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MODULATION BY VERAPAMIL OF VINCRISTINE PHARMACOKINETICS AND TOXICITY IN MICE BEARING HUMAN TUMOR XENOGRAFTS*

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Abstract—The effect of the calcium channel blocker verapamil (VRP) on the accumulation and retention of vincristine (VCR) has been examined in mice bearing xenografts of human rhabdomyosarcomas. The tumors were Rh18, moderately sensitive to VCR, and its subline, Rh18/VCR3, selected *in vivo* for primary resistance to VCR. Administration of VRP by i.p. bolus at dose levels above 75 mg/kg was limited by acute lethality. At this dose, the maximal concentration in plasma was 24 μM, with rapid elimination such that plasma concentrations reported to modulate resistance *in vitro* (approximately 5–10 μM) were maintained for less than 60 min. To sustain a 10 μM plasma concentration, mice were infused with VRP at 6.25 mg/kg/hr (150 mg/kg/day) for up to 7 days using osmotic pumps implanted in the peritoneal cavity. Steady-state plasma levels were ≥ 10 μM for at least 96 hr, and this schedule demonstrated minimal toxicity. Administration of VCR 20 hr after the start of VRP infusion produced significant lethality, requiring an 8-fold reduction in the VCR dose. Pharmacokinetic studies showed that VRP markedly increased the uptake and retention of VCR in small intestine, liver and kidney of mice. In small intestine, 8-fold greater levels of VCR were determined 24 hr after VCR administration, and this was associated with an increase in $T_{1/2}$ for elimination from 350 to 913 min. HPLC analysis of extracts from small intestine showed that > 90% of the radiolabel eluted with VCR or 4-desacetyl-VCR. Modulation of VCR retention was also related to the dose of VCR administered. The VRP-sensitive efflux pathway appeared more effective in certain tissues only at higher concentrations of VCR. In contrast, VRP did not alter significantly the uptake and retention of VCR in either the parent or VCR-resistant human xenografts. The data demonstrated that, in the mouse, VRP modulates the uptake and retention of VCR in several tissues, and this may indicate that drug efflux mediated by a VRP-sensitive mechanism (e.g. GP-170, associated with the multiple drug resistance phenotype) has a protective function against xenobiotics in these tissues.

Tumor cells selected for resistance to one class of cytotoxic agent may become collaterally resistant to other structurally dissimilar agents. The multiple drug resistance (MDR)¶ phenotype has been well characterized in a number of cultured rodent [1–3] and human [4–6] cell lines. One form of MDR is associated with an efflux mechanism for chemotherapeutic agents which prevents accumulation of drugs within the cell [6, 7]. This is thought to be mediated by a membrane glycoprotein (GP-170) which is encoded for by the *mdr1* gene [8–11]. Transfection of this gene has been shown to confer the MDR phenotype [12]. Amino acid homology has been observed between GP-170 and bacterial active transport proteins, and its structure is consistent with its proposed role as a drug efflux pump [13, 14].

Studies *in vitro* have demonstrated that various classes of heterocyclic compounds enhance the cytotoxicity of vinca alkaloids and/or anthracyclines in cell lines exhibiting an MDR phenotype [15–22]. Of these modulators, inhibitors of slow Ca^{2+} channels have received the most attention, although it is not established that Ca^{2+} channel blockage *per se* is responsible for reversing the resistant phenotype [23, 24]. At concentrations which reverse resistance, several of these agents also inhibit binding of a vinblastine analogue to GP-170 [25, 26]. It is probable that verapamil (VRP) competes with the cytotoxic agents for outward transport resulting in greater intracellular drug accumulation. For a modulator such as VRP to potentiate the cytotoxicity of vinca alkaloids or anthracyclines, it appears necessary to maintain modulator intracellular concentrations for the entire time during which cells are exposed to the cytotoxic agent [27]. Hence, in designing therapeutic experiments, maintenance of modulator at levels sufficient for activity is required.

Whereas the data demonstrating reversal of MDR *in vitro* are quite convincing, there is less evidence for the therapeutic value of such modulators *in vivo*.

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¶ Abbreviations: MDR, multiple drug resistance; MTD, maximal tolerated dose; VCR, vincristine; VRP, verapamil; DOX, doxorubicin; and DMDP, *N*-(3,4-dimethoxyphenethyl)-*N*-methyl 2-(2-napthyl-*M*-dithane)-2-propylamine.

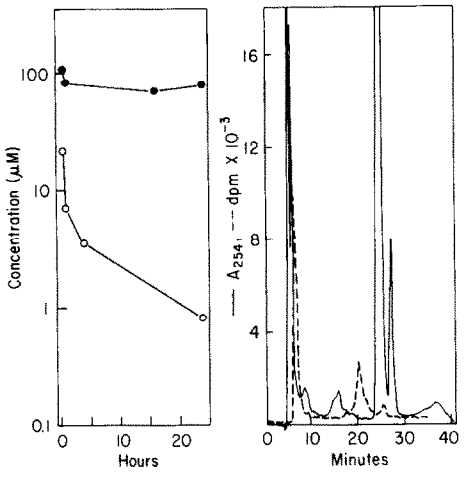

Fig. 1. Clearance and metabolism of [^{3}H-*N-methyl*]VRP in plasma, subsequent to i.p. administration (75 mg/kg). Mice received a single administration of VRP, and blood was drawn by cardiac puncture under metofane anesthesia. Plasma was extracted, using acidified ethanol, and analyzed by reverse-phase HPLC. Left panel: Total radiolabel in plasma (●), and parent VRP (○), measured over 24 hr. Right panel: HPLC profile of metabolites of [^{3}H]VRP in plasma 4 hr after i.p. administration. Verapamil eluted at 26 min. Data are the mean for plasma pooled from three mice per time point.

