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MODULATION BY VERAPAMIL OF VINCRISTINE PHARMACOKINETICS AND SENSITIVITY TO METAPHASE ARREST OF THE NORMAL RAT COLON IN ORGAN CULTURE

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Abstract—The *in-vitro* pharmacokinetics of vincristine (VCR) in normal rat colonic mucosa were studied. Two complementary approaches were adopted using an explant organ-culture system. Firstly [G-^{3}H]vincristine (^{3}HVCR) accumulation, retention and efflux were characterized under basal conditions and compared with measurements made either under energy-depleted conditions, or in the presence of VRP. Secondly, a histological method—the postmetaphase index (PMI)—was used to compare the sensitivity of explants to VCR in the presence or absence of verapamil (VRP). This latter technique involves the measurement, by counting, of the proportion of mitotic figures escaping from metaphase arrest. The studies yielded the following results: ^{3}HVCR accumulation in colonic mucosa showed no evidence of saturability up to the maximum dose studied (130 nM), at a dose of 52 nM accumulation was enhanced in energy-depleted conditions by a factor of 1.8, and in the presence of VRP (6.6 μM) by a factor of 1.4. In the presence of VRP (6.6 μM) retention of ^{3}HVCR was increased by a factor of 1.3 and efflux was reduced by a factor of 0.8 after 2 hr. VRP (6.6 μM) reduced the PMI of colonic mucosal epithelial cells exposed to 11 nM VCR from 18.8% to 11.4% (i.e. 40% reduction) indicating sensitization of the cells to this property of VCR. These results provide evidence that the sensitivity of normal colonic mucosa to vincristine is, at least in part, regulated by drug transport. Qualitatively our observations resemble those described in multidrug resistance. Given that P-glycoprotein has been demonstrated by several groups in colonic mucosal cells, the results support a normal role for this membrane transport molecule in the protection of intestinal cells from plant alkaloids and other xenobiotic agents ingested in the diet.

Increasing attention is being given to the mechanisms regulating the intrinsic drug resistance of many human malignancies. Resistance to the *Vinca* alkaloids, anthracyclines, epipodophyllotoxins and other 'natural product' anticancer drugs may be mediated via the drug-transport mechanism characteristic of the multidrug resistance (MDR§) phenotype, a membrane glycoprotein (P-gp) which is encoded for by the mdr1 gene [1–5]. Alternatively, it may be attributable to alterations in other mechanisms, such as glutathione-*S*-transferase and topoisomerase II activity, described in so-called 'atypical' MDR [6]. Whether studied in cell or organ culture the resistance of colorectal adenocarcinoma cells shows evidence of altered drug transport. In particular, both the accumulation of drug and the cell sensitivity may be modified by VRP [7–11], though other authors were unable to demonstrate VRP-mediated sensitization in primary clonogenic assays of human colorectal adenocarcinomata [12].

Fojo *et al.* [13] showed that expression of the mdr1 gene was present in both normal and neoplastic colonic tissues and P-gp has been demonstrated in colonic tissues directly by immunohistochemistry [14, 15]. In a recent communication Horton *et al.* [16] reported that certain mouse tissues *in vivo* (small intestine, liver and kidney) showed increased uptake and retention of VCR in the presence of VRP. All these tissues are known to express P-gp, and the data presented support the novel hypothesis that the function of this protein in normal tissues involves the elimination of toxic alkaloids and xenobiotics. In the present study we have explored the possibility that sensitivity to VCR in normal colonic mucosa might be regulated in a manner consistent with such a role for P-gp in this tissue. We have developed a method enabling the measurement of both the accumulation and efflux of ^{3}HVCR in explants of colonic mucosa maintained in organ culture. In addition we have used a histological index of sensitivity to VCR, the PMI [17], to study the effect of VRP in normal rat colonic epithelium. Our results support a role for drug-transport in the regulation of the sensitivity of this normal tissue to VCR. Both transport and sensitivity to the drug are modified by VRP.

MATERIALS AND METHODS

Chemicals. Verapamil was kindly donated by

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§ Abbreviations: MDR, multidrug resistance; ^{3}HVCR, [G-^{3}H]vincristine; P-gp, P-glycoprotein; SW, supplemented Waymouths MB752/1 medium (see Materials and Methods); FBS, fetal bovine serum; PMI, postmetaphase index; VCR, vincristine; VRP, verapamil; DAPI, diamidinophenylindole.

