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Plasma pharmacokinetics, tissue disposition, excretion and metabolism of vinleucinol in mice as determined by high-performance liquid chromatography

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Abstract. We investigated the pharmacokinetics of the experimental semisynthetic vinca alkaloid vinleucinol (VileE; O4-deacetyl-3-de(methoxycarbonyl)-3-[[[1-ethoxycarbonyl-2-methylbutyl]amino]carbonyl]-vincaleukoblastine). The study was performed in male FVB mice receiving 10.5 mg/kg VileE i.v. or p.o. Plasma, urine, faeces and tissue samples were analysed by a selective method based on ion-exchange normal-phase high-performance liquid chromatography (HPLC) with fluorescence detection and liquid-liquid extraction for sample clean-up. Apart from the parent drug, two other metabolic compounds were detected. One of these metabolites is vinleucinol acid (VileA; O4-deacetyl-3-de(methoxycarbonyl)-3-[[[1-carboxyl-2-methylbutyl]amino]carbonyl]-vincaleukoblastine), which possesses no cytotoxic activity. The structure proposed for the second metabolite (VileX) was based on tandem mass spectrometry (MS-MS) and infrared (IR) spectroscopy data. Metabolization of VileE to VileX must occur in the amino acid moiety of the molecule, with a (β- or γ-) lactone ring being formed after oxidation of the (β- or γ) carbon of the amino acid. VileX is a major metabolite, which is excreted in faeces and urine after i.v. administration and accounting for up to 23% of the administered dose. The activity of VileX against cultured L1210 cells is four times that of the parent drug VileE and comparable with that of vinblastine (VBL). At 48 h after administration of VileE, the concentration of VileX exceeds that of the parent drug in many tissues. These findings indicate that the metabolite VileX may be at least largely responsible for the activity observed against xenografts in mice after administration of the parent drug, VileE.

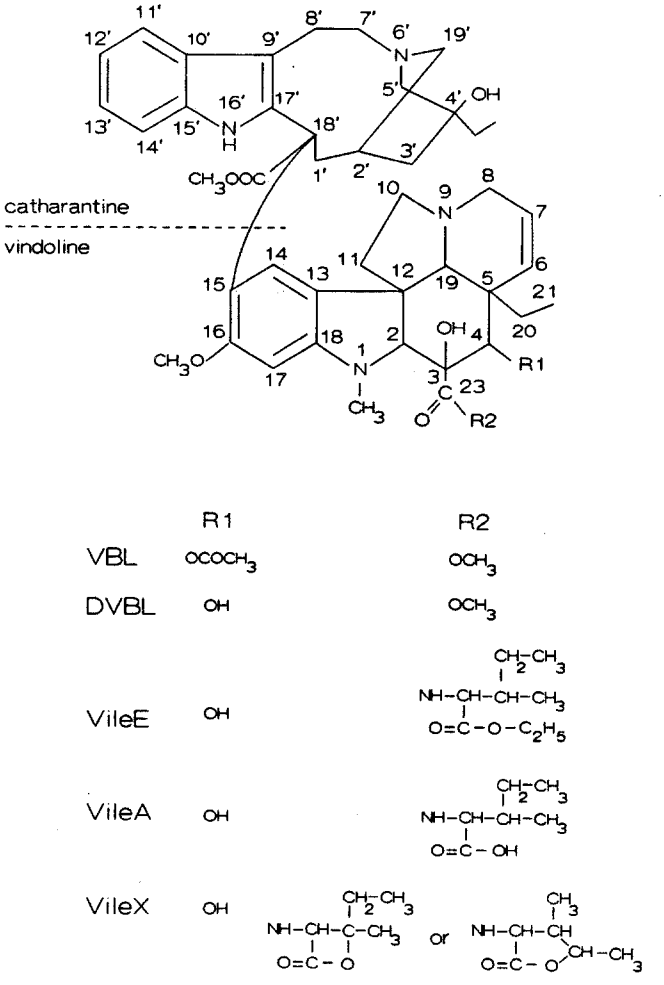

Fig. 1. Molecular structures of vinblastine (VBL), O4-deacetylvinblastine (DVBL), vinleucinol (VileE), and vinleucinol acid (VileA), and a proposed structure for VileX, also a metabolite of VileE

Introduction

Recognition of the cytotoxic properties of the alkaloid fraction of the periwinkle plant Catharanthus roseus G. Don dates back to the late 1950s [3, 8]. Isolation and elucidation of the structure have revealed a large number of structurally closely related alkaloids, composed of a dimeric skeleton of

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two almost identical condensed ring systems, vindoline and catharantine, linked by a carbon-carbon bond (Fig. 1). Despite their structural resemblances these compounds exhibit very different cytotoxic properties, and eventually only vinblastine (VBL) and vincristine (VCR) proved to be clinically useful [7, 9, 13, 14]. Today, these drugs constitute an important class of antitumour agents, belonging to the potentially curative treatment modalities. Since antitumour spectra, as well as clinical efficacies, appear to be determined by only minor structural differences, considerable effort has been devoted to the development of semi-synthetic analogues [18]. Vindesine was the first semi-synthetic analogue shown to be clinically useful [2, 5]. A number of other semi-synthetic analogues are currently under (pre-)clinical investigation [4, 10, 12]. In a series of 21 deacetylvinblastine-23-oyl amino acid derivatives, the conjugates of O^4-deacetylvinblastine (DVBL) and both L-ethyl tryptophan (vintriptol; VtrpE) and L-ethyl isoleucine (VileE) have demonstrated higher antitumour activity than VBL in mice bearing P388 leukaemia or 6C3HED lymphosarcoma [1]. Superior activity of VileE over both VBL and VtrpE against a number of other human solid tumour xenografts in mice has recently been reported [6].

In a previous paper on the pharmacokinetics and tissue distribution of VBL in mice [19], it was reported that, although the uptake of VBL in various tissues was high, its retention in many was minimal. Exceptions, however, were tissues of lymphatic and testicular origin, which showed a remarkably selective retention. Although these findings were obtained in normal tissues, they may help to explain why lymphomas and testicular carcinomas are most responsive to chemotherapy with VBL, while it is not active, or only marginally active, against other malignancies. Apparently, many tissues carry effective mechanisms for rapid elimination of this cytotoxic compound. As tumour cells are known to be only marginally different from the normal cells from which they originate, it may be speculated that after malignant transformation, cells will retain their capability of drug elimination. These results provide a rationale for studying the tissue distribution of new semi-synthetic analogues in normal healthy tissues in order to identify potentially sensitive tumours derived from these tissues. In this paper we report on our investigations with the experimental vinca alkaloid VileE and its metabolites, using a validated, selective and sensitive HPLC method. Structural characterization of an important metabolite was achieved by mass spectrometry and infrared spectroscopy.