Verapamil has been reported to reverse resistance to vincristine (VCR) in murine P388/VCR [15], in VCR-resistant Erhlich ascites tumor [28] and against colon 26 tumors [29]. However, Radel *et al.* [30] were unable to demonstrate an increased therapeutic efficacy of doxorubicin (DOX) against P388/ADR *in vivo* by either VRP or the tiapamil analogue DMDP, and Johnson could not show a reversal of either VCR or DOX resistance in P388 sublines using different schedules of VRP (R. K. Johnson, personal communication, cited with permission). Mattern *et al.* [31] have reported some potentiation of VCR activity against a human epidermal lung cancer xenograft when VCR is administered with a relatively low dose of VRP. However, the significance of this effect was difficult to assess as tumor response was determined over only 6 days.

In humans, the use of VRP is limited by cardiac toxicity. Benson *et al.* [32] reported maximal plasma levels of 0.45 μM when VRP was infused at 0.18 mg/kg/hr for 5.5 days, well below the concentration shown to modulate cytotoxicity *in vitro*. In the study by Ozols *et al.* [33] a median plasma concentration of 2.6 μM (maximal levels being 4.7 μM) during a 3-day infusion was achieved; however, this dose produced unacceptable toxicity. In neither study where VRP was combined with vinblastine [32] or DOX [33] were there objective responses to therapy.

In the current study, our goal was to achieve concentrations of VRP in plasma of mice that have been shown to modulate resistance *in vitro*, and to examine the uptake and retention of VCR in normal as well as neoplastic tissues. The results demonstrated that VRP enhances uptake and retention of VCR in several normal tissues of the mouse, causing a significant increase in toxicity to the host. These data suggest that efflux mechanisms such as GP-170 may play an important functional role as protective mechanisms in non-neoplastic tissues. In contrast, VRP did not potentiate, significantly, the uptake or retention of VCR in a human rhabdomyosarcoma xenograft selected *in vivo* for primary resistance to VCR [34] and cross-resistant to other natural product antitumor agents [35].

METHODS

Chemicals. [G-^{3}H]Vincristine was purchased from Moravek Biochemicals, (Brea, CA) (sp. act. 12 Ci/mmol) or from the Amersham Corp. (Arlington Heights, IL) (sp. act. 6 Ci/mmol). Non-radiolabeled pharmaceutical VCR (Oncovin) was obtained from Eli Lilly & Co. (Indianapolis, IN). NCS and OCS scintillant were from the Amersham Corp. HPLC grade methanol was purchased from Burdick & Jackson (Muskegon, MI). [*N-methyl*-^{3}H]Verapamil was obtained from New England Nuclear (Boston, MA) (sp. act. 68.8 Ci/mmol). Non-radiolabeled VRP was a gift of the Knoll Pharmaceutical Co. (Whippany, NJ).

Immune deprivation of mice. Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, ME), obtained at 4 weeks of age, were immune-deprived by thymectomy followed by i.p. administration of 1-β-D-arabinofuranosylcytosine (200 mg/kg) 3 weeks later. After 48 hr, they received whole body irradiation of 950 cGy at a rate of 170 cGy/min from a ^{137}Cs source as described previously [34]. Alternatively, mice did not receive cytosine arabinoside priming, but were reconstituted with 3×10^6 nucleated bone marrow cells [36].

Tumor lines. Human rhabdomyosarcoma line Rh18 was established directly as a xenograft from an untreated primary tumor [37]. Its VCR-resistant derivative was selected *in vivo* [34] and maintained resistance for > 3 yr without additional drug selection. Both lines routinely grow in > 90% of recipient immune-deprived mice. Rh18 xenografts demonstrate moderate sensitivity to VCR, DOX and actinomycin D. Rh18/VCR-3 tumors are cross-resistant to DOX; hence they demonstrate an MDR phenotype [35]. Tumor fragments were placed bilaterally in the subcutaneous space in the dorsal flanks of mice 2 weeks post-irradiation using an aseptic technique.