Abbott Laboratories (Queensborough, Kent, U.K.). ^{3}HVCR (sp. act. 178 GBq/mmol) was purchased from Amersham (Aylesbury, Bucks, U.K.). Non-radiolabelled VCR (vincristine injection) was purchased from Lederle Laboratories (Gosport, Hants, U.K.). Waymouths MB752/1 medium and Dulbecco's phosphate-buffered saline were purchased from Gibco (Paisley, U.K.), FBS from Northumbria Biologicals (Cramlington, Northumberland, U.K.).

Animals and organ culture. Colonic mucosa was obtained from adult male Wistar rats (University of Newcastle upon Tyne). The animals were aged between 11 and 30 weeks, and weighed between 250 and 500 g. They had been fed on a uniform diet (Breeders Diet No 3, Special Diet Services Ltd, Witham, Essex, U.K.), with unlimited access to tap water. These animals were not known to have been exposed to any xenobiotic agent or plant alkaloid.

The animals were killed by cervical dislocation. Explants of colonic mucosa, measuring approximately 2 mm^2 in surface area, were prepared and placed into an organ culture system as described previously [18, 19]. The colon was opened longitudinally following resection and the lumenal contents gently removed. The colon was then placed into a sterile container with 20 mL of chilled culture medium. After further gentle agitation to remove adherent faeces the specimen was transported to the laboratory on melting ice. In the laboratory the mucosa was stripped from the underlying muscularis propria using fine jewellers forceps. The mucosa used for explantation originated from a region of colon measuring 100 mm in length and situated 40 mm proximal to the anus. The mucosa was then placed, lumenal surface uppermost, onto sterile cork board and was kept moistened with chilled culture medium throughout. Using a dermatome blade portions of mucosa were cut and transferred to cellulose acetate filters (Millipore) as described below. They were plated out with the lumenal surface uppermost. Histologically the explants are composed of the epithelium of the surface and colonic crypts, the lamina propria, the muscularis mucosa and a minimal and variable amount of vascular collagenous submucosal tissue. The explants were cultured, at the gas/liquid interface, in a controlled atmosphere chamber containing a mixture of 95% oxygen and 5% carbon dioxide. The cultures were maintained on a rocking platform to optimize the diffusion of gas and solutes. A supplemented Waymouths MB752/1 medium (SW) was used containing: 10% FBS, 300 μg/mL ascorbic acid, 0.45 μg/mL ferrous sulphate and 3 μg/mL hydrocortisone. Penicillin, streptomycin and mycostatin were added at 100 units/mL for each drug.

Drug transport experiments. The accumulation (uptake and retention) and efflux of ^{3}HVCR from mucosal explants were measured by modification of methods used to study cell lines in suspension culture [7, 20] and tumour xenografts *in vivo* [16]. The use of mucosal explants of an intact tissue raises several problems: (1) the procedures necessary for the physical manipulation of explants during sampling and washing preclude the use of short (i.e. seconds-long) sampling intervals; (2) drug accumulation is usually expressed in terms of accumulated drug per 10^6 cells. The number of cells present in an explant of intestinal mucosa is impossible to measure directly and we used a DNA assay to arrive at an estimate of the amount of cellular tissue in each sample; and (3) incubation of intestinal mucosa, comprising in part acellular stromal tissue, is likely to be associated with greater non-specific adsorbance of drug. We attempted to estimate this component by performing a rapid incubation for 5 min in ice-cold conditions. A mean value was deducted from all the measurements of ^{3}HVCR obtained in the accumulation experiments.

Accumulation of ^{3}HVCR. The explants were cultured in 24-well multiwell plates on 13-mm diameter cellulose acetate filters. Four explants were placed in each well and they were preincubated for 2 hr in SW medium. At each time point duplicate pairs of wells were sampled. The duplicates were analysed separately throughout. The means of these duplicate estimations were used for each time point. Following the 2 hr preincubation period the experiments were begun by changing the culture medium to SW medium containing ^{3}HVCR at the stated dose. At the appropriate time point the samples were washed rapidly in three aliquots of 1 mL of ice-cold PBS. Three washing steps were selected following preliminary experiments showing no change in cpm between the second and third wash effluents, the levels of which were only marginally raised above background cpm (data not shown). After washing, the explants were suspended in 2.5 mL of 0.2 M NaOH solution for overnight solubilization. Aliquots of the supernatant were taken as follows: duplicate samples were taken for scintillation counting and further duplicate samples were taken for estimation of DNA content in a standard spectrofluorimetric assay [21] using DAPI (2.5 μg/mL). The vincristine content of each sample was thus expressed as pg VCR/μg DNA.

