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RELATIONSHIP BETWEEN BINDING AFFINITY, RETENTION AND SENSITIVITY OF HUMAN RHABDOMYOSARCOMA XENOGRAFTS TO VINCA ALKALOIDS*

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Abstract—Xenografts of human rhabdomyosarcoma (RMS) have been derived that differ in their degree of sensitivity to Vinca alkaloids. Lines Rh12 and Rh18 demonstrated, respectively, high and moderate sensitivity to vincristine (VCR), but showed little responsiveness to vinblastine (VLB) *in vivo*. Rh18/VCR-3, a subline of Rh18 selected for resistance to VCR under *in situ* conditions, was insensitive to further challenge with VCR. Resistance was associated with elimination of the agent in a biphasic manner, whereas sensitivity to VCR corresponded to very prolonged drug retention in sensitive neoplastic tissues. The initial half-times for drug retention in tumors *in vivo* ($t_{\frac{1}{2}}\alpha$) correlated with the degree of sensitivity of tumors to Vinca alkaloids, decreasing $t_{\frac{1}{2}}\alpha$ being associated with decreased sensitivity. A single binding species was observed when membrane-free supernatant fractions were incubated at 37° for 15 min with 10.4 nM [^{3}H]VCR and analyzed by gel filtration HPLC. The protein eluted with a retention time of 57 min and corresponded to a molecular weight (M_r) of approximately 113,000 daltons, agreeing very closely with the M_r of dimeric tubulin (≈110,000 daltons). Two fractions were collected and eluted on a one-dimensional denaturing gel. Proteins were transferred subsequently to nitrocellulose and probed with an ^{125}I-labeled monoclonal antibody specific for β-tubulins. Only the fraction containing bound [^{3}H]VCR contained tubulin. Estimates for the dissociation constants (K_d) for the binding affinity of VCR and VLB in crude, membrane-free supernatant fractions from RMS xenografts were obtained by computer curve fitting using a mathematical binding model. Data fitted a two-site binding model, with K_d values for the high-affinity site ranging from 61 to 160 nM, and for the low-affinity site, from 42 to 94 μM. At physiologically achievable drug concentrations, the relationship between binding affinity, drug retention and tumor sensitivity was examined further. A close relationship was apparent between the K_d values for VCR in Rh12, Rh18 and Rh18/VCR-3 tumor supernatant fractions and VLB in Rh12 preparations, and $t_{\frac{1}{2}}\alpha$ values for drug retention. Prolonged drug retention correlated with a low binding constant. As $t_{\frac{1}{2}}\alpha$ decreased, binding affinity also decreased, as demonstrated by an increase in the K_d value. Consequently, the tightness of drug binding in tumors also correlated with the degree of sensitivity of the xenografts to Vinca alkaloids. Data strongly support the contention that intrinsic resistance to VLB and acquired resistance to VCR are related to the affinity of drug binding in RMS under the *in situ* conditions of tumor growth. Small differences in drug concentration and K_d values (<4-fold) have accounted for the loss of drug sensitivity *in vivo* in these human tumor lines and may relate to the level of drug resistance that is observed clinically.

The selective action of vincristine (VCR) has correlated with the selective and prolonged retention of drug in human rhabdomyosarcoma (RMS) xenografts with rapid elimination from normal tissues of host mice [1, 2]. Intrinsic resistance to vinblastine (VLB) and acquired resistance to VCR are associated with decreased drug retention in these tumors [1, 3]. Similarly, the superior efficacy of VCR compared to VLB in cultured cells exposed for short durations has also correlated with retention of VCR and rapid elimination of VLB [4, 5]. Bender *et al.* [6] demonstrated the importance of the binding of VCR in mediating cytotoxicity in a series of murine leukemic cell lines. In these studies, the ratios of influx:efflux velocities and the level of bound VCR correlated with the degree of *in vivo* sensitivity of neoplastic cells to VCR.

Vinca alkaloids appear to mediate cytotoxicity in mammalian cells by binding to the protein subunit of microtubules, tubulin [7–10]. This protein exists as a heterodimer of α and β subunits, each with a molecular weight in the order of 55,000 daltons [11–13]. VCR and VLB thereby prevent the polymerization of tubulin to form microtubules [14, 15], with arrest of cells in mitosis [16–19]. The importance of tubulin as the target for Vinca alkaloids has been suggested in studies where selection for resistance to antimitotic agents including VCR [3], colchicine, colcemid [20, 21], or maytansine [22] in CHO cells or human RMS xenografts *in vivo* has resulted in

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mutants that demonstrate altered patterns of expression of either α [22] or β [20, 21] tubulins, low (1.5- to 3-fold) but significant cross resistance to VLB [20–22], and decreased binding affinity for colcemid [20].

