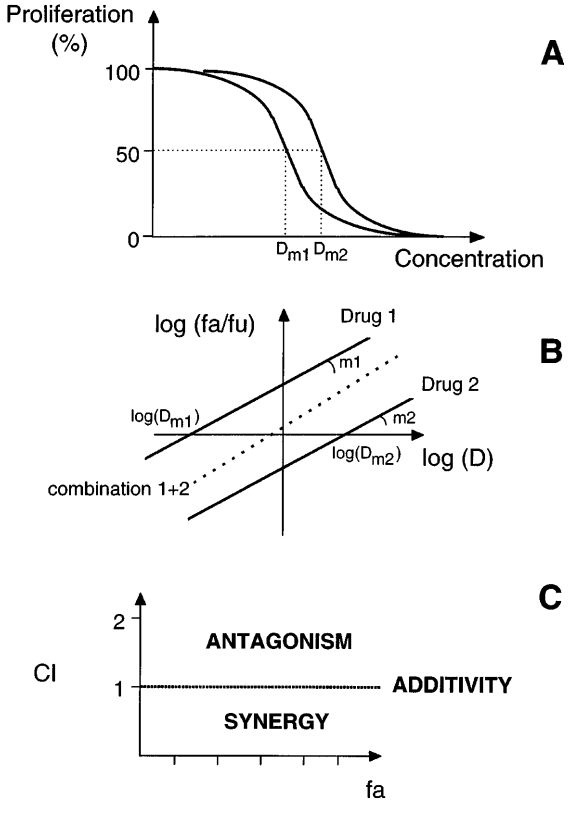

Fig. 2A–C Diagram summarizing the three steps of median-effect analysis. **A** Determination of the cytotoxicity of each drug alone. **B** Transformation of the curves reflecting the cytotoxicity of the drugs tested alone or in combination. **C** Plot of the CI versus the cytotoxicity or fraction affected (fa)

multiplying diluted combinations of the drugs using the "median effect" equation: $fa/fu = (D/Dm)^m$, where D is the dose, Dm is the dose required for 50% effect (e.g. 50% inhibition of cell proliferation at 48 h as compared to the drug-free control), fa and fu are the fractions affected and unaffected, respectively, by the dose D and *m* is a coefficient signifying the sigmoidicity of the dose-effect curve. The dose-effect curve was plotted using a logarithmic conversion of this equation which determines the values of *m* and Dm (Fig. 2B).

The conformity of the data to the median-effect principle can be readily shown by the linear correlation coefficient (*r*). A CI was then determined using the equation: $(D)_1/(Dx)_1 + (D)_2/(Dx)_2 + \alpha(D)_1(D)_2/(Dx)_1(Dx)_2$, where $(Dx)_1$ is the dose of drug '1' required to produce x% effect alone and $(D)_1$ is the dose required to produce x% effect in combination with $(D)_2$. Similarly, $(Dx)_2$ is the dose of drug '2' required to produce x% effect alone and $(D)_2$ is the dose required to produce x% effect in combination. When the drugs are mutually exclusive (i.e. with similar modes of action) $\alpha = 0$, or if they are mutually nonexclusive (i.e. with independent modes of action) $\alpha = 1$. Finally, the CI was plotted as a function of the fraction affected (Fig. 2C). When $CI = 1$, the interaction is considered additive, when $CI < 1$ synergy is indicated and when $CI > 1$ antagonism is indicated. All these calculations were performed using the CalcuSyn program (Biosoft, Cambridge, UK).

Results

Initially, the dose-effect relationships of each drug against A549 cells were subjected to the median-effect plot to determine their potency (Dm), shape (*m*), and conformity (*r*), and the pooled results are shown in Table 1. The correlation coefficients (*r* values) were 0.94 or greater, indicating a good linear relationship and good reproducibility. All ten compounds were potent cytotoxic agents with Dm values ranging from 0.016 to 33 μ*M* and the relative potency against A549 cells was in the order (highest to lowest): gemcitabine, vinorelbine and paclitaxel, camptothecin, vinflunine, doxorubicin, mitomycin C, etoposide, cisplatin and 5-FU. The Dm and *m* values for single drugs and their combinations were used for calculating synergism, additivity or antagonism as described in Materials and methods (Fig. 2).