Infusion of verapamil. Verapamil was dissolved at 150 mg/ml in sterile water with gentle warming to facilitate solution. At this concentration, VRP remained in solution at 37°. Verapamil was infused from Alzet osmotic pumps (model 2001; ALZA Corp., Buena Vista, CA), implanted i.p. in immune-deprived mice. Briefly, mice were anesthetized using metofane, the skin was swabbed with alcohol, and a midline incision was made through the skin. A small opening (5 mm) was made in the peritoneal wall, and the pump was inserted in a diagonal orientation. The peritoneal wall was closed using a surgical suture and the skin closed using a single wound clip. The procedure was carried out in a class B biohazard cabinet. For sham-operated mice, the procedure was identical except that pumps were not implanted. The infusion rate was 1.01 μl/hr, giving a dose rate of

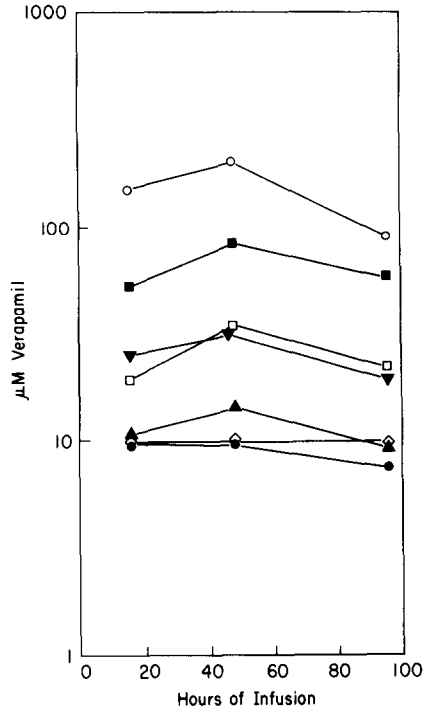

Fig. 2. Levels of VRP in tissues and plasma during i.p. infusion of VRP at 150 mg/kg/day to immune-deprived mice. Tissues were excised and extracted as described in Methods, and then analyzed by HPLC. Data show concentrations of parent VRP calculated in cell water, 16, 48 and 96 hr after start of infusion. Key: (○) liver; (■) kidney; (□) colon; (▼) small intestine; (▲) Rh18/VCR-3; (◇) skeletal muscle; and (●) plasma. Each point represents the mean value for two or three determinations.

6.25 mg/kg/hr (150 mg/kg/day) VRP. After implantation, there was a 4-hr delay before start of infusion; hence, administration of VCR 24 hr after implantation of the pump, corresponded to 20 hr after the start of VRP infusion. All surgical procedures were in accordance with protocols reviewed and approved by the Institutional Internal Review Board for Animal Care and Welfare.

Retention of [G-^{3}H]vincristine in tissues. Immune-deprived mice were implanted i.p. with osmotic pumps (VRP 150 mg/kg/day or saline) and received a single administration of VCR at dose levels between 0.15 and 1.5 mg/kg. To determine total radiolabel, non-neoplastic tissues were excised between 1 and 24 hr after VCR administration. For tumors, the concentration of total radiolabel was determined over 72 hr. Tissues were washed in ice-cold 0.9% NaCl (saline), blotted dry, weighed and digested in NCS tissue solubilizer. For small intestine, lumenal contents were evacuated by washing with saline, tissue was blotted dry, and the mucosal lining was removed from the muscularis by scraping with a microscope slide and processed as described. Radiolabel in the resulting solution was determined by scintillation counting.

Extraction and analysis of [^{3}H-methyl]verapamil. Two schedules of administration were examined. For bolus administration, VRP was injected i.p. at 75 mg/kg (sp. act. 3.29 mCi/mmol) and for infusion at 150 mg/kg/day, the specific activity was 0.70 mCi/mmol. Tissues were rapidly excised, placed on ice, and homogenized (Polytron, Brinkmann Instruments, 4 × 15 sec, 2°) in 4 vol. of ice-cold acidified EtOH (pH 5.0 using 1 M CH_3COOH). The homogenate was centrifuged (3000 *g*, 2°, 10 min), and the pellet was re-extracted with 1 M acetic acid. Washings were combined and centrifuged, and supernatant lyophilized to dryness. Recovery of radiolabel was 88.5% (range 82 to 95%). Under the conditions for extraction and storage (−120° for up to 7 days), [^{3}H-*methyl*]VRP was stable. Verapamil and its metabolites were analyzed by reverse phase HPLC using an RAC ODS-3 column (100 × 10 mm, Whatman, Clifton, NJ) and a gradient from 30% methanol (containing 10 mM KH_2PO_4, pH 5.0, buffer A) to 70% methanol (pH 5.0 using H_3PO_4) over 60 min. The flow rate was 1 ml/min, and absorbance was determined at 254 nm. Fractions were collected at 0.5-min intervals and mixed with 3.5 ml scintillation fluid (ACS), and the radioactivity was determined. Prior to analysis, samples were dissolved in buffer A and mixed with unlabeled VRP. Under these conditions, VRP eluted at 26 min and recovery of radiolabel from the column was > 90%.

Extraction and analysis of [G-^{3}H]vincristine. Mice received a single administration of [G-^{3}H]VCR (1.5 mg/kg; sp. act. 0.6 Ci/mmol). Twenty-four hours later, tissues were excised and homogenized in 4 vol. of ice-cold EtOH (95%, acidified to pH 3.9 using H_3PO_4). Tissues were centrifuged and re-extracted, and supernatant fractions were combined. Samples were concentrated by evaporation on ice under a stream of air, mixed with unlabeled VCR, and analyzed by HPLC as described previously [38]. Separation of VCR from metabolites was similar to the system used for VRP except that buffers were adjusted to pH 3.9, the flow rate was 2 ml/min, and 0.3-min fractions were collected. Radiolabeled VCR eluted at 16 min.