Accumulation of ^{3}HVCR in energy-depleted conditions—comparison with normal conditions. For energy-depleted conditions the explants were preincubated in SW medium for 1.5 hr and then the medium was changed to either SW medium or to Dulbecco's phosphate-buffered saline containing sodium azide (15 mM) and 2-deoxyglucose (50 mM). After a further preincubation period of 0.5 hr the experimental period was initiated by changing the media to include ^{3}HVCR in either SW medium or the supplemented Dulbecco's medium, respectively.

Accumulation of ^{3}HVCR in the presence of VRP. Where VRP was used the explants were preincubated for 2 hr in either SW medium (control explants), or in SW medium with 6.6 μM VRP. ^{3}HVCR was added in SW medium either with or without VRP at 6.6 μM.

Retention and efflux of ^{3}HVCR. Culture conditions were modified for drug-efflux experiments in the following ways: greater numbers of explants were pooled in each dish, and the loading dose of VCR was raised to 130 nM. Both modifications increased the total amount of intracellular drug in each dish available for drug transport. Since cellulose acetate filters show significant non-specific adsorption of VCR and significant drug-efflux (in experiments where filters are incubated alone) the explants were

resuspended, after drug loading and washing, into bare plastic culture dishes for efflux measurements. The experimental procedure adopted was as follows: four 60-mm petri dishes (Nunclon, Karmstrup, Denmark) were prepared containing 36 explants each, on 47 mm diameter cellulose acetate filters. These were incubated for 4 hr in SW medium containing 130 nM ^{3}HVCR. The preloaded explants were then washed rapidly, whilst adherent to the filters, in three changes of ice-cold Waymouths MB752/1 medium (50 mL). The wash effluents were retained for scintillation counting. After washing, six explants were retained from each dish for solubilization in 2.5 mL 0.2 M NaOH to measure the initial intracellular VCR content of the tissue. The remaining 30 explants were transferred from the filters to clean petri dishes and suspended in 6 mL of either SW medium (control explants) or SW medium with 6.6 μM VRP (experimental explants). The efflux of drug into the culture medium was measured by sampling 0.2 mL at 5-min intervals over the first 1 hr. Further samples were taken at 1.5 and 2 hr. At the end of the experiment the explants were washed and solubilized in 12.5 mL 0.2 M NaOH to measure the final intracellular VCR concentration. Estimates were then calculated both of VCR-efflux into the medium and of retention of VCR in the explants.

Statistical analysis of drug-transport experiments. Following preliminary experiments to establish experimental protocols, all experiments were performed as triplicate sets consecutively. For each set of three experiments a similar result was obtained between the replications. The effect of each treatment is illustrated using the log ratio of the accumulation, retention or efflux of ^{3}HVCR between treated and control or comparison explants. In order to illustrate the variability encountered between and within experiments for this novel experimental approach the figures show the log ratios for each experimental replication together with a line fitted to the mean values. The absolute level of accumulation is indicated in the tables at either 4 hr (accumulation experiments) or 2 hr (efflux experiments) to allow comparison between the mean values for each experimental set.

The statistical significance of the effects observed can be determined by comparison of the 95 or 99% confidence interval of the mean log ratio with the value of 1 which would be observed if there was no effect. Thus, if the confidence interval does not include 1 then the effect can be regarded as significant at the 95 or 99% level, respectively. These confidence intervals are shown in the tables and were calculated from the fitted lines. In the accumulation experiments the fitted lines are straight and confidence intervals were calculated by the method of least squares. In the efflux experiment the fitted line is curved (for explanation see text) and the confidence interval was calculated from a quadratic fit to the data.