There is a lack of information in the literature concerning the relationship between the affinity of cellular binding of Vinca alkaloids, drug retention and cytotoxicity in neoplastic cells that demonstrate either intrinsic or acquired resistance to Vinca alkaloids. Several studies using cultured cells selected for high levels of resistance have suggested that reduced cellular retention of drug is associated with altered cellular transport properties [23, 24], although the possibilities for differences in drug binding have not been sought rigorously.

Studies on the binding affinities of Vinca alkaloids for tubulin have generally been conducted using crude [25] or purified [7–10, 26] tubulin preparations derived from mammalian [8–10, 25, 26] or chick [7, 25] brain. However, only one study has compared the binding affinities of VCR and VLB in purified tubulin from porcine brain, where K_d values were 125 and 167 nM respectively [9]. Others have examined the binding affinity of VLB for different brain preparations, with K_d values ranging from 161 nM to 43.5 μM. Such variation may relate either to the age of the preparation or to the source of tubulin used.

As the expression of tubulin isoforms is species specific [27–29] and tissue specific [30, 31], and expression changes during fetal and neonatal development [32, 33], it was of interest to examine the binding of VCR and VLB in human embryonal tumors. The relevance of drug binding to retention within tumors and to the degree of *in vivo* sensitivity to VCR or VLB has therefore been examined in human RMS xenografts using crude, fresh preparations for Scatchard analyses. Data strongly suggest that the affinity of cellular binding is important in influencing intrinsic and acquired resistance to Vinca alkaloids in these tumors.

METHODS

Chemicals. [G-^{3}H]Vincristine (12 Ci/mmole) and [G-^{3}H]vinblastine (10 Ci/mmole) were purchased from Moravek Biochemicals, Brea, CA, and purified by HPLC as previously described [1, 34]. Non-radiolabeled pharmaceutical VCR and VLB were used for *in vivo* studies. For Scatchard analyses purified compounds were a gift from Eli Lilly & Co., Indianapolis, IN. NaI[^{125}I] was obtained from the Amersham Corp., Arlington Heights, IL. NCS was purchased from the Amersham Corp. All other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, or were of reagent grade. DE-81 discs were purchased from Whatman, Inc., Clifton, NJ.

Immune-deprivation of mice. Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, ME), 4 weeks of age, were immune-deprived by thymectomy followed 3 weeks later by i.p. administration of 1-β-D-arabinofuranosylcytosine (200 mg/kg). After 48 hr they received potentially lethal whole-body irradiation of 950 rads at a dose rate of 170 rads/min from a ^{137}Cs source, described previously for heterotransplant studies [1].

Tumor lines. Tumor Rh18 was established s.c. as a xenograft from an RMS resected in a child who had received no chemotherapy. The human specimen was embryonal RMS with small areas showing alveolar histology; the xenograft was moderately differentiated, also with embryonal histology [35]. The RMS xenograft Rh12 was established from a tumor also resected from a previously untreated patient. The histological characteristics of parent tumor and xenograft were also similar and were of embryonal RMS [35]. Line Rh18/VCR-3 was a variant of Rh18, selected *in vivo* for resistance subsequent to a single i.p. administration of VCR at the maximum tolerated dose (3 mg/kg; [3, 36]).

In vivo *sensitivity to Vinca alkaloids.* Mice bearing bilateral s.c. xenografts of Rh12, Rh18 and Rh18/VCR-3 received a single i.p. administration of VCR at the maximum tolerated dose (MTD) level of 3 mg/kg or an equimolar dose of VLB (3 mg/kg; MTD = 4 mg/kg). Tumor volumes were calculated from the measurement of two perpendicular diameters at weekly intervals using vernier calipers, as previously described [1]. Growth inhibition was determined by the difference between the time taken for treated and control tumors to reach four times their treatment volume [1, 37].

Drug retention in tumors. Tumor-bearing mice received i.p. injections of [^{3}H]VCR (3 mg/kg) or an equimolar dose of [^{3}H]VLB (3 mg/kg; 0.1 to 0.2 μCi/g body wt). The concentrations of drug achieved in tumors were determined between 10 min and 72 hr after treatment. Tumors were rapidly excised, washed in ice-cold 0.9% saline, blotted dry, weighed, and digested in NCS. One milliliter of solution was assayed for radioactivity [1, 3]. Intracellular water was determined by measuring the difference between the dry weight and [hydroxy-^{14}C-*methyl*]inulin space of tumors as described previously [1].