Materials and methods

Drugs and chemicals. VileE, VileA, VBL and DVBL were obtained from the Medgenix Group (Fleurus, Belgium). Bovine serum albumin (BSA) originated from Organon Technika (Boxtel, The Netherlands). All other reagents were purchased from E. Merck (Darmstadt, Germany) and were of analytical quality, except for acetonitrile, which was HPLC grade. Blank human plasma was obtained from healthy donors. Water purified by the Milli-Q system (Millipore, Milford, Mass., USA) was used throughout.

Animal studies. All experiments were carried out in male FVB mice aged 5 weeks and given food and water ad libitum. VileE (1 mg/ml dissolved in 5% dextrose) was given at half the 10% lethal dose ($0.5 \times LD_{10}$), i.e., 10.5 mg/kg, as an i.v. bolus injection in the tail vein (average injection time: 5 s) or p.o. by direct administration into the stomach using a blunt needle.

Plasma pharmacokinetics. Blood sampling was performed at 1, 2, 5, 10, 20, 30 min and 1, 2, 4, 6, 8, 16, 24, 31 and 48 h after i.v. drug administration. Blood sampling after oral administration was conducted at 5, 10, 15, 20, 30, 45 min and 1, 2, 4, 6, 8, 15, 24, 31 and 48 h after drug administration. Three animals were used per time point. Blood, obtained by heart punction, was collected in heparinized tubes and was centrifuged immediately after sampling (3 min, 11000 *g*). Plasma was stored at –20°C prior to analysis.

Tissue disposition. Tissues from animals receiving the drug by i.v. injection were collected at 4, 24 and 48 h after drug administration, 4 animals being used per time point. The tissues selected for analysis included: brain, muscle (from the back), abdominal fat, stomach, small and large intestine, appendix, liver, gall bladder, kidney, lung, spleen, heart, testis, epididymis, thymus, mesenteral plus peripheral lymph nodes and plasma. Tissues were homogenized with a tissue homogenizer (Biospec Products, Bartlesville, Okla., USA) at 4°C in 1 to 5 ml of blank human plasma (approximately 0.1–0.2 g tissue/ml) and were stored at –20°C.

Excretion studies. Eight animals ($4 \times$ i.v., $4 \times$ p.o.) were placed in metabolic cages. Urine and faeces were collected in 24-h portions for a total period of 96 h. Each portion of faeces was homogenized in 10 ml of 4% (w/w) BSA in water with a tissue homogenizer. Urine samples and faeces homogenates were stored at –20°C prior to analysis.

Drug analysis. Extractions were carried out in glass tubes fitted with PTFE-covered screw caps. Thawing of samples took place in a waterbath at 20°C.

Plasma: Volumes of 5 to 500 µl of plasma sample were completed to 500 µl with blank human plasma. After adding 50 µl of internal standard (2 mg/l DVBL in acetonitrile) and 4 ml of diethyl ether, the tubes were shaken vigorously for 10 min, followed by centrifugation for 5 min at 1500 g. Next, the aqueous phase was frozen in ethanol/solid carbon dioxide and the organic solvent decanted into a clean glass tube. The aqueous layer was thawed and again extracted with 4 ml of diethyl ether. The combined ether fractions were evaporated to dryness under a gentle stream of nitrogen (37°C) and the residue redissolved in 100 µl of acetonitrile. A volume of 80 µl was subjected to chromatography for the analysis of VileE and VileX. To the remaining aqueous phase, volumes of 50 µl of internal standard (2 mg/l DVBL), 2.5 ml of 0.5 M phosphate buffer pH 5.0 and 5 ml of chloroform:2-propanol (95:5, v/v) were added. The tubes were shaken for 10 min followed by centrifugation for 10 min at 3000 g. The aqueous layer was discarded, the organic layer was transferred to a clean tube and dried in a stream of nitrogen (37°C). The residue was redissolved in 100 µl of acetonitrile and an aliquot of 80 µl was subjected to chromatography for the analysis of VileA.

Tissue, urine and faeces: Quantities of 200 or 500 µl of tissue homogenate were made up to 500 µl with blank human plasma. Extractions of urine samples (50 µl) or faeces suspension (100 µl) were performed after addition of 500 µl of blank human plasma. A volume of 50 µl of internal standard (2 mg/l DVBL in acetonitrile) was used. Sample extraction for the analysis of VileE and VileX was carried out with 4 ml of diethyl ether. For VileA, extraction was carried out with 5 ml of a mixture of chloroform:2-propanol (95:5, v/v) after the addition of 2.5 ml of 0.5 M phosphate buffer pH 5.0 to the sample. The tubes were shaken for 10 min and then centrifuged (10 min, 3000 *g*, 4°C). The organic layers were separated and evaporated to dryness under a gentle stream of nitrogen (37°C). The residues were dissolved in 100 µl of acetonitrile by sonication for 5 min, and aliquots of 80 µl were subjected to chromatography.

High-performance liquid chromatography. The HPLC system consisted of a Spectroflow SF400 pump, a Spectroflow 980 fluorimetric detector, a model 1000S UV-photodiode array (PDA) detector (Kratos, Ramsey, N.J.) and a Model 360 autosampler (Kontron, Basle, Switzerland). The chromatographic analyses were performed with a stainless steel colum (250 mm long × 2 mm i.d.) packed with 5 µm Spherisorb Si material, (Phase Separations, Queensferry, U.K.). The mobile phase comprised a mixture of acetonitrile and 10 mM citrate buffer (pH 3.0) (85/15, v/v) and contained 10 mM tetrabutylammonium bromide. Elution was carried out at ambient temperature with a flow rate of 0.2 ml/min. The excitation monochromator was set at 270 nm, while emission was monitored using a 320 nm long-pass filter. Integration was done using a WINner/286 data station (Spectra Physics, San Jose, Calif.).

Calibration. Calibration curves constructed in blank human plasma in the range of 5–500 ng/ml were used for plasma, urine and tissue samples. The concentrations in faeces homogenates were read from a separate calibration curve, also constructed in plasma but with the addition of 100 µl of blank faeces homogenate to each calibration sample. The ratio of peak areas of VileE, VileX, VileA and internal standard was used for quantitation purposes.

