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Inhibition of Growth of Colon 38 Adenocarcinoma by Vinblastine and Colchicine: Evidence for a Vascular Mechanism

Bruce C. Baguley, Karen M. Holdaway, Lindy L. Thomsen, Li Zhuang and L. Jonathan Zwi

Vinblastine or colchicine, administered intraperitoneally to B6D2F$_1$ mice with advanced subcutaneous colon 38 tumours, induced substantial tumour growth delays with progressive development of haemorrhagic necrosis beginning within 8 hours of treatment. Two multidrug-resistant P388 leukaemia sublines, refractory to vinblastine and vincristine when grown as intraperitoneal ascites, were sensitive to necrosis induction when grown as subcutaneous tumours. Vascular labelling with two fluorescent markers indicated that vincristine substantially reduced tumour blood flow within 4 hours after treatment. The effects of vinblastine, vincristine and colchicine were similar to those of tumour necrosis factor alpha in that: (a) similar tumour necrosis and blood flow changes were induced, (b) coadministration of the serotonin antagonist cyproheptidine prevented tumour necrosis and (c) plasma nitrate levels were elevated, indicative of the stimulation of oxidation of L-arginine to nitric oxide. The results suggest that vinca alkaloids and colchicine act on solid tumours by host cell-mediated vascular effects as well as by direct tubulin-mediated cytotoxicity.
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INTRODUCTION

THE ANTITUMOUR vinca alkaloids were discovered during the course of random screening of plant products against transplantable murine leukaemias. Several were found to have clinical antitumour activity, and vincristine and vinblastine, as well as the semisynthetic analogue vindesine, are now in widespread clinical use, predominantly for the treatment of haematological malignancies [1]. These alkaloids are thought to act by binding to tubulin and inducing mitotic arrest [2]. A number of other plant-derived tubulin-binding mitotic inhibitors are known, with colchicine and its more dose-potent derivative colcemid (demecolcine) of particular interest because of their activity against experimental solid tumours [3].

Recent studies in this laboratory have been concerned with the identification of compounds which induce haemorrhagic necrosis of advanced subcutaneous murine colon 38 tumours in a fashion similar to that induced by tumour necrosis factor alpha (TNF-α) [4]. The effect of the experimental antitumour agent flavone acetic acid (FAA) was first observed to have this effect in our laboratory [5], and while most clinical antitumour agents fail to induce necrosis, the experimental agents fostriecin and homoharringtonine induce both necrosis and growth delays of subcutaneous tumours [6]. In this communication we report the results of further studies which show that vinca alkaloids, as well as colchicine, induce haemorrhagic necrosis of subcutaneous colon 38 tumours. We have also investigated the effects of these compounds on subcutaneous tumours derived from two multidrug-resistant P388 leukaemia sublines. Since FAA has also been demonstrated to reduce tumour blood flow [7, 8] and to elevate plasma nitrate concentrations [9] appropriate studies have been carried out using vinblastine, vincristine and colchicine. The results suggest these drugs act on solid tumours by a novel, host cell-mediated mechanism with similarities to that of TNF-α.

MATERIALS AND METHODS

Drugs

Vincristine, vindesine and vinblastine were obtained as clinical formulations from Eli Lilly and Co (West Ryde, Australia). Colchicine, colcemid, serotonin and Hoechst 33342 (H33342) were from Sigma (St Louis, USA), 9-nonylacridine orange (NAO) was from Molecular Probes (Eugene, USA) and cyproheptidine was from Serva (Heidelberg, Germany).

Tumours

Mice were bred in the laboratory under conditions of constant temperature, lighting and humidity with sterile bedding, food and water, and experiments performed according to institutional ethical guidelines. Colon 38, P/DOX (P388 resistant to doxorubicin) and P/DACT (P388 resistant to actinomycin D) cells were from Mason Research Institute (Worcester, USA). P388 cells were from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, USA). Colon 38 tumours (1 mm^3 fragments) were implanted in $B6D2F_1$ mice anaesthetised by intraperitoneal injection of pentobarbital (90 mg/kg in 0.15 ml H_2O). P388 lines and sublines were inoculated as suspensions of 10^6 cells.