Analysis of [^{3}H]*VCR-binding proteins in tumors.* Tumor Rh12 was homogenized (1 g/ml) in HPLC buffer (200 mM sodium phosphate, pH 6.8, containing 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 5% glycerol). After centrifugation at 100,000 *g* for 60 min at 4°, membrane-free supernatant fractions were incubated with 10.4 nM [^{3}H]VCR at 37° for 15 min to steady state; a 0.6-ml sample containing 14.2 mg protein was analyzed by gel filtration HPLC using 2 TSK SW3000 columns connected in series and a flow rate of 0.5 ml/min. Filtration of proteins was achieved at ambient temperature (21–23°), and ultraviolet absorbance was detected at 213λ; radioactivity was determined in 1-min fractions. Under these conditions, the retention times of myosin (M_r 205,000), β-galactosidase (M_r 116,000), bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), trypsinogen (M_r 24,000) and lysozyme (M_r 14,300) were 45.0, 55.7, 72.5, 78.7, 87.0 and 99.7 min respectively. VCR (M_r 923) eluted at 173 min. The method is similar to that described previously for the examination of [^{3}H]VCR bound to protein in Rh18 tumors [3].

Immunoblotting procedures. Following gel filtration HPLC, aliquots (250 μl) from the peak fraction containing bound [^{3}H]VCR and from a fraction

subsequent to this peak, were boiled for 90 sec with one-fifth volume of sodium dodecyl sulfate (SDS) sample buffer (3.9% SDS, 39% glycerol, 2% β-mercaptoethanol) prior to storage at −20°; 200 μl of each sample was electrophoresed using the discontinuous system of Laemmli [38]. Proteins were transferred to nitrocellulose (0.45 μm, Schleicher & Schuell) in Tris (25 mM)–glycine (192 mM)–methanol (20%), pH 8.3, by elution at 60 V for 3 hr. They were preincubated with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 1 hr, washed with PBS, and subsequently incubated for 2 hr with TUB 2.1 (a monoclonal antibody specific for β-tubulin; [31]) radiolabeled with ^{125}I using the chloramine T method [39]. The incubation mixture consisted of antibody (10^6 dpm/ml) in PBS containing 3% bovine serum albumin and 10% fetal calf serum. Following a subsequent wash with PBS, the nitrocellulose sheet was air-dried, overlaid with Cronex 4L medical X-ray film (Dupont) and exposed at −20° for 42 hr prior to developing.

Scatchard analysis. Scatchard analyses were performed using crude, membrane-free supernatant fractions prepared from Rh12, Rh18 and Rh18/VCR-3 tumors, using the DE-81 disc assay previously described [26, 40]. Homogenates (20%) were prepared on ice in 10 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, containing 10 mM $MgCl_2$ and 0.1 mM GTP (Buffer A), and were centrifuged at 100,000 *g* for 1 hr at 2°. Protein in the supernatant fraction was assayed by the method of Lowry *et al.* [41] and adjusted to 5 mg/ml. Portions of supernatant (500 μl) were incubated at 37° to steady state (15 min) with [^{3}H]VCR or [^{3}H]VLB, each diluted with cold carrier, at concentrations ranging from 50 nM to 100 μM. Aliquots (100 μl) were subsequently filtered through DE-81 discs using Buffer A as the wash buffer [40] to determine the amount of bound drug. Results for each tumor line were adjusted for their respective filtration efficiencies. These were determined by a comparison of the dpm in aliquots of protein-bound [^{3}H]VCR (obtained after isolation from free ligand by gel filtration) both before and after filtration through DE-81 discs. Non-specific binding was determined using the method of Lauzon and Paterson [42], where radiolabeled ligand was displaced from the cellular binding sites by an excess of unlabeled ligand; this value was subtracted from the results obtained for total binding. Three or four determinations were obtained at each concentration of [^{3}H]VCR or [^{3}H]VLB. To help compensate for between-experiment variability, three or four Scatchard analyses were performed for each drug and tumor, and these replicate experiments were pooled in the computer analysis.