Validation. The accuracy and precision of the method and the stability of the compounds in the various tissue samples were determined in blank samples spiked with VileE and VileA. These specimens were analysed during three separate runs with repeated freezing and thawing of the samples.
Blank faeces homogenate, urine and tissue homogenate (a mixture of spleen, liver, kidneys, lungs, intestines and testes) were spiked with VileE, VileA and VileX, and were assayed with all runs, serving as control samples.

Pharmacokinetic data analyses. Pharmacokinetic parameters were calculated by nonlinear regression analysis using PCNONLIN. The plasma concentration-versus-time curve for VileE after i.v. bolus administration was fitted in a three-compartment model:
$C_t = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + C \cdot e^{-\gamma t}$ with weighing proportional to $1/C_t^2$.
The half-lives were calculated from:
$t^1/_2(\alpha) = \ln 2/\alpha$, $t^1/_2(\beta) = \ln 2/\beta$, $t^1/_2(\gamma) = \ln 2/\gamma$
The area under the curve (AUC) from t = 0 to infinity was calculated as:
$AUC = A/\alpha + B/\beta + C/\gamma$
The plasma clearance:
Cl = Dose / AUC
and the volume of distribution:
V_d = Dose /(AUC·γ)
were calculated using classic pharmacokinetic equations.
The linear trapezoidal rule with extrapolation to infinity was applied for calculating the plasma $AUC_{(0-\infty)}$ of VileE after oral drug administration. The relative bioavailability (F) was calculated from:
$F = AUC_{(oral)}/AUC_{(IV)} \cdot 100\%$.
The linear trapezoidal rule was also used for calculating the plasma $AUC_{(0-48\,h)}$ of VileX and VileA after both i.v. and p.o. administration.

Isolation of VileX. VileX was isolated from the faeces of mice. Faeces portions produced between 0 and 24 h after oral administration of 10.5 mg/kg VileE were collected. The combined faeces amounted to approximately 50 g. A total of 18 portions of about 3 g each were homogenized in 10 ml of 0.5 M phosphate buffer, pH 5.0. First, the homogenates were shaken three times with *n*-hexane to remove fat and promote the homogeneity of the specimen. Subsequently, the aqueous phase was extracted three times with 20 ml of diethyl ether. All ether fractions, containing both VileE and VileX, were combined and evaporated under a gentle stream of nitrogen. The residue was dissolved in 1 ml of chloroform followed by dropwise addition of 3 ml of acetonitrile. After centrifugation (3 min, 1000 *g*) the precipitate was redissolved in chloroform and the process of precipitation and dissolution was repeated 5 times. Aliquots of 500 µl of the combined supernatant solutions were subjected to semipreparative HPLC. The HPLC conditions were similar to those used for analytical HPLC.

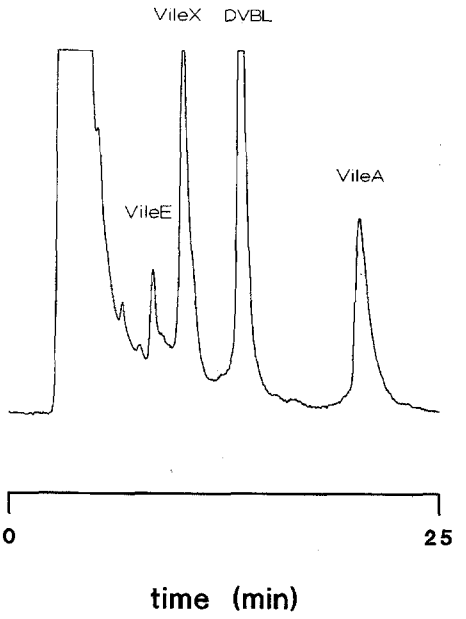

Fig. 2. Chromatogram of a faeces specimen collected 0–24 h after drug administration

Instead of the minibore 2 mm (i.d.) column a conventional 4.6 mm (i.d.) column and a flow rate of 1 ml/min was used. The eluate fractions containing VileX were combined, frozen in liquid nitrogen (–196°C) and lyophilized. After dissolution in 10 ml of 50 mM phosphate buffer pH 7.0, the solution was extracted three times with 10 ml of diethyl ether. The combined ether fractions were dried, redissolved in acetonitrile:water (50:50, v/v) and again subjected to semi-preparative HPLC on 5 µm Hypersil ODS with a mobile phase composed of acetonitrile-50 mM phosphate buffer pH 7.0 (60:40, v/v). The combined and lyophilized eluate fractions were redissolved in ether and washed twice with water to remove buffer salts. The ether was divided into four portions, dried under nitrogen (37°C) and kept at –20°C.

Mass spectrometry. Positive ion spectra were obtained with a JMS-SX/SX102A tandem mass spectrometer (BEBE; JEOL, Tokyo, Japan) operating at an accelerating voltage of 10 kV. A xenon beam with an energy of about 6 keV was applied for obtaining fast atom bombardment (FAB) spectra. In all experiments, glycerol was used as the matrix. MS-MS spectra were recorded at 50% precursor ion reduction, with helium as the collision gas.

Fourier transform infrared (FT-IR) spectrometry. The FT-IR spectra were recorded with a Perkin Elmer Model 1720 (Perkin Elmer Corp., Norwalk, Conn.) equipped with a deuterated triglycine sulphate (DTGS) detector and via an IR microscope with a mercury cadmium telluride (MCT) detector in the external beam. Transmission spectra of the pure solids were obtained from the samples deposited from $CHCl_3$ solution on a ZnSe window and were measured via microspectrometry with a Perkin-Elmer microscope. Solutions of the compounds in CCl_4 were measured in 2 mm CaF_2 liquid cells. The resolution was 4 cm^{-1} and the number of scans was between 100 and 1000, apodization normal. Data manipulation was carried out on a Perkin-Elmer Model PE 3600 data station running the program M1700.

Cell culture. L1210 murine leukemia cells seeded at 20000 and 200000 cells/ml were grown at 37°C in 5% CO_2-95% air in RPMI1640 medium containing L-glutamine (Life Technologies, Paisley, Scotland), 10% (v/v) fetal calf serum (Sebak, Aidenach, Germany), penicillin, streptomycin and β-mercaptoethanol. Cells were exposed continuously to the drugs during the entire test period (3 days).