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Measurement of tumour growth delay

Life extension experiments with P388 leukaemia and its resistant sublines were carried out using 20 control $B6D2F_1$ mice and groups of 6 treated mice, inoculated intraperitoneally on day 0 with 10^6 cells and treated with drug intraperitoneally on days 1, 5 and 9 [10]. Growth delay experiments were carried out using treated and control groups of 5–6 $B6D2F_1$ mice with subcutaneous 5–10 mm diameter colon 38 tumours (10 days after implantation) [6]. Mice were randomised with respect to tumour size and injected intraperitoneally with a single dose of drug. Tumours were measured with digital callipers and tumour volumes calculated as $0.52a^2b$, where a and b were the minor and major axes of the tumour. Means and standard errors were calculated on the basis of the logarithms of tumour volume and the results expressed as a fraction of the initial mean tumour volume (typically 0.3 cm^3).

Measurement of tumour necrosis

Histological assessment was carried out using $B6D2F_1$ mice with subcutaneous colon 38 tumours (10 days after implantation) or with subcutaneous P388, P/DOX or P/DACT tumours (7–9 days after inoculation). Mice were injected with a single dose of drug and were killed by cervical dislocation 24 h later. Tumours were removed and fixed immediately in 10% formalin. Sections taken from the centre of the paraffin-embedded tumours were stained with haematoxylin and eosin. A grid marked at 0.4 mm intervals was placed over the slide and the intersections of the grid scored as either "undamaged" tumour or "necrotic" (pyknotic and fragmented nuclei, altered staining pattern). Grid intersections on blood vessels or obvious stromal elements were not counted. The fraction of tumour necrosis was determined by dividing the number of necrotic intersections by the number of necrotic plus undamaged intersections and expressing the result as a percentage.

Measurement of blood flow

Changes in blood flow in subcutaneous colon 38 tumours (0.2–0.9 g) after treatment with vincristine (5 mg/kg intravenously) were determined using a modification of a double label fluorescent staining technique [8]. Briefly, the first dye (H33342; 32.5 μmol/kg) was injected intravenously immediately before drug treatment and the second dye (NAO; 20 μmol/kg) 30 min or four h later. Frozen sections of the excised tumours were viewed at 100 × magnification using a fluorescent microscope with appropriate filters. Each of 25 microscopic fields was scored from 0–4 for each dye, depending on the fraction of the field that was fluorescent. The percentage of remaining blood flow after treatment for each tumour was calculated by the sum of scores for NAO fluorescence divided by the sum of scores for H33342 fluorescence.

Measurement of plasma nitrate concentrations

Blood was collected after drug treatment (12 h unless otherwise indicated) by cardiac puncture under anaesthesia with a heparinised 20 G needle. Blood samples were immediately centrifuged at 8000 *g* for 5 min and the plasma was removed and stored at −70°C. Nitrate was measured as previously described [9]. Plasma samples were diluted with milli-Q water, the proteins precipitated with 30% $ZnSO_4$ (0.05 ml per ml of plasma), and centrifuged (8000 *g*, 5 min). After reducing NO_3^- in the supernatants to NO_2^- using acid-washed cadmium powder, NO_2^- concentrations were measured using a microplate assay method based on the Griess reaction [11]. The NO_2^-/NO_3^- value

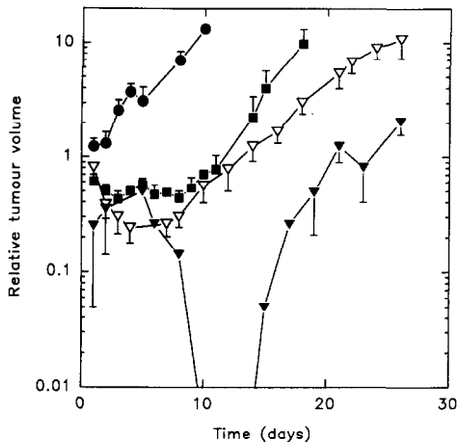


Fig. 1. Growth of subcutaneous colon 38 tumours in mice with no treatment (●), 10 mg/kg vinblastine (▼), 7 mg/kg vinblastine (▽) or 10 mg/kg colchicine (■) administered as a single intraperitoneal dose. At the higher of the two vinblastine doses, 1/6 toxic deaths occurred at day 4, tumours were not palpable in the remaining mice between days 10 and 14, but all tumours eventually regrew. At the lower dose of vinblastine and with colchicine, no toxic deaths or complete remissions were evident.