Statistical analysis. Data were analyzed by the method of Munson and Rodbard [43] using the computer program "Ligand" adapted for use on an Apple IIe computer. Scatchard plots of the bound/free (B/F) ratio versus the total bound (B) drug concentration revealed a curvilinear relationship for all experiments. The two-site binding model described by Feldman [44] was fit to the data. Improvement of this model over the simpler one-site model was tested using the F test for increase of goodness of fit. Estimates for the association constants (K_a) were obtained using nonlinear least squares regression, where initial values were determined from the slopes of the Scatchard curves. For the four tumor–drug groups investigated, all six pairwise tests of equality of the K_a values were determined by *t*-tests, with the significance level adjusted by the Bonferroni method [45]. Thus, for a true 5% significance level, a P value less than 0.0083 would be required for statistical significance in any of the individual pairwise tests. The dissociation constant (K_d) was calculated as the inverse of K_a. The relationship S.E. (K_d) = S.E. (K_a)/K_a^2 was derived by the series expansion of a function of a random variable [46].

RESULTS

The sensitivities of Rh12, Rh18 and Rh18/VCR-3 xenografts to equimolar doses of either VCR or VLB (3 mg/kg) are shown in Table 1. Line Rh12 was exquisitely sensitive to VCR, with advanced tumors demonstrating complete volume regressions and no regrowths, whereas Rh18 tumors were only moderately sensitive to the agent. In contrast, Rh12 and Rh18 tumors demonstrated little sensitivity to VLB and were intrinsically resistant to this drug. In line Rh18/VCR-3 selected for resistance to VCR, virtually no activity with VCR was observed; the response to VLB was consequently not determined.

To evaluate the relationship between tumor sensitivity to Vinca alkaloids and the half-time ($t_{\frac{1}{2}}$) for drug retention in tumors *in vivo*, the concentrations of VCR or VLB achieved after treatment of tumor-bearing mice were examined from 10 min to 72 hr after drug administration. The retention profiles and concentrations of VCR and VLB achieved in Rh12 tumors are shown in Fig. 1. VCR accumulated rapidly, reaching maximal concentrations of 1.4 μM in cell water within 3 hr of drug administration. The drug was tenaciously retained in neoplastic tissues, remaining at levels of 1.2 μM, 72 hr after treatment. In contrast, although VLB also accumulated rapidly,

Table 1. *In vivo* sensitivity and drug retention of Vinca alkaloids in human RMS xenografts

Tumor line	Drug*	Sensitivity†	$t_{\frac{1}{2}}\alpha$‡ (hr)
Rh12	VCR	++++++	723
Rh18	VCR	+++	240
Rh18/VCR-3	VCR	±	40
Rh18	VLB	±	19
Rh12	VLB	+	18

* Drugs were administered i.p. by a single injection of the MTD of VCR (3 mg/kg) or an equimolar dose of VLB (3 mg/kg) to tumor-bearing mice.

† The tumor grading system used was: ±, transient response, with inhibition $<Td_2$ (where Td_2 is the mean volume doubling time); +, growth inhibition $\geq Td_2$; +++, growth inhibition $\geq 3 \times Td_2$; ++++++, complete regression with no regrowth.

‡ $t_{\frac{1}{2}}\alpha$ = the initial half-time for drug retention in tumors *in situ* calculated during the initial elimination phase, which for VLB was between 8 and 24 hr after drug administration. Data were derived, in part, from Ref. 1.

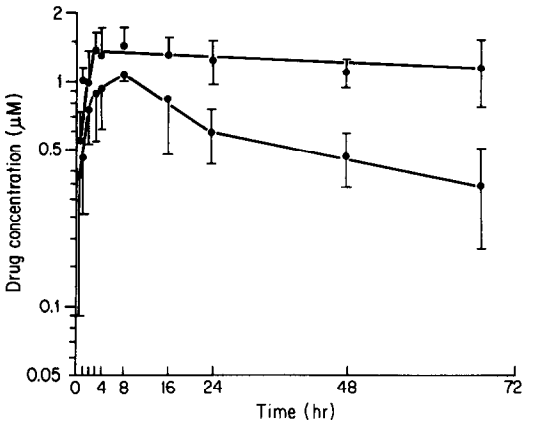

Fig. 1. Tumor retention profiles of VCR and VLB. Mice bearing Rh12 xenografts received a single i.p. administration of [^{3}H]VCR or [^{3}H]VLB (3 mg/kg). Between 10 min and 72 hr after treatment, tumors were rapidly excised, washed in ice-cold 0.9% saline, blotted dry, weighed and digested in NCS. One milliliter of solution was assayed for radioactivity. Drug concentrations in cell water were estimated after determination of the volume of cell water from the difference between the dry weight and [*hydroxy*-^{14}C-*methyl*]inulin space of tumors [1]. Upper curve, VCR; lower curve, VLB. Results represent pooled data from six to twelve tumors at each time point.