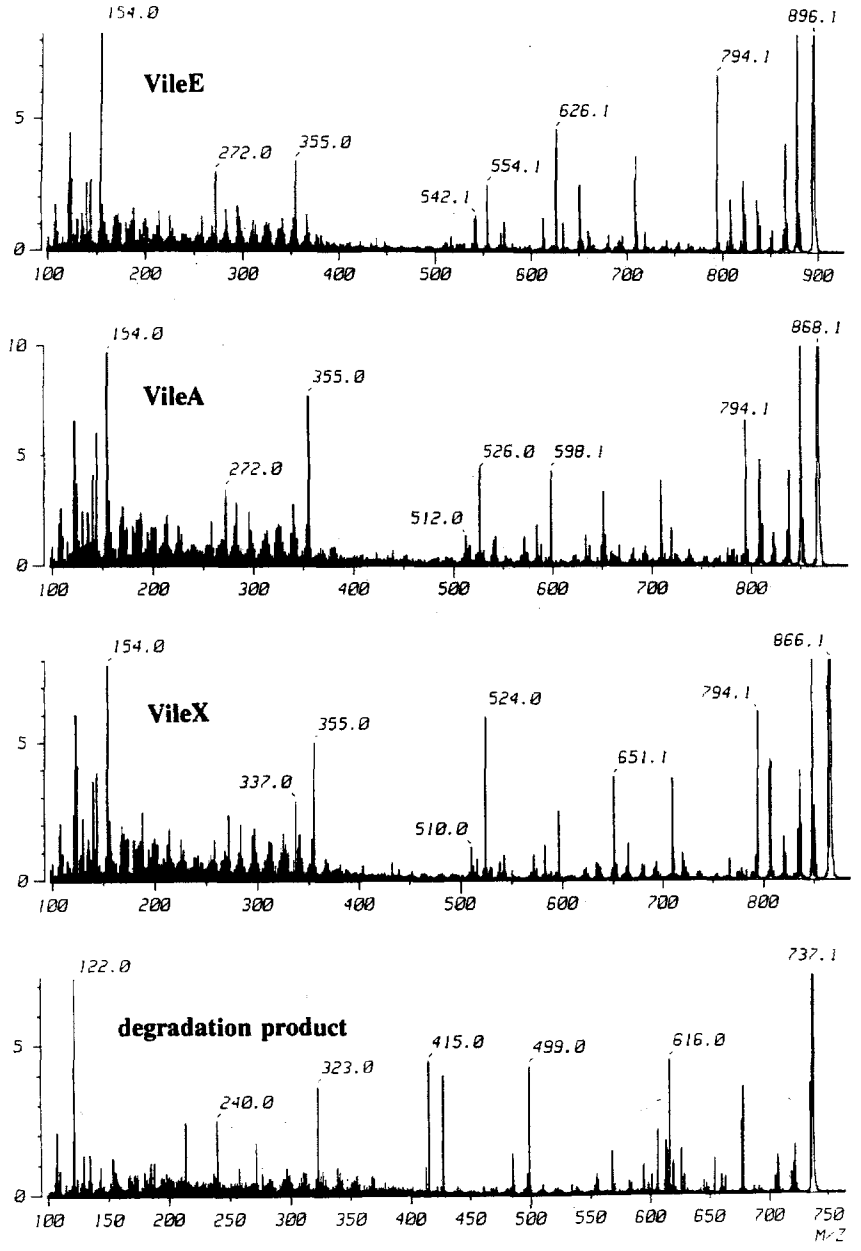

Fig. 3. CID/MS-MS spectra of VileE, VileA, VileX and its degradation product

The drug concentrations tested were 1.6, 6.3, 25 and 100 ng/ml. The 20000 cells/ml cultures were used if the corresponding 200000 cells/ml culture entered plateau phase growth (2000000 cells/ml).

Results

Identification of VileX

In addition to the peaks that co-eluted with VileE and VileA reference compounds, a third component (VileX) was detected with approximately the same capacity factor as VBL in the HPLC system used (Fig. 2). This compound was isolated from the faeces. The purity of the isolated product, judged from the chromatographic trace obtained with UV photodiode array (PDA) and fluorescence detection was over 98%. FAB-MS of VileX showed the protonated molecular ion at m/z 866, whereas this ion occurs at m/z 896 for VileE and at m/z 868 for VileA. The collision-induced dissociation (CID)/MS-MS spectra of the protonated molecules of VileE, VileA and VileX show similar fragmentation patterns (Fig. 3, Table 1). The infrared spectrum of VileX (Fig. 4) shows a carbonyl band at 1786 cm^{-1} (in CCl_4 solution; 1778 cm^{-1} in the reflection spectrum), which is absent from the spectra of both VileE and VileA. VileX appeared to be unstable when stored in acetonitrile at –20° C. A degradation product was formed with a capacity factor 1.2 times that of VileX in the HPLC system. The FAB-MS spectrum showed a protonated molecular ion peak at m/z 737; its CID/MS-MS spectrum differs markedly from those of VileE, VileA and VileX. Its UV spectrum is also different from the spectra of the other compounds (Fig. 5) and its fluorescence intensity has diminished fourfold. In the IR spectrum the carbonyl band at 1786 cm^{-1}

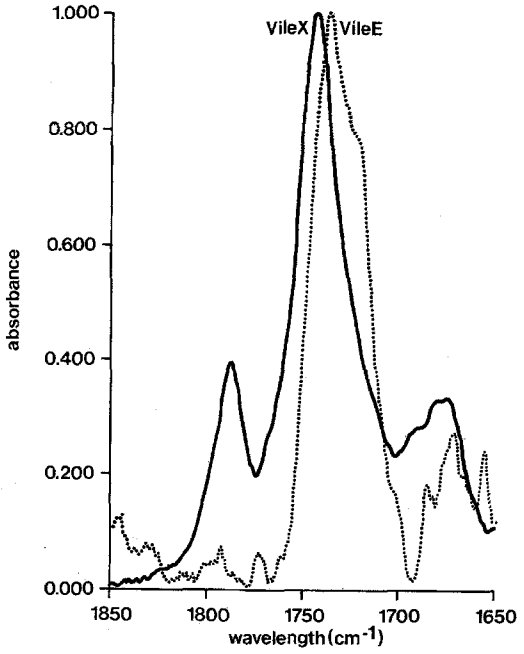

Fig. 4. Infrared spectra (carbonyl region) of VileE and VileX

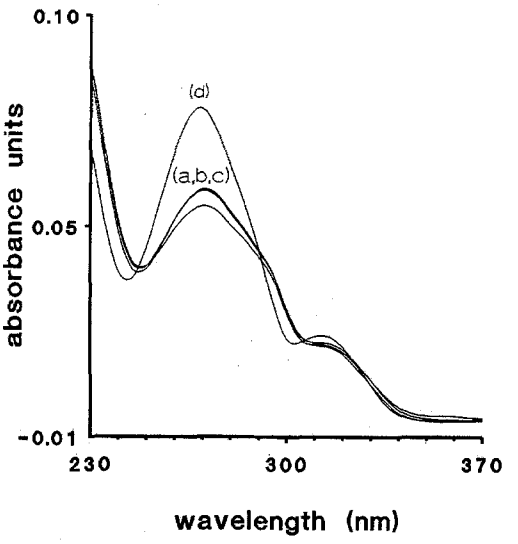

Fig. 5. UV spectra of VileE (*a*), VileA (*b*), VileX (*c*) and its degradation product (*d*)