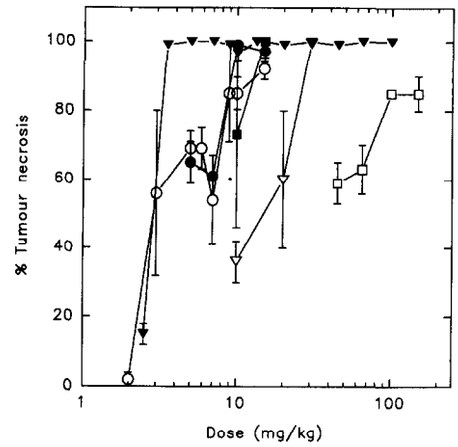


Fig. 2. Relationship between the administered intraperitoneal dose and the percentage necrosis of subcutaneous colon 38 tumours for vinblastine (○), vindesine (■), vincristine (●), colchicine (▼), colcemid (▽) and serotonin (□). The percentage necrosis in tumours from untreated mice was 12 (3)%.

for each plasma sample was obtained in duplicate assays from experiments using at least three mice per data point. In selected samples, NO_2^- concentrations were assayed without prior reduction of NO_3^- by cadmium and were found to be below the level of detection.

Statistics

Results are presented as means (standard error of the mean). Differences between groups are calculated using Student's *t* test.

RESULTS

Induction of tumour growth delays by vinblastine and colchicine

Mice with subcutaneous colon 38 tumours, when injected with the maximum tolerated dose of vinblastine (10 mg/kg) showed ruffled fur and diarrhoea after 4 days. There was one toxic death. The mice underwent tumour regression with a growth delay of 23 days (Fig. 1) Vincristine at the maximum tolerated dose (2.9 mg/kg) was ineffective. Vinblastine (7 mg/kg) and colchicine (10 mg/kg) induced a growth delays of 15 and 11 days, respectively (Fig. 1).

Induction of haemorrhagic necrosis in colon 38 tumours

Vinblastine, vindesine and vincristine induced tumour necrosis 24 h after intraperitoneal administration of a range of doses (Fig. 2). Vincristine (5 mg/kg intravenously) and vinblastine (10 mg/kg intravenously) also induced more than 85% necrosis. Colchicine induced complete tumour necrosis at doses at or above 3.5 mg/kg and was more than 10 fold more potent than was its analogue colcemid (Fig. 2). Serotonin, tested for reasons which are explained below, induced significant but incomplete tumour necrosis (Fig. 2).

The time course of induction of haemorrhagic necrosis was examined for vincristine (10 mg/kg), vinblastine (10 mg/kg) and colchicine (10 mg/kg). Similar results were obtained for all drugs and representative sections are shown in Fig. 3. After 2 and 4 h little or no evidence was obtained for increased tumour necrosis, although a substantial increase in the number of mitotic cells was observed (Fig. 3b). After 8 h a significant proportion of the tumour was found to be necrotic. By 24 h very little evidence of mitotic cells remained and the whole tumour section contained necrotic cells with pyknotic and often fragmented nuclei (Fig. 3c).