RESULTS

Toxicity and pharmacokinetics of VRP. Reversal of resistance to VCR *in vitro* requires maintenance of between 4 and 13 μM VRP. In mice, VRP caused acute lethality when administered at dose levels exceeding 75 mg/kg (Table 1). At the maximal tolerated dose (75 mg/kg i.p.), analysis by HPLC of plasma levels of [^{3}H]VRP demonstrated rapid metabolism and clearance of the parent compound (Fig. 1). The major metabolite eluted at 5 min, whereas VRP eluted at 26 min. The target concentration (≈ 10 μM) was maintained for less than 1 hr. Thus, because of the acute toxicity and failure to maintain adequate plasma levels of modulator, alternative schedules for VRP were examined.

Continuous infusion via i.p. osmotic pump. Verapamil was dissolved in water at its limit of solubility (150 mg/ml) and infused from osmotic pumps, surgically implanted into the peritoneal cavity. Implantation of osmotic pumps filled with either saline or VRP caused similar weight loss, with no deaths in either group of seven mice. Analysis, over 96 hr, of

Table 1. Toxicity of verapamil and vincristine

(A) Bolus administration of VRP*

Dose (mg/kg)	Deaths/Total
50	0/7
75	0/7
100	1/7†
150	6/7†

(B) Administration of VRP from osmotic pump

Surgical procedure	VCR (mg/kg)	Deaths/Total
None	3.0	2/7
None	2.0	0/7
None	1.0	0/7
None	0.75	0/7
None	0.375	0/7
Sham-operated	3.0	0/7
Sham-operated	2.0	1/7
Sham-operated	1.0	0/7
Osmotic pump (saline)	3.0	2/7
Osmotic pump (saline)	2.0	3/7
Osmotic pump (saline)	1.5	2/14
Osmotic pump (saline)	0.75	0/7
Osmotic pump (VRP, 150 mg/kg/day × 8)	3.0	Acute toxicity
Osmotic pump (VRP, 150 mg/kg/day × 8)	1.5	5/7‡
Osmotic pump (VRP, 150 mg/kg/day × 8)	0.75	3/7
Osmotic pump (VRP, 150 mg/kg/day × 8)	0.375	3/6
Osmotic pump (VRP, 150 mg/kg/day × 8)	0.187	1/7

* Single administration i.p.
† Acute lethality (<24 hr).
‡ Lethal in two mice within 4 hr.

parent VRP in plasma, tissues, and Rh18/VCR-3 xenografts is presented in Fig. 2. In Rh18/VCR-3 xenografts, the concentration of VRP was relatively constant, being ≈ 15–20 μM (calculated in cell water) during this period. The greatest concentrations of VRP were determined in liver, kidney and small intestine of tumor-bearing mice.

Intraperitoneal implantation of osmotic pumps increased the toxicity of VCR and reduced the MTD from 3 to 1.5 mg/kg (2/14 deaths, Table 1). When VCR was administered 20 hr into the VRP infusion (150 mg/kg/day), toxicity was enhanced significantly. In the presence of VRP, the MTD for VCR was 0.187 mg/kg, an 8-fold potentiation. At each dose level of VCR, deaths occurred within 11 days of drug administration.

Modulation of [^{3}H]VCR pharmacokinetics in non-neoplastic tissues. The pharmacokinetics of VCR in non-neoplastic tissues of the mouse in the presence or absence of VRP infusion was examined. Non-tumor-bearing, immune-deprived mice were implanted with osmotic pumps and infused with VRP (150 mg/kg/day) or saline. Mice received a single i.p. injection of [G-^{3}H]VCR 24 hr after implantation of each pump, and tissues were sampled over 48 hr (0.2 mg/kg; Table 2) or 24 hr after VCR administration (1.5 mg/kg) (Fig. 3). At 0.2 mg/kg VCR, VRP caused no significant changes in accumulation or retention (Table 2). Only at the higher dose of VCR in mice receiving VRP infusion was there a marked increase in both accumulation and retention of total radiolabel in small intestine and kidney, whereas clearance from plasma was relatively unchanged. In mice receiving VRP infusion, VCR (1.5 mg/kg) exerted an acute toxic effect characterized by ptosis, unsteady gait and death (2/12 mice) within 4 hr.

Determination of metabolites in non-neoplastic tissues. Pharmacokinetic studies indicated that [G-^{3}H]VCR or metabolite(s) was retained to a greater degree in mice receiving VRP infusion. Greatest retention was determined in small intestine, liver and kidney. To examine whether radiolabel was associated with VCR, mice received VCR (1.5 mg/kg) 20 hr after start of infusion of VRP or saline. Tissues were excised 24 hr after injection of [G-^{3}H]VCR, extracted using acidified ethanol, and analyzed by HPLC (Fig. 4). Extracts of small intestine and liver from mice receiving VRP infusion each demonstrated two major species. In extracts of small intestine, authentic VCR eluted at fraction 57. Two radiolabeled species with peak elution at fractions 42 and 57 were detected and were consistent with 4-desacetyl VCR and VCR respectively. Authentic