Table 1. Concise list of CID/MS-MS fragments in vinleucinol (VileE), vinleucinol acid (VileA) and a second metabolite of VileE, VileX, and their possible origin

Fragments (m/z) found in:			
VileE	VileA	VileX	Possible identity
896	868	866	$[M + H]^+$
878	850	848	$[M + H]^+ - H_2O$
866	838	836	$[M + H]^+ - CH_2O$
864	836	834	$[M + H]^+ - CH_3OH$
794	794	794	[CATH + VIN + NH(-CH)-C = O + H]$^+$
709	709	709	[CATH + VIN]$^+$ – (C = O)-R2 after cleavage of C_3-C_{23}
626	598	596	[VIN + C(-CH_2)-$COOCH_3$ + R2 + H]$^+$ after cleavage C_{18},-C_{17}, and C_1,-C_2,
612	584	582	[VIN + C-$COOCH_3$ + R2 + H]$^+$ after cleavage C_{18},-C_{17}, and C_{18},-C_1,
540	512	510	[VIN + R2]$^+$ after cleavage C_{15}-C_{18},
355	355	355	[CATH]$^+$ after cleavage C_{15}-C_{18},

CATH, catharantine part; VIN, vindoline part
For enumerations of the C-atoms and R2 substituent see Fig. 1

Table 2. Accuracy and precision of the assay in various biological matrices

Matrix	Spiked concentration (ng/ml)	VileE		VileX		VileA	
		Accuracy	RSD	Accuracy	RSD	Accuracy	RSD
Brains	50	141.0%	2.3%			62.7%	21.2%
Muscle	50	110.2%	2.6%			81.8%	16.7%
Eolon	50	96.2%	4.6%			103.8%	8.2%
Appendix	50	98.0%	10.8%			115.0%	7.2%
Large intestine	50	98.6%	0.3%			119.6%	8.7%
Stomach	50	74.0%	15.8%			93.4%	12.4%
Liver	50	106.4%	3.0%			111.2%	14.4%
Kidneys	50	103.6%	7.1%			114.4%	16.1%
Spleen	50	103.2%	6.4%			89.0%	13.2%
Testis	50	101.8%	1.2%			86.4%	12.0%
Tissue	50	87.1%	7.9%	101.4%	5.2%	91.3%	7.6%
	200	89.4%	3.5%	99.3%	3.1%	104.2%	1.7%
Urine	1000	89.8%	6.5%	104.9%	5.8%	104.1%	2.4%
Faeces	1000	98.9%	8.3%	96.4%	7.5%	93.4%	7.4%

RSD, relative standard deviation

has disappeared. Both VileE and VileA are stable in acetonitrile at –20° C. Degradation proceeded to a far lesser degree when VileX was stored in plasma at –20° C, with only about 5% formed over a period of 8 months. The degradation product was not found in any of the in vivo samples.

Analytical procedures

The UV spectra of VileE, VileA and VileX are virtually superimposable (Fig. 5), allowing us to calculate the purity and concentration of reference standard solutions of the compounds from peak areas of the HPLC-UV chromatograms by using VileE as a reference. VileE itself was found to be over 99% pure, containing only a minor amount (0.5%) of VileA as an impurity. VileA, as supplied by Medgenix, was only 55% pure, but judged from the HPLC trace these impurities were not vinca alkaloid-related compounds. In addition to the VileA peak, only a small second peak coeluting with VileE (2.2%) was found. The purity of VileX was over 98%.

There were marked differences between VileE, VileX and VileA in their extraction behaviour. As we were unable to find a universal extraction solvent, it was decided to extract VileA separately from VileE and the metabolite VileX. Owing to the small volumes of the plasma samples available, the same specimen was used for the consecutive extraction of VileE plus VileX, and VileA.

A standard solution containing 1000 ng/ml of all three compounds were prepared by dilution of the reference standard solution in blank human plasma. Aliquots were

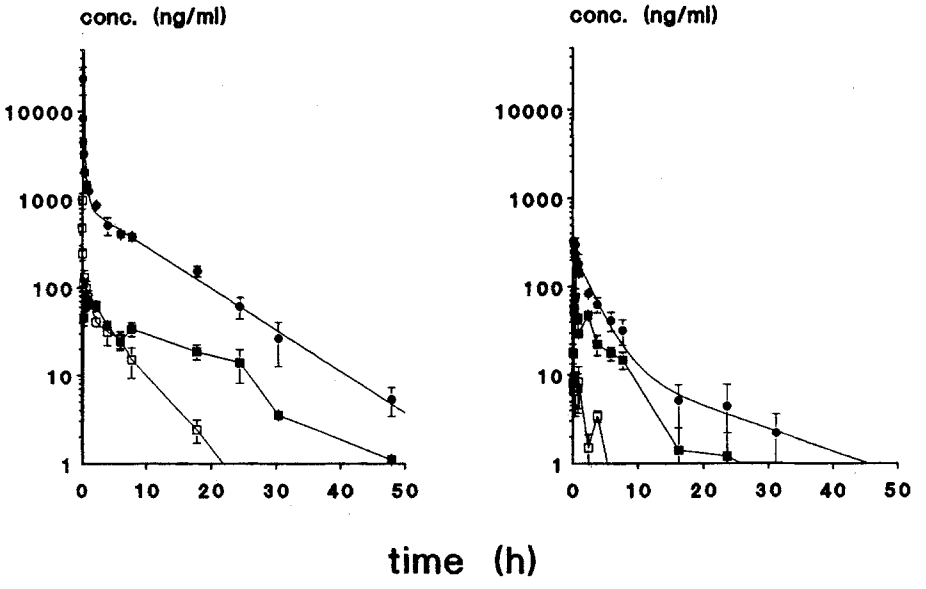

Fig. 6. Plasma concentration-versus-time curves for VileE (●), VileA (□) and VileX (■) after i.v. (*left*) or oral (*right*) administration of VileE. (*Error bars*, SD)