Coincubation of vinflunine with one of the other drugs was first evaluated against the A549 cells. Thus, cells were simultaneously treated with the two drugs for 48 h, then cytotoxicity was evaluated. Each CI determination resulted from at least three independent experiments containing at least five different dilutions of the two drugs. These dilutions were established as ratios of the Dm values indicated in Table 1. Thus, the combination ratio was designed to approximate the IC_{50} ratio of the component drugs, so that the contribution of the effect for each drug in the mixture would be about the same (i.e. equipotency ratio). The results are summarized in Fig. 3 which shows, for each combination, the computer-calculated CI for 50% cytotoxicity ($fa = 0.5$). This value was selected to define synergism, additivity or antagonism since its distorted error distribution, as a consequence of the linearization of the experimental data, is theoretically minimal [21]. The envelope of additivity was arbitrarily enlarged to 0.8–1.2, since the original method does not take into account the precision of the data as discussed by Greco et al. [10].

The following combinations showed additive effects or moderate synergism only: vinflunine + camptothecin, vinflunine + etoposide, vinflunine + gemcitabine, vinflunine + paclitaxel and vinflunine + vinorelbine. On the other hand, highly synergistic effects were found with vinflunine + cisplatin, vinflunine + doxorubicin, vinflunine + 5-FU and vinflunine + mitomycin C. Details of three of these synergistic combinations are shown in Fig. 4 as examples. All experimental points and the corresponding curve which indicate, for each fractional effect, the CI values (±1.96SD) generated by the median-effect analysis are shown. As shown in Fig. 2, the lower the CI value, the higher was the extent of synergy, and conversely the higher the CI value, the greater was the antagonism, whilst additivity was established when the CI value was around 1, indicating that the experimental points were superimposable on those of the calculated curve of additivity. Thus, in Fig. 4, most CI values were in the

Table 1 Dose-effect relationship parameters for the ten drugs tested against the proliferation of A549 cells in vitro

Drug	Dm (μ*M*)	*m*	> *r*
Vinflunine	0.27	0.80	0.96
Camptothecin	0.056	0.87	0.95
Cisplatin	16	0.98	0.98
Doxorubicin	0.81	0.61	0.94
Etoposide	7.4	0.72	0.97
5-FU	33	0.69	0.96
Gemcitabine	0.015	0.74	0.99
Mitomycin C	1.4	0.51	0.94
Paclitaxel	0.016	0.29	0.94
Vinorelbine	0.016	0.61	0.94

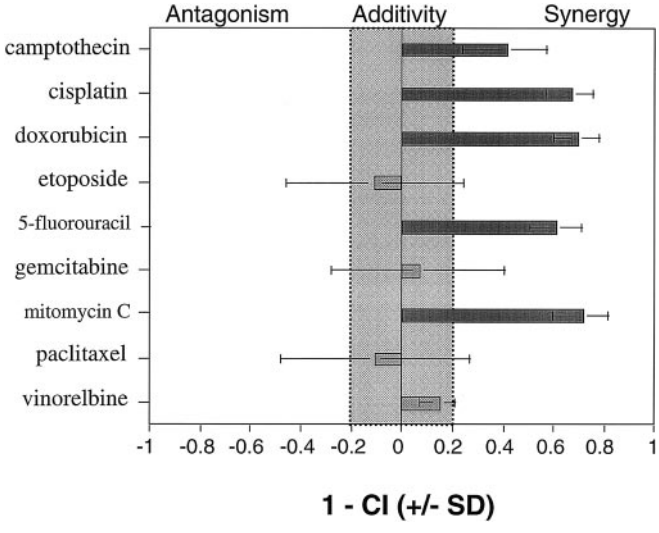

Fig. 3 Histogram summarizing the CI calculated for 50% cytotoxicity ($fa = 0.5$) with the combinations of vinflunine and each of the other nine drugs tested

range 0.2–0.6, confirming the high levels of synergism of the combinations vinflunine + cisplatin, vinflunine + doxorubicin or vinflunine + 5-FU, irrespective of the drug concentration used.

To compare these results with those obtained using another vinca alkaloid tested concurrently, similar experiments and CI determinations were carried out, as detailed in Fig. 5, with vinorelbine instead of vinflunine. The high levels of synergy, already determined for vinflunine combined with cisplatin, doxorubicin or 5-FU were also observed when vinorelbine was coincubated with these three drugs (Fig. 5). The synergy was slightly more pronounced when vinorelbine was combined with cisplatin or 5-FU, with CI values in the range 0.1–0.3 (Fig. 5A,C). However, the synergism observed with the combination vinorelbine + doxorubicin (Fig. 5B) was comparable to that calculated for the combination vinflunine + doxorubicin (Fig. 4B), with CI values around 0.3 in each case.