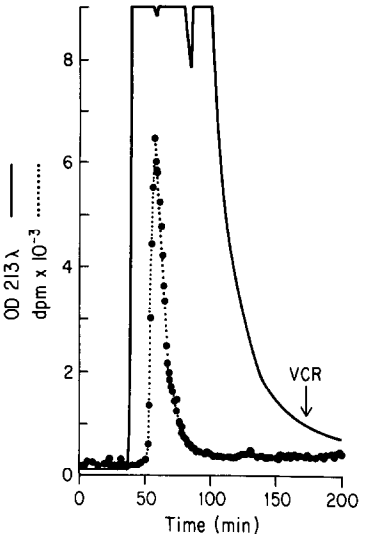

Fig. 2. Gel filtration HPLC analysis of [^{3}H]VCR–protein complexes derived in membrane-free 100,000 *g* supernatant fractions (50% homogenates) of Rh12 tumors. Complexes were formed using 10.4 nM VCR at 37° for 15 min. The single binding species eluted at 57 min (M_r approximately 113,000 daltons), which corresponds to binding to the tubulin dimer (M_r 110,00 daltons).

reaching maximal levels (1.1 μM) at 8 hr after administration, it was, in addition, rapidly eliminated between 8 and 24 hr after treatment ($t_{\frac{1}{2}}\alpha$). The elimination pattern was biphasic, with a more prolonged β-phase between 24 and 72 hr after drug administration as the drug approached an apparent steady-state level. The ratio of VCR:VLB at 72 hr was 3.4. Drug retention in Rh18 tumors after treatment with VCR or VLB was similar to the data presented for line Rh12 [1, 3]. The pattern of retention observed in Rh18/VCR-3 tumors after treatment with VCR was similar to that observed in either Rh12 or Rh18 tumors after treatment with VLB [3].

The initial half-time for *in vivo* retention ($t_{\frac{1}{2}}\alpha$) of VCR and VLB in neoplastic tissues is shown in Table 1. Rh12 tumors demonstrated prolonged retention of VCR ($t_{\frac{1}{2}}\alpha \approx 723$ hr), and rapid elimination of VLB ($t_{\frac{1}{2}}\alpha = 18$ hr). With decreasing drug sensitivity, the duration of $t_{\frac{1}{2}}\alpha$ also decreased, the lowest values always occurring after treatment with VLB.

To determine the M_r of the major binding species of VCR at physiologically achievable drug concentrations, 100,000 *g* supernatant fractions prepared from Rh12 tumors were incubated with 10.4 nM [^{3}H]VCR at 37° for 15 min, and complexes were separated using gel filtration HPLC (Fig. 2). A single binding species was observed, eluting at 57 min, with an approximate M_r of 113,000 daltons. All available VCR was bound, as shown by the absence of a second peak of radioactivity corresponding to free VCR (173 min), suggesting tight binding. When a similar sample was incubated with 1000-fold excess of unlabeled VCR, no peak of [^{3}H]VCR bound to protein was detected (data not shown). In addition, after the elution of two fractions from the HPLC analysis on denaturing gels, only the fraction that contained bound [^{3}H]VCR also contained tubulin (Fig. 3).

The binding affinity of VCR or VLB in membrane-free supernatant fractions prepared from Rh12, Rh18 and Rh18/VCR-3 tumors was subsequently examined. For each tumor line and each drug, between 62 and 87 data points were pooled from three or four separate experiments. Data from representative experiments for VCR or VLB in Rh12 supernatant fractions are shown in Fig. 4, and demonstrate the approximate 2-fold difference in scale on the abscissa between the two studies. Data were found to adequately fit a two-site binding model by computer analysis, demonstrating both high-affinity and low-affinity binding sites for Vinca alkaloids. The two-site model provided a significantly better fit to the data than the one-site model (e.g. for Rh12-VCR, $F = 6.4$ with 2 and 20 degrees of freedom respectively; $P = 0.007$). The K_d values and one standard error for K_d calculated for both sites are shown in Table 2. In preparations from different tumor lines, values for K_d at the high-affinity site ranged from 61 to 160 nM; at the low-affinity site, these were in the μM range (42 to 94 μM). Values for low-affinity binding appeared relatively similar, and as these were achieved at non-physiological drug concentrations (> 1 μM), only the relationship between high-affinity binding and drug retention *in vivo* was examined further. This is demonstrated in Fig. 5, where the K_d has been plotted against $t_{\frac{1}{2}}\alpha$. It is evident that a close relationship exists between these two parameters ($r^2 = 0.97$). The ratio of the binding constants for VCR and VLB was 2.6 in Rh12 tumors, and after statistical analysis of the estimates gave a P value of 0.016, which was at the 10% level of significance (Bonferroni method). A P value of