Induction of haemorrhagic necrosis in multidrug-resistant tumours of P388 leukaemia cells

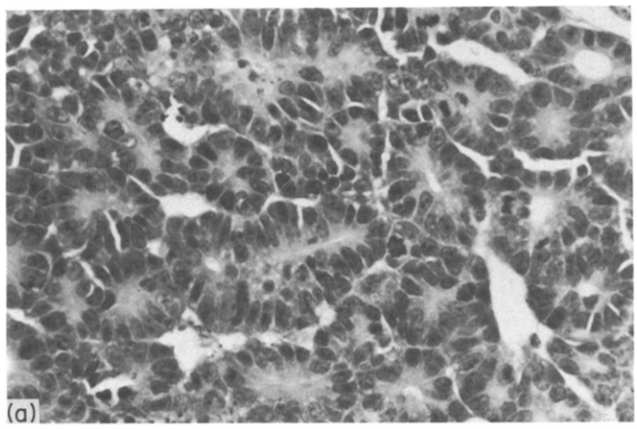
Vincristine (2.6 mg/kg/dose) and vinblastine (2.7 mg/kg/dose), when tested in standard life extension assays [10] employing intraperitoneal tumour and drug administration, induced life extensions of 70% and 85%, respectively, in mice inoculated with the P388 line, but failed to give a significant response (25% life extension) against the two resistant P388 sublines (P/DOX and P/DACT). P388 cells and their resistant sublines were also inoculated subcutaneous and tumours allowed to grow until palpable (7–9 days). Mice were treated with vincristine, vinblastine or colchicine (10 mg/kg), the tumours removed 24 h later, and histological sections subjected to examination. All tumours were found to undergo necrosis to a significantly greater extent than that of untreated tumours (Fig. 4, Table 1).

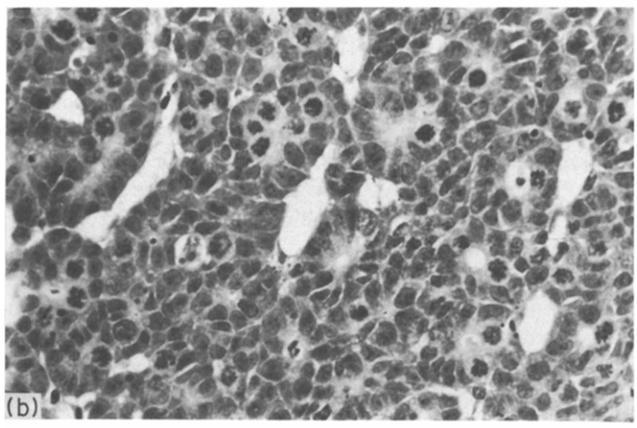
Inhibition of tumour blood flow

Vincristine (5 mg/kg) was administered to colon 38-bearing mice, followed by a fluorescent dye to mark areas of blood flow. Either 30 min or 4 h later, mice were injected with a second fluorescent marker, and the tumour subsequently removed for analysis of frozen sections [8]. After 30 min, 89 (3.2)% of the tumour areas labelled with the first dye was also labelled with the second dye. This was not significantly different to the value obtained [93.5 (2.3)%] for tumour-bearing mice which received no vincristine. After 4 h, only 27.3 (7.7)% of the areas labelled with the first dye were also labelled with the second, significantly lower ($P < 0.001$) than the value obtained [92.8 (4.1)%] for untreated mice.

Reversal of haemorrhagic necrosis by the serotonin antagonist cyproheptidine

Cyproheptidine has been reported to prevent TNF-α from inducing haemorrhagic necrosis in the Meth A murine fibrosar-