VRP modification of sensivitity to VCR. Four separate experiments were performed, each using colonic mucosa from one animal. Explants were maintained in the organ culture system overnight to allow stabilization of cell proliferative activity [22]. From each animal 10 plates of four explants were prepared. At the start of the experiment five plates were changed to SW medium containing 11 nM VCR. The other five were changed to SW medium including 11 nM VCR and 6.6 μM VRP. After 2.5 hr the explants were fixed simultaneously in buffered formaldehyde solution. They were processed to paraffin wax and histological sections were prepared at 4 μm thickness. Counting was performed after routine H + E staining. The rationale of the PMI technique is described in detail elsewhere [17]. Briefly VCR prevents the formation of the mitotic spindle by inhibiting the polymerization of tubulin and the proportion of cells which are sufficiently unaffected to give rise to normal anaphase and telophase mitotic figures falls rapidly with increasing dose of VCR. At any given dose of VCR this proportion of unarrested mitotic figures is a measure of the overall sensitivity of the tissue to VCR, and reflects the sum of the varying sensitivities of all the individual cells in mitosis in the tissue section. The technique allows quantification of the sensitivity of different tissues (e.g. normal vs neoplastic) to the same dose of VCR, or of the effect of a modifier of sensitivity. The total number of mitotic figures is counted, together with the subtotal of unequivocally normal postmetaphase figures. Stepped sections of the explants were examined microscopically and mitotic figures were identified in the epithelium of the surface or of colonic crypts. All the mitotic figures in each section were examined up to and including the section containing the fiftieth mitotic figure. Using step-sections the problem of including the same mitotic figure in adjacent serial sections is avoided. The PMI is calculated as the proportion of all mitotic figures comprising unequivocally normal anaphase and telophase figures. The results from all four animals were pooled and the data for VRP-treated and untreated tissue compared by analysis of deviance.

RESULTS

Drug-transport studies

Non-specific adsorbance. Background scintillation counts for this system were in the range 60–70 cpm. The mean level for ^{3}HVCR accumulation in rapid low temperature incubations was 100 cpm above background. Both of these values were deducted from each measurement.

Pilot studies. Preliminary experiments were performed to assess the suitability of the protocols adopted. Firstly experiments were performed in which explants were cultured as described in multiwell plates in SW medium. Viability was assessed using the following criteria: (i) the presence of mitotic figures and (ii) the absence of necrosis of crypt epithelial cells. These histological preparations indicated that there is no evidence of loss of viability during 30 hr in culture. Secondly, an experimental series using ^{3}HVCR was performed to compare accumulation after a preincubation period of either 2 or 6 hr. These studies showed that drug accumulation was initially lower in the explants subjected to delayed incubation. This difference declined over the period studied such that after 4 hr (either 6 or 10 hr in culture, respectively) the

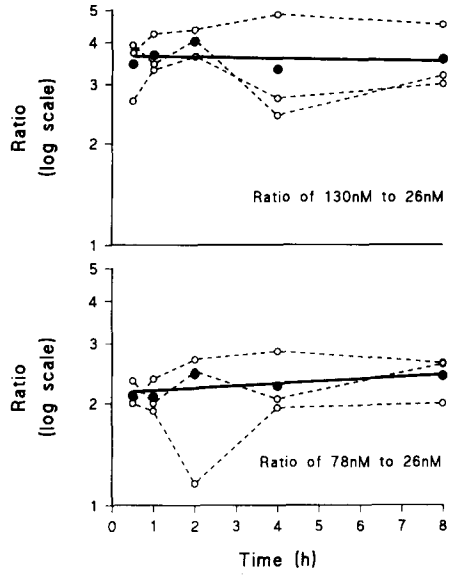

Fig. 1. Dose–response relationship. Log ratios of accumulation of ^{3}HVCR by colonic explants over a period of 4 hr. Explants were incubated in medium containing VCR at one of three doses (26, 78 and 130 nM). The graphs show three experimental replicates (○) together with the mean values (●) at each time point. Fitted line to these mean values. For statistical comparisons and absolute level of accumulation (4 hr) see Table 1.

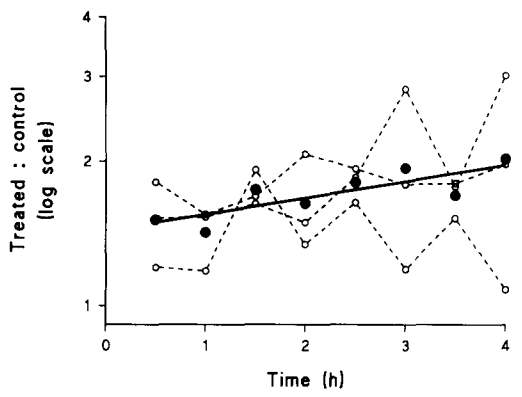

Fig. 2. Effect of energy depletion on accumulation of ^{3}HVCR. Log ratios of accumulated VCR in explants exposed to energy-depleted conditions (Dulbecco's PBS with azide and deoxyglucose) compared with normal conditions (SW medium). Explants were exposed to ^{3}HVCR at 52 nM. The graph shows three experimental replicates (○) together with the mean values (●) for each time point. Fitted line to the mean values. For statistical comparison and absolute level of accumulation (4 hr) see Table 2.