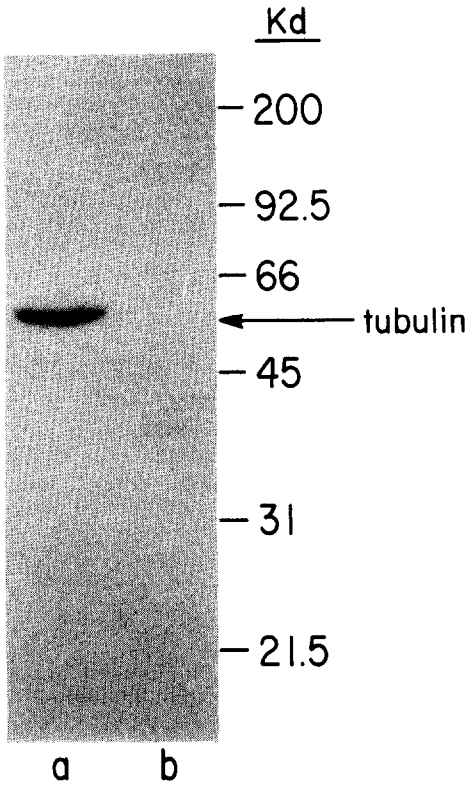

Fig. 3. Immunoblot of fractions obtained from gel filtration HPLC analysis of [^{3}H]VCR–protein complexes derived from Rh12 supernatant fractions and probed with monoclonal antibody specific for β-tubulin, according to the procedures described in Methods. Fraction: (a) peak protein fraction containing bound [^{3}H]VCR and tubulin; (b) eluting subsequent to bound [^{3}H]VCR and containing no tubulin.

0.08 was obtained when Rh12 (VCR) was compared to Rh18 (VCR), and 0.03 when Rh12 (VCR) was compared to Rh18/VCR-3 (VCR). All other comparisons yielded P values > 0.34.

DISCUSSION

Of agents that are known to bind to tubulin and inhibit cells in mitosis, including colchicine, maytansine, griseofulvin, podophyllotoxin, nocodazole and taxol, only Vinca alkaloids have demonstrated high therapeutic selectivity in the treatment of neoplastic disease. It has been of interest, therefore, to elucidate the mechanisms(s) responsible for the selective action of VCR in human RMS xenografts, and examine factors that determine intrinsic and acquired resistance to Vinca alkaloids under *in vivo* conditions. The derivation of a series of human rhabdomyosarcomas as xenografts that demonstrate sensitivity to VCR, intrinsic resistance to VLB and acquired resistance to VCR, and therefore differ in their degrees of sensitivity to Vinca alkaloids *in vivo*, would appear to be an appropriate model system for such evaluations. In Rh12 tumors that are extremely sensitive to VCR, the dose–response curve for this agent is steep [1]. For a 2-fold change in the dose level of VCR administered to tumor-bearing mice, the degree of response decreased from complete tumor regressions (1.5 mg/kg) to slight growth inhibition (0.75 mg/kg). Consequently, small changes in drug concentration may have profound biological consequences when tumors are maintained under the *in situ* conditions of growth.