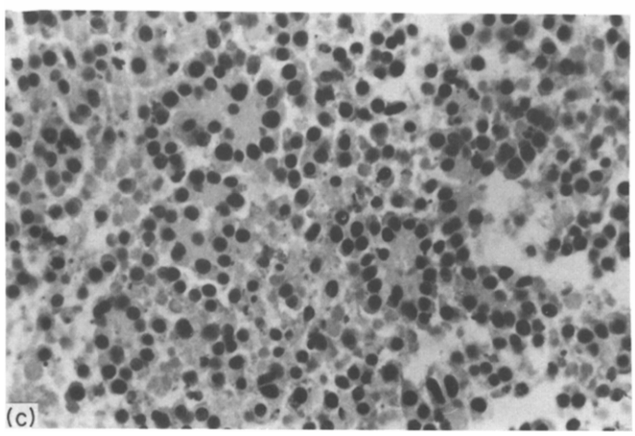
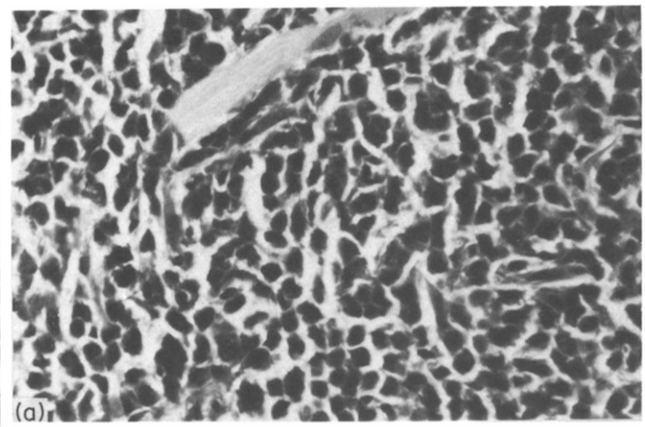


Fig. 3. Histologic appearance (×100) of subcutaneous colon 38 tumours either before (a), 4 h after (b), or 24 h after (c) a single dose of vinblastine (10 mg/kg intraperitoneally). The control tissue is organised into acini with numerous blood vessels. After 4 h numerous mitotic cells are visible. After 24 h almost all cells are necrotic and the tumour contains interspersed erythrocytes.



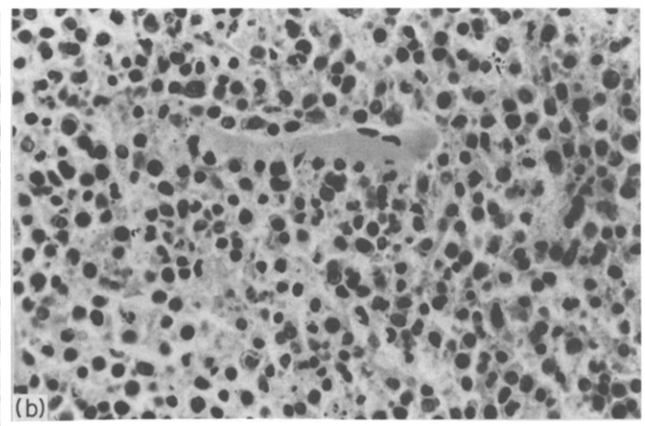


Fig. 4. Histologic appearance (×100) of subcutaneous tumours of P/DACT leukaemia cells either before (a) or 24 h (b) after a single dose of vinblastine (10 mg/kg intraperitoneally). Control cells show tightly packed lymphocytic cells which have surrounded a muscle fibre, and numerous small blood vessels. After 24 h cells became necrotic with pyknotic or fragmented cell nuclei and interspersed erythrocytes. Sections of subcutaneous tumours of P388 and P/DOX leukaemia cells showed the same appearance.

coma [12]. When injected intraperitoneally (20 mg/kg) into colon 38-bearing animals simultaneously with vinblastine, vincristine or colchicine, it significantly decreased the degree of tumour necrosis (Table 2) but had no effect on drug-induced mitotic arrest. Cyproheptidine alone had no effect on the histological appearance of the tumour after 24 h. Serotonin-induced tumour necrosis (see Fig. 2) was completely inhibited by coadministration of cyproheptidine.

Increase in plasma nitrate concentrations

TNF-α has been reported to increase plasma nitrate concentrations as a result of the oxidation of L-arginine to citrulline and nitric oxide, which is subsequently converted to nitrite and nitrate [9]. To determine whether these drugs had a similar effect, colon 38-bearing mice were treated with vinblastine (10 mg/kg) and plasma analysed for nitrate. A significant increase ($P < 0.001$) was found and the elevation was maximal 8–16 h after administration. Vinblastine, vincristine and colchicine were compared after 12 h and found to increase plasma nitrate concentrations significantly (Table 2). Similar elevation were observed in tumour-free mice. The increase in plasma nitrate was almost abolished by the coadministration of cyproheptidine (Table 2).