Table 3. Plasma pharmacokinetic parameters of VileE and metabolites

		Administration i.v.	Administration p.o.	
VileE	$t^1/_2(\alpha)$	0.0080 ± 0.0020		h
	$t^1/_2(\beta)$	0.297 ± 0.064		h
	$t^1/_2(\gamma)$	6.38 ± 0.22		h
	$AUC_{(0-\infty)}$	10.4 ± 0.5	0.87	mg/l×h
	Cl	1.01 ± 0.05		l/h per kg
	V_d	9.32 ± 0.75		l/kg
	F		8.4	%
VileX	$AUC_{(0-48h)}$	0.79	0.30	mg/l×h
VileA	$AUC_{(0-48h)}$	0.47	0.021	mg/l×h

Table 4. Tissue concentration of VileE given as mean concentration (ng/g) ± SD. Percentages in parenthesis show the fraction of drug remaining relative to the 4 h specimen

	4 h	24 h		48 h	
Brains	107 ± 44	129 ± 70	(121%)	39 ± 9	(36%)
Muscle	1340 ± 170	205 ± 41	(15%)	23 ± 17	(2%)
Abdominal fat	3070 ± 580	1350 ± 220	(44%)	360 ± 139	(12%)
Colon	3390 ± 500	311 ± 41	(9%)	84 ± 15	(2%)
Appendix	4840 ± 1850	291 ± 90	(6%)	84 ± 38	(2%)
Large intestine	3660 ± 730	417 ± 128	(11%)	121 ± 27	(3%)
Stomach	3810 ± 710	733 ± 24	(19%)	301 ± 46	(8%)
Liver	11360 ± 940	703 ± 58	(6%)	99 ± 7	(0.9%)
Gall bladder	4060 ± 1540	ND		ND	
Kideny	13250 ± 840	887 ± 50	(7%)	227 ± 52	(2%)
Lung	10280 ± 2130	1740 ± 66	(17%)	159 ± 24	(2%)
Spleen	11090 ± 1980	1580 ± 50	(14%)	434 ± 64	(4%)
Heart	1640 ± 170	142 ± 21	(9%)	36 ± 6	(2%)
Testis	800 ± 70	1050 ± 94	(131%)	905 ± 14	(113%)
Epididymis	2960 ± 390	1740 ± 330	(59%)	884 ± 113	(30%)
Thymus	6000 ± 380	3170 ± 420	(53%)	1600 ± 330	(27%)
Lymph nodes	9410 ± 770	1900 ± 360	(20%)	167 ± 51	(2%)
Plasma	504 ± 115	67 ± 7	(13%)	7 ± 2	(1.4%)

ND, not detectable

stored at –20° C and these stock solutions were used for the daily construction of a calibration curve in blank human plasma. The concentrations in the other biological matrices could also be determined with acceptable accuracy using the calibration curves constructed in plasma (Table 2).

The compounds were stable in plasma, urine and in BSA-homogenized faeces samples upon repeated freezing and thawing. In tissue samples homogenized in BSA, however, the concentration of the compounds decreased with repeated freezing and thawing. This occurred rapidly in stomach tissue, where only 10% of the initial drug concentration was recovered after one cycle of freezing and thawing. Except for the stomach, where 74% of the spiked concentration was recovered after the third time of freezing and thawing of the sample, this phenomenon did not occur when tissues were homogenized in blank human plasma.

Plasma pharmacokinetics

The plasma concentration versus time curves of VileE, metabolite and VileA after IV and oral administration are depicted in Fig. 6 and the calculated pharmacokinetic parameters are tabulated in Table 3. The bioavailability of VileE after oral administration is low (8.4%). The large differences in plasma concentrations found between samples obtained at the same time points indicate substantial mouse-to-mouse differences in drug uptake. These large differences mean that computerized fitting of the plasma concentration-versus-time curve was not accurate. The AUC of VileA after p.o. drug administration was only about 5% of the AUC after i.v. administration, whereas the AUC of VileX was approximately 40%.

Tissue kinetics

The tissue concentrations of VileE and metabolites found at serial time points are tabulated in Tables 4–6. Extensive distribution of both VileE and VileX is noticed in all tissues. Prolonged retention of VileE is observed mainly in

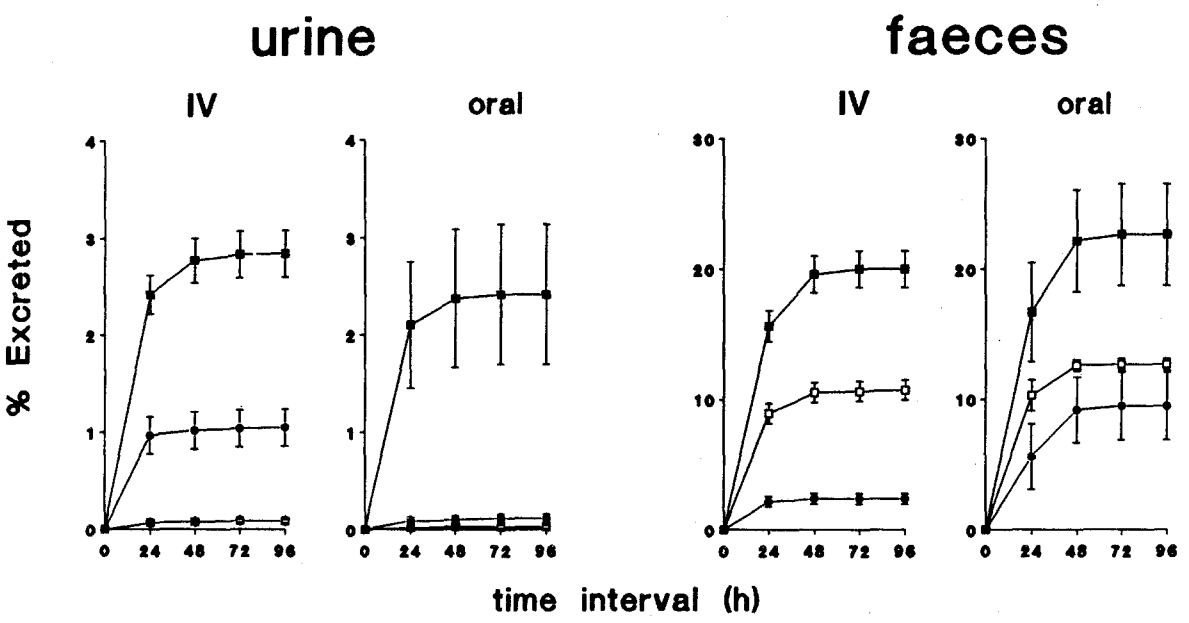

Fig. 7. Cumulative excretion in urine faeces of VileE and metabolites as percentage of the administered dose of VileE after IV and oral administration. VileE (●), VileA (□) and VileX (■). (*Error bars*, SD)