Large differences in values for $t_{\frac{1}{2}}\alpha$ (Table 1) for equimolar dose levels of either VCR or VLB have resulted in small changes in drug concentration at 72 hr after treatment. For VCR in Rh12 (Fig. 1) and Rh18 [1, 3] tumors, VCR was tenaciously retained, whereas VLB was rapidly eliminated between 8 and 24 hr after treatment, until a new steady-state level was approached. A 40-fold difference in VCR and VLB $t_{\frac{1}{2}}\alpha$ values in Rh12 tumors was associated with a 3.4-fold decrease in drug concentration for VLB at 72 hr after treatment. The prolonged retention of VCR in these xenografts was in contrast to rapid drug elimination in many normal tissues including ileum, liver, kidney, skeletal muscle and plasma [1], and forms the basis for the selective action of VCR in this *in vivo* system. The rapid accumulation and retention of drug in tumors at a time when plasma levels were declining suggested that VCR was rapidly and tightly bound in these neoplastic tissues. However, intrinsic resistance to VLB appeared to be associated with a failure to retain drug (Fig. 1). Similarly, the loss of selective drug retention also appeared to be associated with acquired resistance to VCR in Rh18/VCR-3 tumors, and may be associated with the appearance of a more acidic form of β-tubulin [3]. It is clear from Table 1 that a decrease in the $t_{\frac{1}{2}}\alpha$ for drug elimination was associated with a decrease in the sensitivity of RMS xenografts to Vinca alkaloids. It was subsequently of importance to elucidate the relevance of drug binding in mediating the cytotoxicity of Vinca alkaloids in human RMS xenografts.

The binding of physiological concentrations of [^{3}H]VCR in Rh12 tumors demonstrated a single binding species of approximate M_r 113,000 daltons, corresponding to the M_r of the tubulin heterodimer (110,000 daltons; [11]). No radioactive ligand appeared bound to microtubules ($M_r > 200{,}000$ daltons). Similar data have been obtained for Rh18 tumors [3]. Additional data that support the binding of [^{3}H]VCR to tubulin include (a) the ability of unlabeled ligand to eliminate the peak of protein-bound [^{3}H]VCR from HPLC analysis, suggesting direct competition for the binding site, (b) the detection of tubulin from immunoblotting procedures in the fraction containing protein-bound [^{3}H]VCR (Fig. 3), and (c) the altered expression of β-tubulins in Rh18/VCR-3 tumors. It was therefore of interest to determine the dissociation constants for VCR and VLB in human RMS xenografts of differing sensitivities to these agents by Scatchard analysis.

Studies performed using preparations derived from mammalian or chick brain have utilized crude supernatant fractions [25] or, alternatively, tubulin at different levels of purification [7–10, 26]. In the current analysis, tubulin purified to homogeneity was not evaluated, as such preparations are devoid of the microtubule associated proteins and other proteins that may influence the binding of Vinca alkaloids

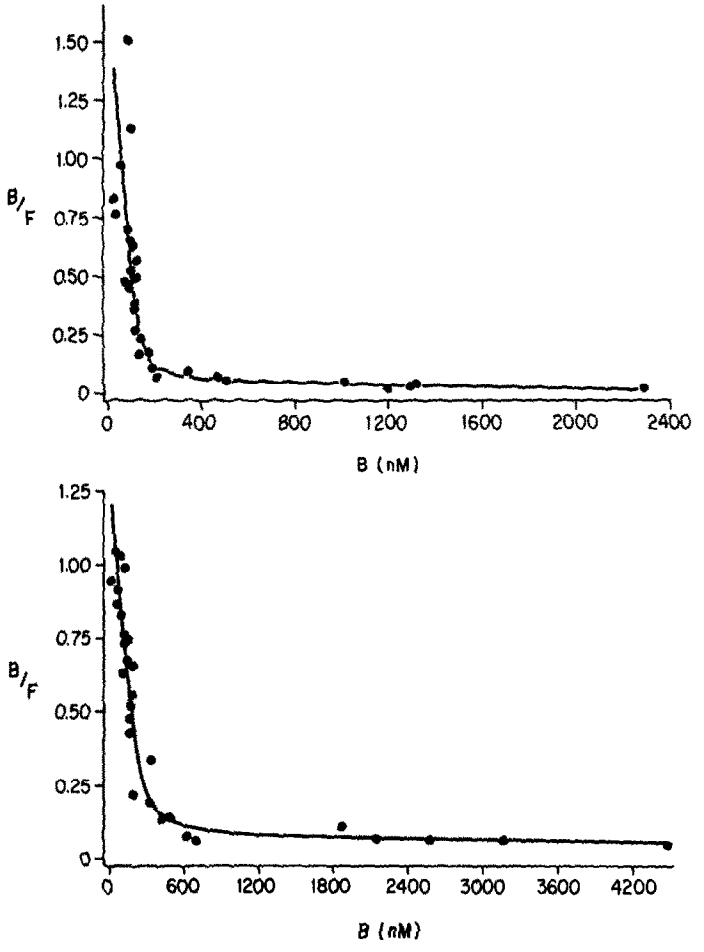

Fig. 4. Scatchard analysis of the binding of VCR (upper panel) or VLB (lower panel) in crude supernatant fractions derived from Rh12 tumors. Representative experiment comprised 29 data points and in each case were analyzed using the computer program "Ligand" developed by Munson and Rodbard [43], adapted for use on an Apple IIe computer.

under *in situ* conditions. In addition, tubulin partially purified by several cycles of assembly–disassembly, which contains the associated proteins, was not utilized, since the binding properties of tubulin may change upon aging [26], and care must be taken to characterize and ensure the freshness of the preparation. For Scatchard analyses in RMS xenografts, crude, membrane-free supernatant fractions were prepared and utilized immediately.