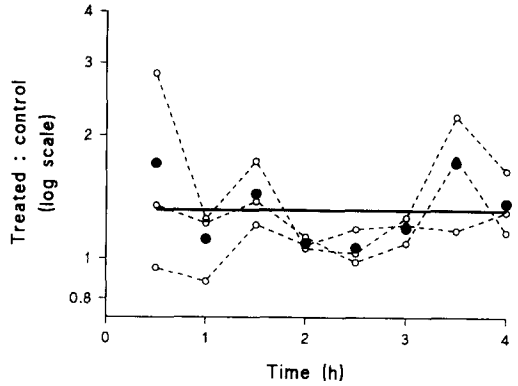

Fig. 3. Effect of verapamil (6.6 μM) on accumulation of ^{3}HVCR. Log ratios of accumulated VCR in VRP-treated and untreated explants. The explants were exposed to ^{3}HVCR at 52 nM. The graph shows three experimental replicates (○) together with the mean values (●) for each time point. Fitted line to these mean values. For statistical comparison and absolute levels of accumulation (4 hr) see Table 3.

experiments converged. Other experiments measuring uptake into dead explants (e.g. heated tissue) showed rapid uptake in excess of that reported here. These results were interpreted as follows. There is no loss of viability in culture over the period studied. During the initial 4–6 hr following explantation the metabolic disturbance sustained is associated with a slight reduction in the ability of the explants to exclude VCR. Thus, the ability of the explants to minimize drug accumulation actually improves during the experimental period selected for this series of experiments. Since each experiment includes internal comparisons this minor change in baseline transport does not affect interpretation of the results. Similar consequences of explantation, with gradual recovery, have been observed for mitotic activity [22].

Effect of VCR-dose on accumulation. In the initial series of experiments the effect of increasing the extracellular concentration of VCR was studied (Fig. 1). Mean accumulation of ^{3}HVCR at 4 hr in the presence of 26, 78 and 130 nM is shown in Table 1. The fitted lines for the log ratios of the comparisons 78:26 nm and 130:26 nM are horizontal indicating a constant ratio of accumulation over the period of the experiment. Increasing the external concentration of VCR by a factor of 3 (78:26 nM) results in a mean increase in accumulation in the explants of 2.3× (95% confidence limits 2.18–2.42). Similarly when the external concentration is increased by a factor of 5 (130:26 nM) accumulation increases by a factor of 3.7× (95% confidence limits 3.47–3.91). Thus, the explants appear to be able to restrict increasing accumulation below the expected value if transport were mediated only by passive diffusion. There is no evidence of saturation of transport at the highest dose studied.

Effect of energy depletion on accumulation. In energy-depleted explants there is marked enhancement of VCR accumulation (Table 2) indicating an element of energy-dependent exclusion under the normal comparison conditions used. An external concentration of 52 nM was selected for this and the VRP experiment (see below). Figure 2 shows the log ratios of accumulation in the three experimental replicates together with mean values. The fitted line appears to rise during the course of the experiment. This is likely to occur because in energy-depleted explants the ability to exclude VCR

Table 1. Accumulation of ^{3}HVCR at initial extracellular concentrations of 26, 78 and 130 nM. Mean accumulated VCR at 4 hr and ratios of accumulation (78:26 nM, 130:26 nM)

VCR (nM) (in medium)	VCR (pg/μg DNA) (accumulated)	Ratio (95% confidence limits)
26	9.4	1
78	21.9	2.3 (2.18–2.42)
130	34.9	3.7 (3.47–3.91)

Table 2. Effect of energy-depleted conditions on accumulation of ^{3}HVCR. Mean accumulated VCR at 4 hr in 52 nM VCR

Treatment	VCR (pg/μg DNA) (accumulated)	Ratio (95% confidence limits)
SW	13.8	1
DPBS	25.4	1.84 (1.67–2.01)

DPBS, Dulbecco's phosphate-buffered saline including azide and deoxyglucose (see text).
SW, supplemented Waymouth's medium (see text).

Table 3. Effect of verapamil on accumulation of ^{3}HVCR. Mean accumulated VCR at 4 hr in 52 nM VCR

VRP (μM)	VCR (pg/μg DNA) (accumulated)	Ratio (95% confidence limits)
0	11.9	1
6.6	16.7	1.4 (1.32–1.48)

diminishes with time as their viability declines. After 4 hr the mean log ratio of accumulation in energy-depleted conditions compared with normal conditions is 1.84 (95% confidence limits 1.67–2.01) indicating a major impairment of drug handling.