Table 5. Tissue concentration of VileX given as mean concentration (ng/g) ± SD. Percentages in parenthesis give the fraction of drug remaining relative to the 4 h specimen

	4 h	24 h		48 h	
Brains	ND	27±20		ND	
Muscle	259±42	100±7	(39%)	41±6	(16%)
Abdominal fat	200±7	100±17	(50%)	63±40	(32%)
Colon	1670±520	495±83	(30%)	234±93	(14%)
Appendix	440±220	950±121	(216%)	388±155	(41%)
Large intestine	1990±410	610±110	(31%)	271±27	(14%)
Stomach	1240±140	630±95	(51%)	473±46	(38%)
Liver	4140±160	930±200	(22%)	224±27	(5%)
Gall bladder	13180±3390	1760±510	(13%)	970±370	(7%)
Kidney	2950±250	560±100	(19%)	360±30	(12%)
Lung	1590±280	321±61	(20%)	120±19	(8%)
Spleen	3050±500	1575±224	(52%)	850±240	(28%)
Heart	526±101	220±10	(42%)	74±6	(14%)
Testis	62±9	155±10	(250%)	149±16	(240%)
Epididymis	370±30	381±62	(103%)	256±82	(69%)
Thymus	516±30	870±140	(169%)	626±183	(121%)
Lymph nodes	1060±120	830±135	(78%)	193±36	(18%)
Plasma	37±5	11±2	(30%)	2±1	(5%)

ND, not detectable

Table 6. Tissue concentration of VileA given as mean concentration (ng/g) ± SD

	t = 4 h	t = 24 h	t = 48 h
Colon	1060±540	189±83	53±93
Appendix	4651±2270	364±121	139±155
Large intestine	352±120	113±110	53±27
Stomach	122±62	20±95	ND
Liver	60±11	ND	ND
Gall bladder	10300±3880	440±510	ND
Kidney	38±2	ND	ND
Lung	33±2	ND	ND
Lymph nodes	51±1	ND	ND
Plasma	30±3	ND	ND

ND, not detectable

testicular tissue, whereas in most other tissues the concentration of VileE declines in parallel with the plasma levels. Compared with VileE, the metabolite VileX also appears to be retained longer in a number of other tissues. VileA is only found in considerable concentrations in tissues of the gastrointestinal tract and the gall bladder.

Excretion

VileE and metabolites are excreted predominantly in the faeces (Fig. 7). The excretion of unchanged drug following i.v. or p.o. administration is minimal. Although the amount of metabolite excreted in urine after oral administration varies considerably, it is almost the same as the amount excreted after i.v. administration. The total amount of drug recovered as VileE, VileX or VileA in a period of 96 h is only 36.9% and 47.4% of the administered dose after i.v. and p.o. administration, respectively.

Cell culture

The experiment with cultured L1210 cells reveals differences between the tested compounds in their potency for inhibiting cell growth (Fig. 8). In this cell line, VileA possesses no growth inhibitory properties. VileE is clearly a less potent growth-inhibiting vinca derivative than VBL. VileX was found to be as potent as VBL, whereas its in vitro degradation product retained cytotoxic properties.

Discussion

The naturally occurring vinca alkaloids VBL and VCR have been used as antineoplastic agents for over three decades. Although these compounds are successfully used

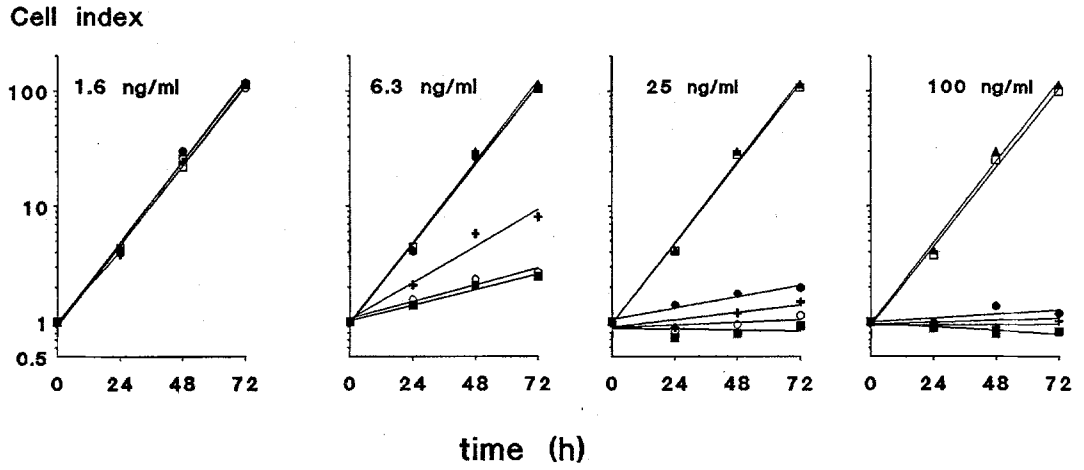

Fig. 8. Growth inhibition of L1210 cells by vinca alkaloids. L1210 cells were grown under continuous exposure to various drug concentrations. The cell index is defined as the ratio of the number of cells present during the test period relative to the number present at the beginning. ○ VBL; ● VileE; □ VileA; ■ VileX; ◆ degradation product; ▲ control (no drug)

in a number of potentially curative treatment modalities, their therapeutic use is limited to a number of relatively rare malignancies (e.g. lymphomas and testicular cancer). In the 1960s, the considerable influence of minor alterations of the molecular structure on the cytotoxic activity was recognized, and this observation has stimulated the development of a large number of semi-synthetic derivatives with the aim of optimizing or extending the therapeutic usefulness [18].