Data fitted a two-site binding model, with K_d values for high-affinity binding in the nM range (physiologically achievable), and those for low-affinity binding in the μM range. The importance of the tightness of binding at the high-affinity site in

Table 2. Dissociation constants for high-affinity and low-affinity binding of Vinca alkaloids in membrane-free preparations of human RMS xenografts

Tumor line	Drug	High-affinity site $K_d \pm 1$ S.E. (nM)	Low-affinity site $K_d \pm 1$ S.E. *(μM)
Rh12	VCR	61 ± 15	94 ± 37
Rh18	VCR	117 ± 27	42 ± 14
Rh18/VCR-3	VCR	139 ± 23	86 ± 35
Rh12	VLB	160 ± 36	54 ± 15

* While the values for high-affinity binding are considered accurate (since tight-binding ligands would remain associated during washing procedures), those for low-affinity binding may be less accurate since ligand associated with low-affinity sites may be more susceptible to dissociation during washing of the DE-81 filters.

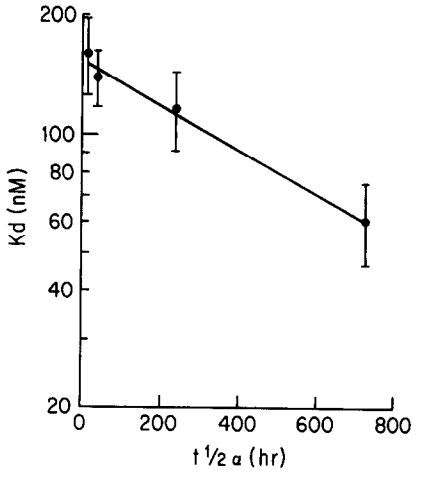

Fig. 5. Relationship between the dissociation constant ($K_d \pm 1$ S.E.) for the binding of Vinca alkaloids in crude supernatant fractions prepared from human RMS xenografts and initial half-time for drug elimination ($t_{\frac{1}{2}}\alpha$) from neoplastic tissues *in vivo*. The line is the least squares curve ($r^2 = 0.97$). L to R: Rh12-VLB (N = 87 from four separate experiments; $t_{\frac{1}{2}}\alpha \times 18$ hr). Rh18/VCR-3-VCR (N = 62 from three separate experiments; $t_{\frac{1}{2}}\alpha = 40$ hr). Rh18/VCR (N = 82 from four separate experiments; $t_{\frac{1}{2}}\alpha = 240$ hr). Rh12-VCR (N = 66 from three separate experiments; $t_{\frac{1}{2}}\alpha = 723$ hr).

influencing drug retention was evident from the close relationship between K_d and $t_{\frac{1}{2}}\alpha$ (Fig. 5). The greatest differential was between VCR and VLB in Rh12 supernatant fractions, where a 3.4-fold difference in drug concentrations within tumors 72 hr after treatment was reflected in a 2.6-fold difference in K_d values. For Rh18 and Rh18/VCR-3 tumors, the difference in dissociation constant for VCR was small (20%), being higher for Rh18/VCR-3. However, from the relationship presented in Fig. 5 this difference would correspond to the determined change in $t_{\frac{1}{2}}\alpha$ for VCR and to the differences in drug sensitivity. A low K_d value (high binding affinity) correlated with prolonged drug retention *in vivo*. As binding affinity decreased, drug retention and sensitivity also decreased. It is of interest, therefore, that drug binding appears to influence intrinsic resistance to VLB and acquired resistance to VCR, characterized under *in situ* conditions. Differences between tumor lines are small, but appear to relate to the responsiveness of RMS xenografts to Vinca alkaloids *in vivo*. Such data may have relevance to the level of drug resistance observed clinically which may be quite low (2- to 4-fold). Factors that may influence the binding of VCR and VLB to tubulin are currently being explored.

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