All these data were obtained using the A549 cell line. Since it is well known that the cytotoxicity of anticancer drugs varies depending on the cell line tested and that such variation may in turn influence the effects of drug

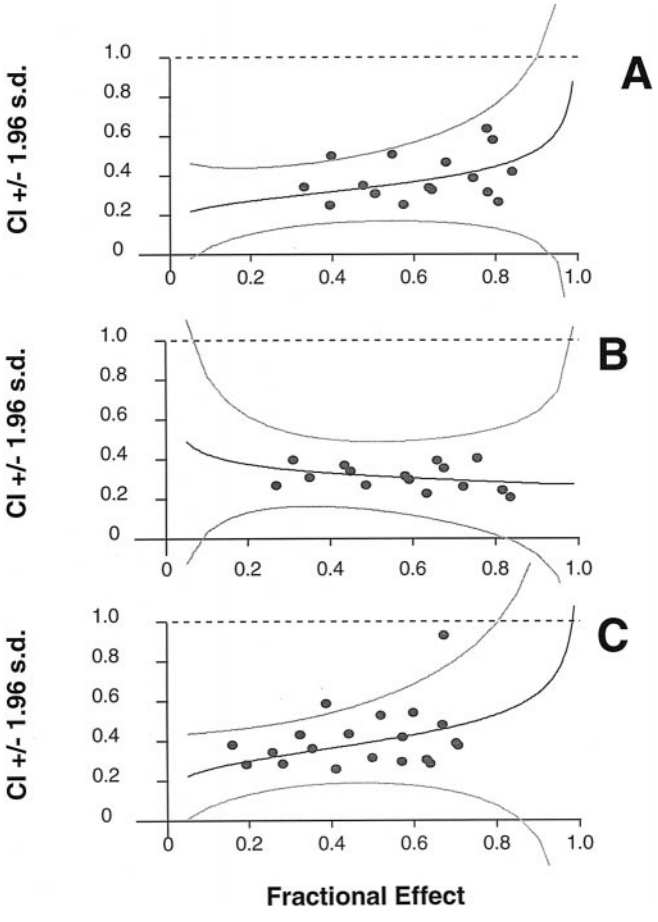

Fig. 4A–C Data resulting from the combinations of vinflunine and cisplatin (**A**), doxorubicin (**B**) and 5-FU (**C**) against A549 cells analysed using the median-effect analysis program (Biosoft, Cambridge, UK)

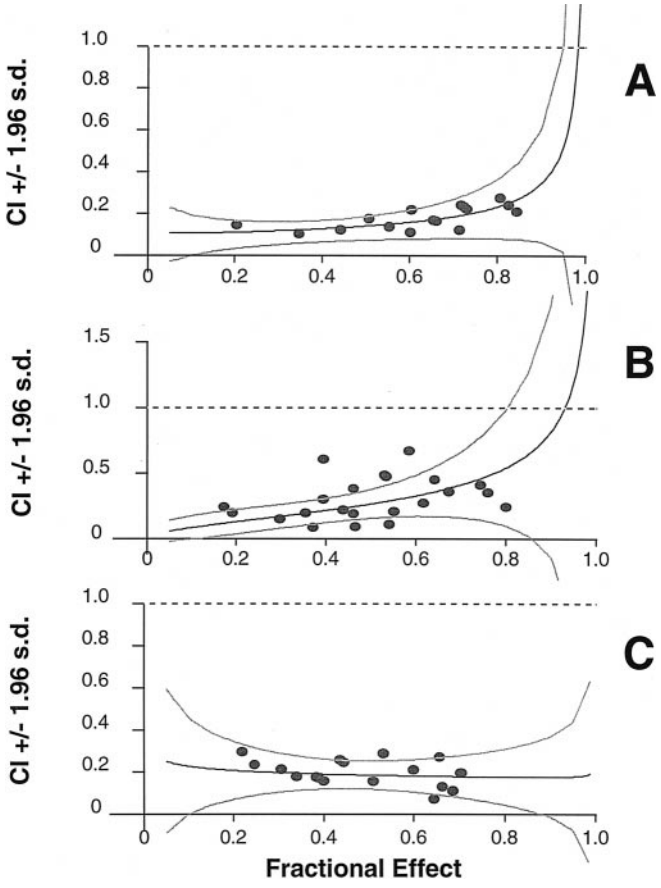

Fig. 5A–C Data resulting from the combinations of vinorelbine and cisplatin (**A**), doxorubicin (**B**) and 5-FU (**C**) against A549 cells analysed using the median-effect analysis program