Table 2. Concentration of vincristine and metabolites in tissues of mice receiving constant infusion verapamil (150 mg/kg/day) or saline and i.p. administration of vincristine (0.2 mg/kg) 20 hr after start of infusion

	Time (hr)	Concentration (pmol/g tissue) + Verapamil	+ Saline
Liver	1	165 ± 30	144 ± 15
	4	150 ± 21	111 ± 20
	24	87 ± 5	89 ± 15
	48	109 ± 3	83 ± 8
Small intestine	1	181 ± 77	180 ± 80
	4	132 ± 48	86 ± 44
	24	46 ± 7	52 ± 12
	48	61 ± 10	31 ± 2
Colon	1	68 ± 20	72 ± 30
	4	132 ± 70	112 ± 39
	24	54 ± 18	50 ± 14
	48	65 ± 15	42 ± 15
Skin, muscle	1	75 ± 9	66 ± 4
	4	69 ± 10	64 ± 8
	24	56 ± 4	58 ± 3
	48	58 ± 1	47 ± 3
Plasma	1	116 ± 54	92 ± 14
	4	99 ± 12	99 ± 30
	24	74 ± 3	72 ± 5
	48	73 ± 5	67 ± 1

Values are means ± SD (N = 4–6).

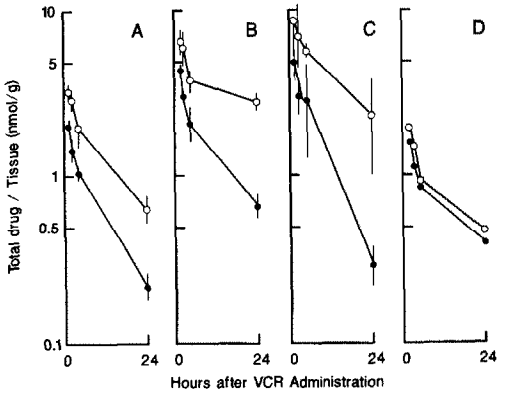

Fig. 3. Accumulation and retention of [G-^{3}H]VCR in tissues of mice receiving VRP (○) or saline (●) infusion. VCR was administered i.p. at 1.5 mg/kg, 24 hr after implantation of osmotic pumps. Tissues and blood were sampled at 1, 2, 4 and 24 hr after VCR administration. (A) Kidney; (B) liver; (C) small intestine; (D) plasma. Data are mean ± 1 SD for three determinations.

VCR mixed with extracts from liver eluted at fraction 45, and two radiolabeled species were detected, one corresponded to VCR, and a second late eluting peak at fraction 56. In extracts of tissues from mice that received saline infusion, the level of radiolabel was far lower. In extracts from small intestine, the highest level of radiolabel eluted at fraction 60 and was probably VCR. Levels of radiolabel in extracts of liver and kidney from mice receiving saline infusion or extracts of kidney from mice receiving VRP infusion were too low to analyze.

Modulation of VCR retention as a function of VCR dose. We have shown previously that levels of VCR retained in tumor tissue were directly related to the dose of VCR administered over a 100-fold range (0.3 to 30 mg/kg; [39]). Preliminary experiments indicated that, when VCR was administered at 0.2 mg/kg, there was no difference in retention of VCR in normal tissues in mice receiving VRP or saline infusion (Table 2). This could have indicated that the VRP-sensitive efflux mechanism in normal tissues was active only at higher concentrations of VCR (e.g. a low-affinity transport system). To examine this, mice were infused with VRP (150 mg/kg/day) or saline and, 20 hr after start of infusion, received [G-^{3}H]VCR at 0.15 to 1.5 mg/kg. Tissues were excised 24 hr later, and total radiolabel was determined. Results are presented in Fig. 5. In each tissue, there was a clear influence of VCR dose upon the degree of modulation by VRP. Modulation of VCR retention in small intestine and kidney was greater at higher dose levels of VCR.

Modulation of [^{3}H]VCR pharmacokinetics in neoplastic tissues. Accumulation and retention of [^{3}H]VCR were examined at 0.2 or 1.5 mg/kg in Rh18 xenografts and its VCR-resistant subline Rh18/VCR-3. The lower dose of VCR which would be therapeutically ineffective against Rh18 tumors would be tolerable in the presence of VRP infusion. Infusion of VRP did not modulate significantly the accumulation or retention of VCR in either tumor line (Table 3). At the higher dose level of VCR (1.5 mg/kg), VRP slightly increased retention of VCR in Rh18/VCR-3 tumors, although differences between VCR levels in VRP-infused tumors were not significantly greater than in mice that received the saline infusion (Table 3).

DISCUSSION

The initial observation that *in vitro* VRP reversed resistance to VCR in cells that exhibited a multidrug phenotype [15] has been confirmed in many human and non-human cell lines. The exact mechanism by which reversal of resistance occurs is not well understood, but there are data to indicate that VRP competes for binding to a membrane protein, GP-170, which may act as a drug efflux pump [25, 26]. Contrary to its impressive activity in sensitizing cells to natural products (vinca alkaloids, anthracyclines, actinomycins) *in vitro*, there is less convincing evidence that VRP can act as a modulator *in vivo*.