Effect of VRP on VCR accumulation. Verapamil also increases the accumulation of ^{3}HVCR compared with normal conditions although the effect is rather less than was seen in energy-depleted conditions (Table 3). The log ratios for each experimental replicate together with the mean values are shown in Fig. 3. The fitted line is clearly horizontal indicating no change in the ratio during the experimental period. The effect of VRP is to increase accumulation by a factor of 1.4 (95% confidence interval 1.32–1.48).

Retention of ^{3}HVCR. The levels of ^{3}HVCR associated with the solubilized explants at the beginning (from six explants) and end (from 30 explants) of the efflux period are shown for each experiment in Table 4. There was enhanced retention in the VRP-treated explants in each experiment compared with control values. The retention within the VRP-treated tissue after 2 hr is 1.3× that found in controls. This result corresponds closely with that obtained in the accumulation experiment using VRP and suggests that altered drug efflux (either directly or via altered intracellular drug binding) is a major factor in determining VCR accumulation.

Drug-efflux of ^{3}HVCR. After exposure to VRP for a period of 2 hr the mean efflux of ^{3}HVCR fell from 6.1 pg/μg DNA to 5.1 pg/μg DNA (Table 5).The log ratio of efflux is shown in Fig. 4 for each experimental replicate together with the mean values. Efflux under VRP-treated conditions begins at a similar rate to control conditions but then falls over the first hour. This is because, in contrast to the equivalent accumulation experiment where explants were preincubated in VRP, these explants were only exposed to VRP at the start of the observation period. This protocol was adopted so that the loading of the two groups of explants would be the same. The fitted line was generated from a quadratic fit to the data. At 2 hr efflux from VRP-treated explants is only 0.84× (95% confidence limits 0.79–0.89) that from control explants.

Morphological studies

Sensitivity of mucosal cells to VCR. The degree of metaphase arrest was studied in mucosal explants in either the presence or absence of VRP using a VCR concentration of 11 nM. The results of the four

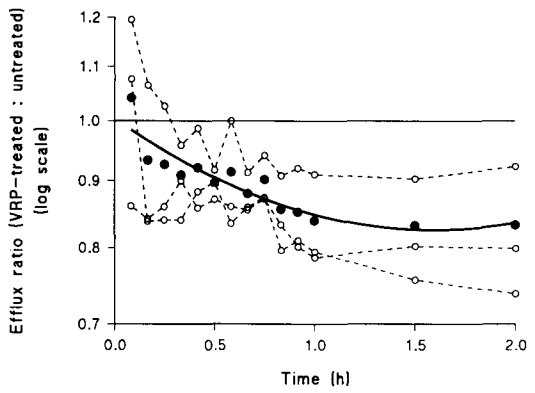

Fig. 4. Effect of verapamil (6.6 μM) on efflux of ^{3}HVCR. Log ratios of efflux of VCR from VRP-treated and untreated explants. All the explants were preloaded, in the absence of VRP, with ^{3}HVCR at 130 nM for 2 hr. The result is expressed as VCR pg/μg DNA to compensate for variation in the total amounts of tissue in the different culture dishes (see text). The graph shows three experimental replicates (○) together with the mean values (●) at each time point. Fitted line to these mean values. For statistical comparison and absolute level of efflux (2 hr) see Table 5.

Table 4. Retention of VCR in mucosal explants after preincubation in 130 nM ^{3}HVCR and resuspension in drug-free medium for 2 hr

	Retention % (VCR [pg/μg DNA] 2 hr/0 hr)	
	Control	VRP-treated (6.6 μM)
Experiment 1	47% (7.3/15.8)	73% (8.7/11.9)
Experiment 2	49% (4.7/9.6)	56% (6.0/10.7)
Experiment 3	39% (6.0/15.2)	51% (8.7/17.1)
Mean	45%	60%*

* VRP-treated vs control, $P < 0.05$.

replicate experiments, together with the mean values are shown in Table 6. Previous characterization of this assay has shown a 'native' mitotic index in rat colonic mucosa in the absence of VCR of approximately 20% [23]. In the presence of VRP

Table 6. Effect of VRP (6.6 μM) on the stathmokinetic effect of VCR (11 nM) measured using the Postmetaphase Index

	PMI (%)	
Experiment	Control	VRP-treated
1	16.1	9.8
2	20.3	10.2
3	18.8	15.4
4	19.8	10.3
Mean (±SE)	18.8 (0.9)	11.4 (1.4)*

Explants were incubated overnight in drug free medium followed by VRP (or control medium) for 0.5 hr and the combined drugs for 2.5 hr.