Bhushana Rao et al. reported on the synthesis and preclinical activity of a series of deacetylvinblastine-23-oyl amino acid derivatives [1]. It was assumed that the coupling of an amino acid to vinca alkaloids might facilitate the cellular uptake by a change of the selectivity of the drug by modifying the overall polarity or by increasing the affinity of the drug to membrane-associated transport systems for amino acids. Compared with the other compounds in these series, both the L-ethyl tryptophan ester (VtrpE) and the L-ethyl isoleucine ester (VileE) of DVBL demonstrated promising antitumour activities in pre-clinical studies [1]. VtrpE has been tested in clinical trials, but unfortunately the phase II trial has shown that this compound lacks clinical efficacy against any of the solid tumours tested [15]. Although these disappointing results are a restraining factor for further clinical testing of VileE, a recent study on human tumour xenografts demonstrated superior activity of VileE over both VBL and VtrpE against several solid tumour cell lines [6].

The present comprehensive pharmacokinetic study was undertaken to gain a better insight into the disposition and metabolic fate of VileE. In a previous report we demonstrated the importance of tissue distribution studies with new semi-synthetic derivatives to identify potentially sensitive tissue types [19]. Since vinca alkaloids are extensively metabolized, the use of a selective analytical method is mandatory. HPLC, in particular ion-exchange HPLC on unmodified silica in combination with fluorescence detection, has proven to be powerful tool for the analysis of this type of compounds [16].

On the basis of previous investigations with VtrpE, the metabolic formation of VileA was expected [17]. Apart from VileA, however, the chromatograms revealed the presence of another metabolite (VileX), which appeared to be present in substantial amounts in all biological samples. Since it was not possible to synthesize VileX in in vitro conditions in liver homogenate and because relatively large amounts were excreted in the faeces after oral administration of the drug, it was decided to isolate the compound from the faeces. The chromatographic behaviour, the UV spectrum, the fluorescence properties, and the cytotoxic activity led us to the assumption that the basic catharantine-vindoline structure of the molecule has remained intact. This is confirmed by the fragment peaks found by CID/MS-MS. All three spectra show a peak at m/z 355 (the catharatine moiety), m/z 651 ($M+H^+$ from which C_3-C_4R1-$C_{23}R2$ has been removed [11]), m/z 709 (arising from cleavage of C_3-C_{23}) and m/z 794. The even mass ion at m/z 794 suggests that the nitrogen of the amino acid is still present in this fragment. VileE shows peaks at m/z 540, 612 and 626, which occur at m/z 512, 584, 598 for VileA and m/z 510, 582, 596 for VileX. The fragments at m/z = 355 and 709 found in VileE, VileA as well as in VileX and the series of fragments, which show a typical difference in mass relative to VileE (VileA m/z-28 and VileX m/z-30), strongly indicate that no changes have occurred in the basic structure and, therefore, the changes must be in the amino acid moiety. The difference between the capacity factors of VileX and VileA indicates the absence of a polar free carboxylic acid group in VileX. Furthermore, an ethyl ester moiety in VileX, as in VileE, is not likely as this metabolic unstable function would almost certainly lead to the formation of an acid, by analogy with the formation of VileA from VileE, as a secondary metabolite. Such a compound has not been detected. Indications for the presence of a lactone ring in VileX have been obtained from the IR spectrum. In addition to C = O bands at 1740 ($COOCH_3$) and 1667 cm^{-1}, the IR spectrum of VileX (in CCl_4) shows a C = O band at 1786 cm^{-1}. This frequency is high for a five-

membered ring (γ-lactone) and low for a four-membered ring (β-lactone). However, structural effects, such as intramolecular hydrogen bonding and inductive or field effects, may influence the band maximum of the C = O stretching vibration. In VileX, as in VileE, the C = O has an α-NH group which could shift the C = O band to a lower frequency. VileE shows C = O bands at 1739 cm^{-1} ($COOCH_3$), 1729 cm^{-1} ($COOC_2H_5$), and 1669 cm^{-1} cm (CONH). In aliphatic esters the C = O band lies around 1745 cm^{-1}. This indicates that the αNH group lowers the C = O frequency with at least 15 cm^{-1} (possibly a combined effect of hydrogen bonding and inductive field effects). Extrapolating this to VileX, the C = O frequency (without an -NH group) would be at about 1800 cm^{-1}, suggesting that VileX is a β-lactone. The observations and the MS and IR data are in agreement with the structure proposed for VileX in Fig. 1. This product may be formed after oxidation of the β- (or γ-) carbon atom of the amino acid, followed by hydrolysis of the ethyl ester and simultaneous ring closure.

The UV spectrum, the fluorescence properties and the CID/MS-MS spectrum of the degradation product of VileX are different from those of the parent compounds, indicating that changes have taken place in the vinca skeleton. The odd value for the protonated molecule indicates that the degradation product has lost a nitrogen atom, probably in the amino acid residue. Surprisingly, the degradation product is still active against L1210 cells. Its structure has not yet been established, but its absence in any of the in vivo samples indicates that it is not a biologically relevant metabolite. The ability of VileX to inhibit cell division in vitro is similar to VBL, whereas the parent drug VileE is only approximately a quarter as active as VBL. Furthermore, it is shown that both VileE and VileX are distributed extensively to most tissues. Prolonged retention of VileE is observed mainly in the testis, whereas VileX is also retained in a number of other tissues. The qualitative pattern of retention of vileX resembles that of DVBL in mice [19]. These observations indicate that the metabolite VileX may be at least largely responsible for the observed antitumour activity against human xenografts in mice after administration of VileE. We observed that the LD_{10} of VileE in mice after i.v. administration is almost equal to that after oral administration. However, the bioavailability after oral administration is minimal. Despite large variations in the concentrations found after oral administration, the plasma concentrations and, in particular, the urinary excretion of VileX are almost the same as those found after i.v. administration. This indicates that the low bioavailability may be caused by a high first-pass effect rather than by limited uptake from the gastrointestinal tract.

VileA appears to play no role in the toxicity and/or activity of the drug. Except for the tissues of the intestinal tract and the gall bladder, VileA is present in low or non-detectable concentrations in all other tissues, including the liver.

VileE and metabolites are excreted predominantly in the faeces, as observed for other vinca alkaloids. The total recovery of the drug and its detected metabolites is far from quantitative. This was also found for VBL and is caused by the occurrence of other very polar – and presumably inactive – metabolites [19].

In conclusion, the (cyto)toxic action of VileE in mice appears to be effected – at least to a substantial degree – by an active metabolite. The exact structure (β- or γ-lactone) could not be established with certainty, but the results of spectroscopic analysis are indicative for the β-lactone. The decision to start a clinical trial with VileE should be preceded by the elucidation of the exact structure of the metabolite, followed by its synthesis and by toxicological studies. Pharmacokinetic studies in other animals are necessary to find out whether conversion to VileX is a species-related phenomenon.

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