Studies *in vitro* have indicated that, to enhance sensitivity of resistant cells, the intracellular concentration of the modulator has to be maintained; once the modulator is removed, there is efflux of the cytotoxic agent. In this study, we have optimized administration of VRP to maintain ≈ 10 μM in plasma. Initial determinations showed that VRP given as a bolus at greater than 75 mg/kg caused acute lethality, death occurring within 1 day. At 75 mg/kg, the maximal plasma level of parent VRP was 24 μM, with rapid elimination. Plasma levels exceeded 10 μM for less than 1 hr, and within 4 hr

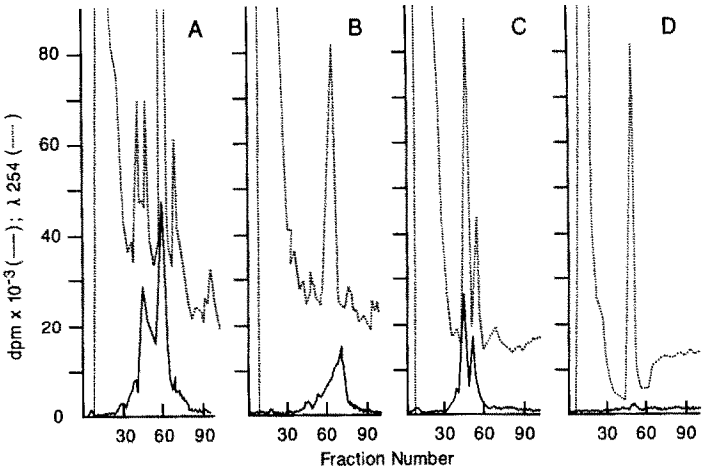

Fig. 4. Analysis of VCR and metabolites in non-neoplastic tissues of mice receiving VRP or saline infusion. Mice received a single i.p. administration of [G-^{3}H]VCR (1.5 mg/kg) 24 hr after implantation of osmotic pumps. Tissues were excised after a further 24 hr, extracted and analyzed by HPLC. Panels A and C show the elution profiles of radiolabeled metabolites from mice receiving VRP infusion (150 mg/kg/day) and B and D from saline-infused mice. A,B: small intestine; C,D: liver. Tissues were pooled from three mice and extracted.

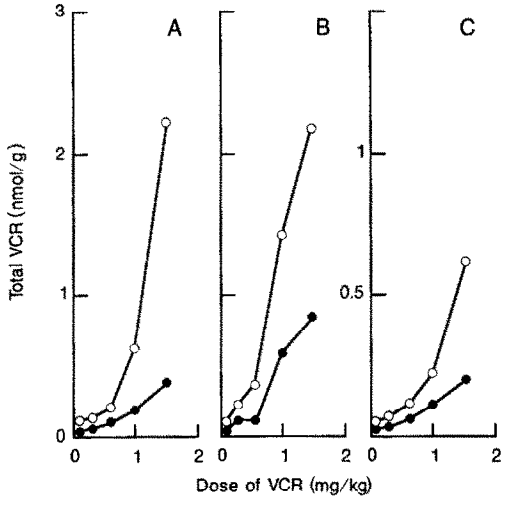

Fig. 5. Modulation of VCR retention as a function of the dose of VCR administered. Mice received infusion of VRP (○) or saline (●) by osmotic pump. After 20 hr of infusion VCR was administered at dose levels between 0.15 and 1.5 mg/kg. Tissues were excised after a further 24 hr, and total drug was determined. (A) Small intestine; (B) liver; (C) kidney. Each point represents tissues pooled from two or three mice.

were 2–3 μM, below the concentration required for *in vitro* modulation. Thus, it is likely that failure to potentiate significantly the effects of DOX and VCR *in vivo* in previous studies was due to inadequate exposure to modulator when given on a once daily schedule.

In contrast to bolus administration, infusion of VRP from an osmotic pump implanted in the peritoneum resulted in only slight toxicity. Analysis of plasma from 20 hr after implantation of the pump (i.e. 16 hr into the infusion) showed that relatively constant levels could be achieved for at least 96 hr. Given at a dose level of 150 mg/kg/day, a 10 μM concentration was achieved in plasma of both tumor-bearing (Fig. 2) and non-tumor-bearing mice (data not shown). Levels of VRP in liver exceeded 50 nmol/g (≈ 100 μM in cell water) with high concentrations also being determined in kidney and small intestine. In the human rhabdomyosarcoma xenograft examined (Rh18/VCR-3), 15–20 μM VRP was maintained over 96 hr. Thus, continuous administration from the i.p. pump allowed an adequate concentration and exposure to examine modulation of VCR accumulation and retention in tissues.

Toxicity data, presented in Table 1, showed that administration of VCR caused greater toxicity in mice in which saline-filled osmotic pumps had been implanted. However, there was a more marked increase in VCR toxicity in mice receiving VRP infusion. In the presence of VRP, the MTD for VCR was reduced from 1.5 to 0.187 mg/kg to give equivalent lethality. When VCR was administered at 1.5 mg/kg in mice receiving VRP, toxicity was acute and symptomatic of neurotoxicity. Mice that survived this phase died within 11 days of VCR administration. These patterns of toxicity suggested that VRP was potentiating VCR effects rather than VCR modulating the toxicity of VRP.