* VRP-treated vs control, $P = 0.014$.

the mean proportion of mitoses escaping metaphase arrest decreased from 18.8% to 11.4% ($P = 0.014$). Verapamil alone has no effect on either the PMI or the rate of entry into mitosis [23]. These results indicate that the epithelium is sensitized to the stathmokinetic effect of VCR by a factor of 0.6.

DISCUSSION

The membrane-transport mechanism characterizing 'classical' MDR was discovered through experiments on transformed cells and tumour cell lines in which high levels of drug resistance were induced by exposure [1]. More recently it has been shown that the molecule responsible, P-gp, is a normal cell constituent present to varying degrees in normal cell populations in man [13]. The molecular biology and pharmacological effects of P-gp clearly demonstrate that it is a transmembrane located drug-efflux pathway [1]. Its presence in normal tissues raises two major questions of relevance to clinical oncology: firstly what is its function in normal cells, and secondly will modifiers of P-gp activity used therapeutically be associated with increased toxicity in those normal tissues in which it is expressed?

There is evidence that P-gp may be important in the protection of the organism from the toxic effects of accidentally ingested plant alkaloids and other xenobiotic agents. Intermediate level expression of mRNA was demonstrated in the human colon, kidney, liver and lung [13], which are all sites

Table 5. Effect of verapamil on efflux of ^{3}HVCR following a 2 hr preincubation in 130 nM. Mean efflux of VCR at 2 hr

VRP (μM)	VCR (pg/μg DNA) (efflux)	Ratio (95% confidence limits)
0	6.1	1
6.6	5.1	0.84 (0.79–0.89)

Efflux of VCR is expressed as pg/μg DNA. This correction was used to allow for unavoidable differences in the total amount of tissue present in each dish.

associated with excretion or detoxification. Arceci *et al.* [24] have reported the presence of P-gp in normal liver and small intestine in the mouse, although Sugawara *et al.* [25] were unable to confirm this. Croop *et al.* [26] studied extracts of mRNA from a variety of normal mouse tissue using probes specific for the three mdr genes described in this species. A probe which detects all three showed the highest level of expression in normal adrenal gland and 'intestinal' tissue. Using the specific probes there was no evidence of either mdr1 or mdr2 expression in the intestine and mdr3 appears to be the only form of P-gp mRNA expressed in this tissue. As yet transfection experiments to test the potential of this gene to convey drug resistance have not been reported.

In several tissues the localization of P-gp by immunohistochemistry shows restriction to lumenal surfaces [14]. Such sites include the biliary canalicular surface of hepatocytes, the lumenal surface of proximal tubular cells in the kidney, and the lumenal surface of cells lining the small pancreatic ducts. Similar localization has been demonstrated by immunoelectron microscopy in KB carcinoma cells [27]. In the adrenal gland the entire cell surface expresses P-gp and in this tissue it has been postulated that P-gp may play a role in steroid hormone transport. The demonstration of P-gp in brain capillaries [15, 28] may relate to a protective function at the blood–brain barrier but a more specific transport role at this site is possible.

Recently Horton *et al.* [16] have reported that VRP reduces the elimination of VCR from normal mouse tissues *in vivo*, including small intestine, kidney, liver and colon. Whilst this probably reflects the interaction of VRP with P-gp these authors were unable to exclude other factors (particularly those associated with the local and systemic cardiovascular effects of VRP in intact animals) and these may be relevant to the interpretation of our previous studies performed *in vivo* [29]. However, our present results add to the data supporting an interaction of VRP with P-gp.

We have confirmed that VRP increases the sensitivity of normal colonic mucosal cells to VCR. Our biochemical data, in keeping with those of Horton *et al.* [16], suggest that this is due to a reduction in one component of rapid elimination of VCR. The present results differ, however, in that we did not demonstrate a dosage threshold for the modifying effect of VRP. In the PMI experiments the data are derived exclusively from mucosal cells and we detected a sensitizing effect on VCR at 11 nM, a very low concentration. Since there is no reported difference in either the genetic or functional properties of P-gp derived from normal or neoplastic cells, our results raise the possibility that strategies aimed at overcoming tumour resistance may also cause increased toxicity in normal tissues.