combinations [13, 19, 24], median-effect analyses were also performed on data from experiments using CEM human leukaemia cells. In these studies, the combinations vinflunine + cisplatin, vinflunine + doxorubicin and vinflunine + 5-FU were evaluated. The cytotoxicity of each of the four compounds tested alone for 48 h was determined and Dm values of 0.095, 0.80, 0.012 and 0.21 μ*M* were obtained for vinflunine, cisplatin, doxorubicin and 5-FU, respectively. The correlation coefficients (*r* values) were 0.96 or greater, indicating a good linear relationship and thus validating the use of the median-effect analysis. The synergism observed with the combinations vinflunine + cisplatin (Fig. 6A), vinflunine + doxorubicin (Fig. 6B) and vinflunine + 5-FU (Fig. 6C) was slightly less pronounced in CEM cells than in A549 cells (Fig 4A–C). Nevertheless, these results provide evidence that such synergism is not restricted to a single tumour cell type.

Discussion

A high level of synergy was identified with combinations of vinflunine and antitumour compounds having completely different modes of action, namely the DNA-

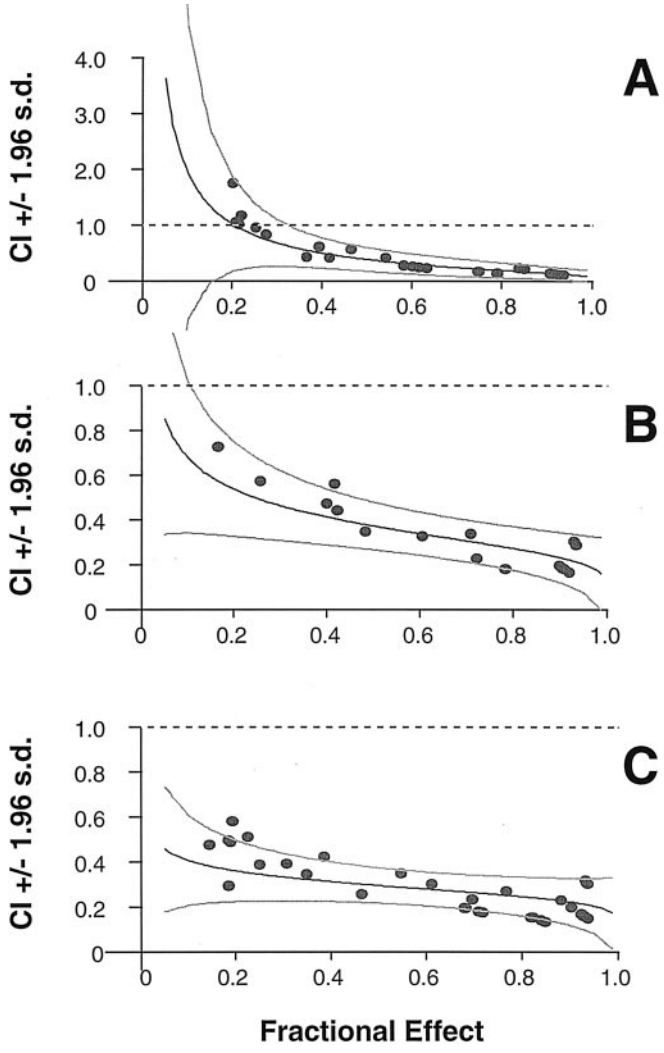

Fig. 6A–C Data resulting from the combinations of vinflunine and cisplatin (**A**), doxorubicin (**B**) or 5-FU (**C**) against CEM cells analysed using the median-effect analysis program

damaging agents cisplatin and mitomycin C, the DNA-intercalating agent doxorubicin, and the antimetabolite 5-FU. Moderate synergy was also identified with the combination of vinflunine and the topoisomerase I poison camptothecin. These findings suggest broad possibilities for including vinflunine in drug combinations. On the other hand, only additivity was identified with the combinations of vinflunine and the topoisomerase II poison etoposide, the antimetabolite gemcitabine, and either of the two tubulin-interacting agents paclitaxel and vinorelbine. However, it is interesting to note that these findings provide no evidence of any antagonism with simultaneous combinations including vinflunine, suggesting that vinflunine might be combined with a large panel of anticancer drugs, and in particular, combinations with DNA-damaging agents may prove synergistic.