Analysis of tissues in mice infused with VRP demonstrated enhanced concentrations and retention of VCR. This modulation was greatest in small intestine, kidney and liver where the increase in VCR levels over those in mice implanted with saline-filled pumps was 8.2-, 2.9- and 2.8-fold, respectively, 24 hr after administration of 1.5 mg/kg VCR. There was increased retention in large intestine also; however, clearance from plasma was similar in mice receiving VRP or saline. If VRP is inhibiting efflux

Table 3. Accumulation and retention of [^{3}H]vincristine in rhabdomyosarcoma xenografts in mice infused with verapamil (150 mg/kg/day) or saline

Treatment and tumor line	Time (hr)	Concentration (pmol/g tissue) + VRP	+ Saline
Vincristine, 0.2 mg/kg i.p.			
Rh18	1	92 ± 20	76 ± 20
	4	76 ± 20	76 ± 20
	24	62 ± 10	75 ± 20
	48	62 ± 10	58 ± 10
Rh18/VCR-3	1	84 ± 10	73 ± 10
	4	78 ± 20	75 ± 10
	24	70 ± 4	70 ± 20
	48	66 ± 3	57 ± 4
Vincristine, 1.5 mg/kg*			
Rh18/VCR-3	24	521 ± 46	481 ± 65
	48	627 ± 11	502 ± 183

Values are means ± SD, N = 4–6.
* Toxic schedule.

of VCR from normal tissues by competing for GP-170-mediated outward transport, it would suggest that this glycoprotein acts as a normal protective mechanism in non-neoplastic tissues of the mouse.

Analysis of VCR metabolites in tissue extracts from mice receiving VRP infusion showed two major peaks in small intestine and liver. Elution profiles in extracts from small intestine were consistent with 4-desacetyl-VCR and VCR. In liver, VCR and a less polar (unidentified) metabolite were observed. In immune-deprived mice, the maximum tolerated dose ($\approx LD_{10}$) of 4-desacetyl VCR was 6 mg/kg compared to 3 mg/kg for VCR (unpublished data). Thus, both VCR and 4-desacetyl VCR presumably contribute to toxicity in mice receiving a VRP–VCR combination. Levels of VCR and metabolites in mice that received saline infusion were too low to analyze.

Preliminary studies demonstrated that VRP infusion (150 mg/kg/day) did not alter significantly the uptake and retention of VCR, when administered at a low dose (0.2 mg/kg) consistent with acceptable toxicity. We have shown previously that the level of VCR retained in tumor tissue is a linear function of dose administered [39]. Hence, modulation by VRP at higher dose levels of VCR suggested that VRP may have altered the slope of the dose–retention curve, or possibly only influenced the retention of VCR when higher concentrations were achieved. It is suggested that, at low concentrations of VCR, efflux is predominantly through a "non-VRP-sensitive" mechanism (e.g. passive diffusion), whereas at higher concentrations efflux is mediated via a "VRP-sensitive" pathway. This would be consistent with a relatively low affinity (high K_m) for VCR transport by the VRP-sensitive pathway. To examine whether the effect of VRP was dependent upon the dose of VCR used, VCR retention was examined over a 10-fold dose range (0.15 to 1.5 mg/kg). In small intestine and kidney the modulation of VCR retention was a function of the dose of VCR administered. Retention of VCR 24 hr after drug treatment at a dose level of 0.3 mg/kg was increased 38% in kidney and 94% in small intestine in the presence of VRP, whereas at 1.5 mg/kg VCR retention was increased by 221% and 489% compared to that in saline-treated controls.

We have postulated that the therapeutic selectivity of VCR in the rhabdomyosarcoma xenograft model is due to selective retention of drug in neoplastic tissue and rapid elimination from many non-neoplastic tissues (e.g. small intestine, kidney) [40]. Rapid elimination from these tissues in part relates to the formation of a relatively unstable complex between VCR and tubulin [41]. Current work suggests that a second component in drug loss may be inhibited by VRP. The data are consistent with an efflux mechanism similar to that mediated by GP-170, associated with a multiple drug resistance phenotype in neoplastic cells. There have been several studies of the distribution of GP-170, or expression of *mdr1*, the gene which encodes this protein [11] in normal human tissues. Expression of *mdr1*, as determined by steady-state levels of poly(A)$^+$ RNA, was found to be elevated in adrenal medulla, kidney, colon, lung and jejunum and at low but detectable levels in many other tissues [42]. The distribution of GP-170, determined by immuno-blotting or by immuno-cytochemistry, revealed less consistent patterns. In autopsy specimens, GP-170 was detected by immuno-blotting using C219 monoclonal antibody in normal liver and small bowel mucosa [43]. Thiebaut *et al.* [44], using MRK16, a monoclonal which recognizes an extracellular epitope on GP-170, detected P-glycoprotein in jejunum, colon, hepatocytes, kidney and adrenal glands. In contrast, Sugawara *et al.* [45] also using MRK16 did not detect GP-170 in small bowel or liver, but did detect it in full-term placenta. In the mouse all tissues were found to have considerable levels of expression of the *mdr* genes, with increased *mdr* mRNA levels particularly in the pregnant uterus [46], and also in the adrenal gland and small intestine (J. Croop, personal communication, cited with permission).