DISCUSSION

We have demonstrated here that vinblastine and colchicine induce significant delays in the growth of advanced subcutaneous colon 38 tumours (Fig. 1). Several observations suggest that these drugs act indirectly by a mechanism which is additional to that normally associated with tubulin-binding agents. Drug treatment is followed by extensive haemorrhagic necrosis (Table 2, Fig. 3) similar to that observed with TNF-α and FAA [6],

Table 1. Induction of haemorrhagic necrosis in subcutaneous P388, P/DACT and P/DOX tumours by vinca alkaloids

Compound	Dose mg/kg	% Tumour necrosis P388	P/DACT	P/DOX
Vinblastine	10	99.7 (0.3)	96.5 (2.2)	88 (7)
Vincristine	10	99.5 (0.5)	99.5 (0.5)	96 (3)
Colchicine	10	100 (0)	99 (0)	100 (0)
No treatment		0 (0)	14 (3)	47 (3)

Mean (S.E.)

in addition to mitotic arrest, and the serotonin antagonist cyproheptidine inhibits drug-induced necrosis (Table 2) but not drug-induced mitotic arrest. The multidrug-resistant leukaemia lines P/DOX and P/DACT, when grown as subcutaneous tumours, undergo necrosis in response to these drugs in a manner similar to that observed with the parent P388 line (Table 1). Both P/DOX and P/DACT are thought to exhibit increased drug efflux since they contain P-glycoprotein and since their resistance *in vitro* to vincristine is overcome by the efflux inhibitor verapamil [13], and both are unresponsive to vincristine and vinblastine when grown intraperitoneally as an ascites. Growth delay experiments to test the effect of vinblastine on subcutaneous P/DOX and P/DACT tumours were not possible since deaths occurred when the subcutaneous tumours were only of moderate size (approx. 10 mm diameter). It is likely that deaths resulted from systemic infiltration with tumour cells which resist drug treatment.

A further indication of the additional effects of vinblastine, vincristine and colchicine is the induction of increased plasma nitrate (Table 2). The likely major source of plasma nitrate is nitric oxide, which is produced from L-arginine by arginine oxidase [14] and subsequently converted to nitrite and nitrate. Since arginine oxidase is present in both platelets [15] and endothelial cells [16] the observed elevation of plasma nitrate may result from nitric oxide production by these cells. The stimulation of nitrate elevation is not as high as those observed following treatment with FAA [9]. Unlike FAA, vinblastine and colchicine do not stimulate nitric oxide synthesis by activated macrophages *in vitro* (unpublished results). It is thus possible that vinblastine and colchicine differ from FAA in being unable to stimulate activated macrophages *in vivo* to release nitric oxide.

Table 2. Effect of cyproheptidine on the induction of haemorrhagic necrosis in subcutaneous colon 38 tumours and on the elevation of plasma nitrate concentrations

Drug(s)	Dose mg/kg	Tumour necrosis (%)	Plasma nitrate μmol/l
No treatment	–	12 (3)	18 (2)
Vinblastine	10	87 (5)	35 (6)
Vinblastine + cyproheptidine	10 20	31 (18)	24 (6)
Vincristine	10	99 (0.4)	53 (10)
Vincristine + cyproheptidine	10 20	8 (1)	30 (5)
Colchicine	10	97 (2.4)	94 (4)
Colchicine + cyproheptidine	10 20	35 (5)	19 (1)