Several previous studies have demonstrated that the effects of combinations of antitumour drugs can vary depending on the tumour cell line tested [13, 19, 24]. In this study, a high level of synergy with the combinations of vinflunine and cisplatin, doxorubicin or 5-FU was observed not only in A549 human non-small-cell lung cancer cells but also in CEM human leukaemia cells. Similar experiments could be carried out in other cell lines to evaluate more fully the potential of such combinations against different types of tumour cells. However, our results provide evidence that such synergism is not restricted to a single tumour cell type. Moreover, these observations provide background information vis-à-vis the potential use of vinflunine in combination chemotherapy regimens.

In vitro combinations involving vinca alkaloids and, particularly vinorelbine, have already been studied using several methodologies and various cell lines [4, 22, 25]. However, comparison of published data is complicated by the differences in so many parameters, namely the method for evaluating the synergy, the cell line used, the technique for measuring the cytotoxicity and the schedule of the drug addition. For these reasons, combinations involving vinorelbine were evaluated concurrently according to the same protocols used with vinflunine. Interestingly, comparable synergism against A549 cells was obtained with both vinflunine and vinorelbine in combination with each of the eight other clinically useful antitumour drugs, suggesting that vinflunine retains the advantage, already demonstrated for vinorelbine, of producing synergistic antitumour activity in combination chemotherapy protocols.

In conclusion, vinflunine appears to be a promising candidate for combining with other anticancer drugs, since no antagonism was observed when it was combined simultaneously with each of nine classical antitumour agents. Particularly potent synergism was identified with the DNA-damaging agents cisplatin and mitomycin C, the antimetabolite 5-FU and the DNA-intercalating agent doxorubicin. Interestingly, all these drugs induce DNA damage directly or indirectly [6, 15, 26] and induce cell death predominantly via a p53-dependent pathway [14]. On the other hand, several recent reports have suggested that tubulin-interacting agents such as taxoids and vinca alkaloids induce cell death predominantly via another pathway [9, 12, 29], since p53 disruption or mutation does not affect the sensitivity of lymphoma, lymphoblastoid cells, breast and colon carcinoma cells to Taxol or vincristine [9]. Thus, the pathway induced by tubulin-interacting agents leading to apoptosis would be complementary to that induced by DNA-damaging agents. This could explain at least in part the synergistic effects observed when vinflunine, a tubulin-interacting agent, was combined with DNA-damaging agents such as cisplatin, mitomycin C, doxorubicin, 5-FU or camptothecin. Nevertheless, this hypothesis remains to be explored by specific studies on the cell cycle and apoptosis.

Direct comparison of the results of in vitro studies with those of clinical studies is always problematic since the definition of synergy is generally different in in vitro studies and in clinical trials. Moreover, in vitro experimentation does not take into account factors such as pharmacokinetics, pharmacodynamics and the toxicity

of drugs, which all obviously influence the effects of drug combinations in vivo. Nevertheless, combinations including cisplatin, mitomycin C or doxorubicin and another vinca alkaloid such as vinorelbine have been tested against lung and breast cancers in clinical trials with encouraging results in terms of response rates and survival benefit [3]. Thus, for example, a very large study comparing vinorelbine alone with combinations of vinorelbine and cisplatin or vindesine and cisplatin in a total of 612 patients has demonstrated significantly higher response rates and a survival advantage for the combination of vinorelbine and cisplatin [18].

Preclinical data suggest that vinflunine may have a much broader spectrum of antitumour activity than vinorelbine [11, 17]. Thus, evaluation of vinflunine in combination with these other drugs could be extended to other tumour types and perhaps to in vivo experimentation so as to provide further preclinical evidence that vinflunine too might have potential in combination chemotherapeutic approaches in the clinic.

Acknowledgements The authors wish to thank Anne Limouzy and Nathalie Cabrol for their technical assistance.

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