Many studies have been published regarding the transport of *Vinca* alkaloids into both drug-sensitive and MDR cell lines (for review see Ref. 1). We have attempted to apply similar methods to intact mucosal explants although we are fully aware that this imposes certain limitations on the interpretation of the resulting drug-transport data. In particular the contribution of drug uptake into the various non-epithelial compartments in our material (muscularis mucosae, inflammatory and stromal cells etc.) has to be considered. Our approach is similar to that of Horton *et al.* [16]. We attempted to estimate non-specific adsorption using rapid, low-temperature conditions subtracting this from the observed measurements of accumulation. Use of uniform cell cultures facilitates accurate assessment of the numbers of cells used in drug transport experiments. To study intact mucosa we used a DNA assay as the most accurate available measure of the amount of cellular tissue within explants, rather than simple wet weight of tissue samples. The only circumstance likely to grossly distort this measurement would be the sampling of mucosa from the region of a lymphoid (Peyer's) patch but these are readily identifiable macroscopically prior to explantation and are avoided. Considerable experience with histological studies of mucosal explants has reassured us that lymphoid patches are rarely present.

No attempt was made to characterize the nature of the radiolabelled component of the tissue extracts and efflux media. Previously reported studies have demonstrated no significant metabolism of VCR either *in vivo* [16] or *in vitro* [20, 30] during short term experiments. Thus, it is unlikely that there is any significant breakdown of VCR in this organ-culture system over the short experimental periods used here.

Controversy exists regarding the mechanisms of VCR transport in mammalian cells and it is not certain whether uptake occurs by passive diffusion or by a saturable low-affinity carrier molecule [20]. Similarly the operation of the efflux pathway, associated with P-gp, may be either directly from the cell membrane [1], or indirectly via drug-sequestration in exocytotic vesicles [31, 32]. These problems reflect the methodological difficulties besetting drug-transport experiments even using purified cell suspensions. However, our findings of dose-dependent accumulation of VCR which is enhanced by energy-depletion and by VRP, are qualitatively consistent with an MDR-type drug-transport system in the epithelial component of the tissue studied, similar to that demonstrated in colonic tumour cells showing *de novo* resistance [9] and in MDR cells. The finding of decreased efflux of VCR from the explants in the presence of VRP provides an explanation for these results.

We undertook the morphological study to determine whether the effect of VRP on VCR transport was associated with any measurable change in the functional and toxic properties of VCR. Once again the nature of the tissue studied precluded conventional assays based on cell survival or *in-vivo* toxicity. We have previously developed an assay based on the stathmokinetic property of VCR, which we have used to demonstrate VRP-mediated sensitization of tumour cells to VCR [10, 29]. The present results indicate similar sensitization of normal colonic mucosa and we infer that this is mediated, at lest in part, via increased cytoplasmic accumulation of vincristine. This confirms our previous impression, based on observations *in vivo*, that VRP has an effect on the sensitivity of normal

intestinal epithelium as well as tumour tissue [10, 29]. These morphological observations help to illuminate the pharmacokinetic data and are of interest because they relate to the epithelial component only, the identity of the cells counted being known with absolute certainty. As the epithelial compartment shows a measurable response in terms of mitotic progression it is likely that the observed effect of VRP on VCR transport mainly involves this tissue compartment.

Our results do not provide direct evidence that P-gp constitutes the pathway of drug efflux giving rise to the biochemical observations that we describe. In particular we have not obtained data which might distinguish conclusively between altered drug efflux or an alteration in intracellular drug binding. Such a mechanism has been postulated as an alternative explanation for reduced accumulation of VCR [30]. The use of organ culture and intact tissue explants precludes experiments to address that issue.

Several studies have demonstrated either mdr1 mRNA or immunoreactive P-gp in the intestine of man [13, 15, 33] and mouse [24, 26], and it is likely to be present in rat. Preliminary studies in our laboratory have indicated low-level expression in rat colon using immunoblotting [23]. Given the evidence linking P-gp to MDR, and the similarity of drug-transport between MDR and our findings, it is reasonable to speculate that P-gp does contribute to these phenomena. These results support the findings of Horton *et al.* [16] and provide evidence for a role for P-gp in normal cells. Our findings support the hypothesis that this protein may participate in the protection of gut epithelium from ingested plant alkaloids and other xenobiotic agents.

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