The observed growth delays of colon 38 tumour appears to result from the induction of vascular changes within the tumour. Treatment with vincristine is accompanied by changes in blood flow similar to those observed with FAA [8]. A possible mechanism for this change is through the induction of serotonin, which is produced by activated platelets and may damage vascular endothelial cells [17]. Serotonin induces haemorrhagic necrosis (Fig. 2 and ref. 12) and the serotonin antagonist cyproheptidine inhibits necrosis induction by vinblastine, vincristine and colchicine (Table 2) as well as that of TNF-α [12]. The mechanism for the activation of platelets in the tumour vasculature is not yet clear. Vinca alkaloids and colchicine induce a rapid reduction in the number of TNF-α receptors on macrophages and endothelial cells [18]. One interpretation of this result is that the drugs induce TNF-α directly, thus initiating vascular damage. The presence of anti-TNF-α antibody does not prevent the reduction in TNF-α receptors, arguing against this hypothesis [18]. Nevertheless, these drugs may stimulate the release of cytokines from endothelial cells, macrophages or other cells and may thus initiate a cascade of events including the activation of platelets, the release of serotonin, and subsequent vascular damage within the tumour.

In conclusion, our results suggest that three inca alkaloids, as well as colchicine and colcemid, have an indirect mechanism against solid tumours which has many similarities to that of TNF-α. Since these drugs are all tubulin binders, it is tempting to conclude that this action is mediated by drug binding to tubulin of host effector cells. However, this conclusion may be premature, since the testing of a number of mitotic inhibitors in our laboratory has shown that not all are active in the induction of tumour necrosis (manuscript in preparation). Furthermore, homoharringtonine, a drug with some structural similarities to colchicine but which is not a mitotic inhibitor, induces haemorrhagic tumour necrosis [6]. Vinblastine, which is considerably less toxic than vincristine, is only slightly less active than vincristine in the necrosis assay (Fig. 2). It thus has a dose "window" in which substantial growth delays can be observed. This dose window is higher than that used clinically and there is little evidence in the clinical literature for vinblastine-induced tumour necrosis [19]. It is possible that analogues of vinblastine exist which, like colchicine, have a larger dose window. Such compounds will be useful to probe the mechanism of this effect, or to exploit it clinically.

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Anthropometric Indicators of Endometrial Cancer Risk

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The relation between selected anthropometric indicators and the risk of endometrial cancer was evaluated using data from a case-control study conducted in Northern Italy on 562 cases and 1780 controls in hospital for acute, non-neoplastic or hormone-related disease. There was no appreciable association between height and endometrial cancer: compared with the lowest quintile, the multivariate relative risks (RR) were 0.9, 0.9, 0.7 and 0.8 for each subsequent quintile. Weight was directly associated with risk (RR=2.7 for top vs. bottom quintile), and the positive association was even stronger when indices of body mass which make allowance for height were considered: the relative risks for extreme quintiles were 3.4 for W/H^2 (Quetelet's index, weight and height), 3.8 for $W/H^{1.5}$ and 3.5 for $W^{0.33}/H$. Surface area, which was positively correlated both with height and weight, showed a weaker direct association (RR=2.4 between extreme quintiles). The relations with measures of body weight were apparently stronger in postmenopause, but the point estimates for the upper quintile were also around 2 in premenopausal women. Although the major findings of this study are not new, they provide more detailed information than was hitherto available on the relation between various anthropometric indicators and endometrial cancer risk. In relation to height, with the sample size of this study it was possible to exclude, at the conventional 95% probability, relative risks above 1.0 for the fourth and above 1.1 for the fifth as compared with the lowest quintile. This provides indirect evidence against the hypothesis that nutritional status early in life is related to the subsequent development of endometrial cancer.
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INTRODUCTION

OVERWEIGHT AND obesity are well-defined risk factors for endometrial cancer, most of the published evidence being based on simple measures of weight or relative indices of body mass (chiefly, Quetelet's index) [1–9].

At least two case-control studies of endometrial cancer, however, have found that not only body weight, but also height is directly associated with risk [4,10]. This is consistent with findings for other sex-hormone related neoplasms (breast and prostate [11,12]) and, in aetiological terms, could be related to a potential role of nutritional factors in childhood and adolescence on the subsequent risk of the neoplasms [13].

The issue is of theoretical interest (as well as of practical relevance for prevention) in order to understand the role of nutrition and overweight within the framework of the process of endometrial carcinogenesis, i.e. whether it has essentially a