At present, therefore, the distribution and func-

tion of GP-170 in normal tissues of humans remain uncertain. However, if the mouse serves as a useful model, certain aspects of the data presented may have therapeutic implications. In the presence of VRP, the toxicity of VCR was enhanced approximately 8-fold, and histological examination of small bowel indicated an increased level of pyknotic cells in crypts during the first 72 hr following administration of VCR (data not presented). The combination also produced acute symptoms such as ptosis and staggered gait, which may indicate enhanced neurotoxic effects of VCR in combination with VRP.

There was no significant effect of VRP on accumulation and retention of VCR in neoplastic tissue. In this study, the human rhabdomyosarcoma line Rh18 was chosen as it demonstrates intermediate sensitivity to VCR [34], whereas its VCR-resistant subline (Rh18/VCR-3), which was selected *in vivo*, is completely resistant to treatment with VCR and is cross-resistant to DOX [35]. Thus, Rh18/VCR-3 demonstrates an MDR phenotype. Although we have no data to indicate involvement of the *mdr1* gene ([35]; unpublished data), the most sensitive method for detecting the involvement of the efflux pathway may be modulation by VRP. Hence, these tumors were used for the initial studies. When VCR was administered at dose levels consistent with acceptable toxicity to mice receiving VRP infusion, there was no increase in accumulation or retention of VCR in either Rh18 or Rh18/VCR-3 tumors. At the higher dose level of VCR (1.5 mg/kg), again there was no significant change in VCR pharmacokinetics over 48 hr in tumors in mice receiving VRP.

These data suggest that in Rh18/VCR-3 there is not a VRP-sensitive efflux mechanism associated with MDR. However, an alternative explanation may be proposed, which may have more general application to therapeutic modulation using agents that compete for GP-170-mediated transport. In certain non-neoplastic tissues, modulation of VCR by VRP was observed particularly at higher dose levels of VCR (e.g. 1.5 mg/kg). In these tissues (e.g. small intestine), peak levels of VCR were approximately 5000 pmol/g. In contrast, maximal levels achieved in tumor tissue are approximately 400 pmol/g [40]. Thus, the possibility exists that the concentration of VCR achieved in solid tumors is too low to allow GP-170 to function effectively as an efflux mechanism.

Data presented are consistent with the existence of a VRP-sensitive efflux mechanism and elevated levels of *mdr1* mRNA in normal tissues of the mouse. However, alternative explanations are possible; for example, VRP has been demonstrated to increase blood flow to tumors [47, 48]. The failure to significantly modulate VCR levels in tumor tissue, or in normal tissues at low doses (e.g. 0.2 mg/kg), suggests that our results are not due to increased blood flow. Further, higher concentrations of VCR in tissues appear to be a consequence of increased retention specifically in these tissues and not in plasma. In tissues the $T_{1/2}$ for the secondary phase of elimination of total radiolabel (between 4 and 24 hr after VCR administration) increased from 350 to 913 min, 633 to 1125 min and 529 to 726 min in small intestine, liver and kidney respectively. In contrast, the $T_{1/2}$ in plasma was increased from 995 to 1148 min in the presence of VRP infusion. It is of interest that, in the presence of VRP, elimination of VCR from tissues appears to parallel loss of radiolabel from plasma, whereas in the absence of VRP the rate of elimination from small intestine, liver and kidney was 2.8-, 1.6- and 1.9-fold more rapid.

Verapamil has also been shown to competitively inhibit the N-demethylation of aminopyrine by hepatic microsomes [49]. Inhibition of microsomal metabolism of VCR (to *N*-desformyl-VCR) by VRP remains to be examined, although if this did occur, then equivalent elevations in tissue levels of VCR would be anticipated at each dose level. Such an inhibition appears inconsistent with the VCR dose-dependent modulation by VRP in kidney and small intestine.

In summary, the data presented suggest that one component of rapid elimination of VCR from non-neoplastic tissues of the mouse may be inhibited by VRP. Although rigorous confirmation is required, data are consistent with a VRP-sensitive efflux mechanism, possibly involving GP-170, and are in agreement with the results of Ince *et al.* [50]. Inhibition of this system results in a significant increase in VCR toxicity, and modulation of VCR by VRP may, in some tissues, be a function of the VCR dose administered. The data presented suggest that, in certain normal tissues (e.g. small intestine, kidney), two mechanisms are responsible for drug loss from cells. At low concentrations of VCR, loss may be predominantly through passive diffusion, whereas, at higher concentrations, a VRP-sensitive system may be important. Thus, inhibition of efflux by agents such as VRP may significantly potentiate toxicity of vinca alkaloids if they are administered by bolus at levels used in conventional protocols. However, in the Rh18 and Rh18/VCR-3 xenograft systems, such manipulations would be anticipated to result in a decreased therapeutic efficacy of VCR. Whether VRP modulates VCR in xenografts that significantly overexpress *mdr1* [35] remains to be determined.

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