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RELATIONSHIPS BETWEEN TUMOR RESPONSIVENESS, VINCRISTINE PHARMACOKINETICS AND ARREST OF MITOSIS IN HUMAN TUMOR XENOGRAFTS*

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Abstract—Tumor responsiveness to vincristine (VCR) was determined in xenografts of human rhabdomyosarcoma (RMS), in sublines of RMS selected *in vivo* for VCR resistance, in a KB line (KB-Ch^{R}8-5) selected *in vitro* for colchicine resistance, and in a colon adenocarcinoma (GC_3). Sensitivity to VCR was associated with prolonged retention of VCR by the tumors after a single i.p. injection, whereas in tumors with acquired or intrinsic VCR resistance the drug was eliminated more rapidly. The sensitive tumors with prolonged retention of drug also showed increased levels of mitotic accumulation for up to 72 hr following VCR administration. There were good correlations between VCR sensitivity, VCR retention and the proposed mechanism of VCR cytotoxicity–mitotic arrest. A model has been developed consistent with data obtained that can explain the responsiveness to VCR of a series of human tumor xenografts irrespective of their tissue of origin.

The kinetics of cell kill by vinca alkaloids have been well characterized. Most data support cell cycle phase specificity [1], with cells being arrested in mitosis. *In vitro*, exposure for short periods yields biphasic survival curves [1], whereas cell kill increases progressively with exposure time.

The cytotoxicity of vincristine (VCR)‡ is thought to be mediated by binding to microtubule protein, resulting in inhibition of tubulin polymerization to form the spindle apparatus at mitosis [2–4]. It is considered that only a small percentage of cells thus inhibited will re-enter the cell cycle [5].

It is therefore of interest that VCR, but not vinblastine (VLB), given as a single i.p. bolus causes complete regression of advanced human rhabdomyosarcoma (RMS) when these tumors are heterografted into mice [6]. These tumors have relatively slow growth rates, and cell cycle times in the order of 50–60 hr *in vivo* [7] and similar doubling-times when adapted to growth *in vitro* [8]. To explain this therapeutic selectivity, we developed a model (shown in Fig. 1), in which sensitivity is determined by prolonged retention of VCR above some critical concentration required to inhibit mitosis. Thus, although *in vivo* vinca alkaloids are rapidly cleared from the plasma and most non-neoplastic tissues, the drug is tenaciously retained in sensitive tumors. Sensitive tumor cells exposed even in early G_1 phase can retain anti-mitotic concentrations until mitosis (50–60 hr) at which point their transit is arrested. The model presented is thus consistent with that proposed by Gout *et al.* [10, 11].

In the present study we compared the pharmacokinetics of VCR in sensitive RMS lines and sublines selected *in vivo* for VCR resistance, in a KB subline selected *in vitro* for colchicine resistance (acquired resistance), and in a human colon adenocarcinoma line intrinsically resistant to VCR. Relationships between drug accumulation, retention, mitotic accumulation, and tumor response have been examined.

METHODS

Chemicals. [G-^{3}H]Vincristine was purchased from Moravek Biochemicals, Brea, CA (sp. act. 12 Ci/mmol) or 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).

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 total at a rate of 170 cGy/min from a ^{137}Cs source as described previously [12].

Tumor lines and selection of resistance. Establishment of RMS Rh12, Rh18 and Rh28 as s.c. xenografts has been described previously [13, 14]. The tumors grow routinely in >90% of recipient mice and are human as determined by karyotype and

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‡ Abbreviations: VCR, vincristine; VLB, vinblastine; and RMS, rhabdomyosarcoma.

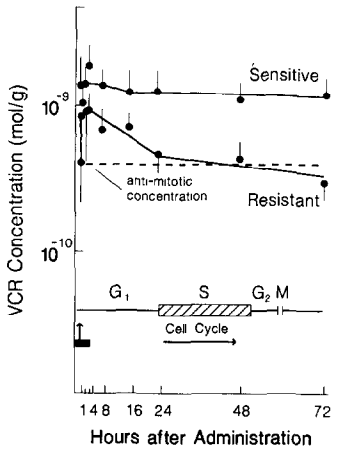

Fig. 1. Proposed model for VCR sensitivity of human tumor xenografts. Bolus administration (arrow) of VCR results in rapid clearance from plasma allowing only a transient exposure (depicted as heavy bar) for tumor cells which are distributed throughout the cell cycle. Uptake and retention of VCR in sensitive and resistant tumor are shown relative to the cell cycle (50–60 hr for RMS). Key: (---) concentration of VCR required for mitotic arrest (data from Ref. 9).

species specific isozyme patterns. The line Rh18/VCR-3 was selected *in vivo* for resistance subsequent to a single i.p. administration of VCR at the maximum tolerated dose (3 mg/kg) [9]. For the Rh12 resistant variant, Rh12/VCR-3, mice bearing Rh12 first received a single i.p. administration of VCR (1.5 mg/kg), and the tumor demonstrating the poorest response was transplanted. After seven cycles, the dose of VCR was increased to 3 mg/kg for six treatments [15].

Human KB cells were obtained from Dr. I. Pastan, Laboratory of Molecular Biology, National Cancer Institute. KB-Ch^{R}8-5, 4-fold resistant to colchicine, was isolated in a two-step procedure with treatment with ethyl methane sulfonate to enhance the mutation rate [16]. These cells were routinely passaged in 10 ng/ml colchicine. Tumor xenografts were obtained by s.c. injection into immune-deprived mice.

The development of the colon adenocarcinoma xenograft GC_3 has been described previously [17].

Tumor sensitivity to VCR. Mice bearing bilateral subcutaneous xenografts were given a single i.p. administration of VCR (3 mg/kg) when tumors were approximately 1 cm in diameter. Tumor volumes were calculated from the measurement of two perpendicular diameters at 7-day intervals using vernier calipers as described previously [14]. Growth inhibition was defined as the difference between the time taken for treated and control tumors to reach four times their treatment volume. Quantitation of the repopulating fraction (P), subsequent to drug treatment, was calculated from the formula ($P = 0.5^{t/c}$) × 100, where t is the mean growth delay and c is the mean tumor-volume doubling time during logarithmic growth [18].

Drug retention in tumors. Tumor-bearing mice were administered single i.p. injections of [^{3}H]VCR (3 mg/kg; 200 μCi/kg). Tumors were excised 1–72 hr after drug treatment and stored at −20° until assay of drug concentration. After washing in 0.9% saline and blotting dry, tumors were weighed and digested in NCS tissue solubilizer overnight at 37°. Radioactivity in the resulting solution was determined by scintillation counting.

In vitro *metabolism of* [*G*-^{3}H]*VCR.* Tumors were homogenized in 2 vol. KH_2PO_4/K_2HPO_4 (40 mM containing 150 mM KCl, pH 7.4; 20 strokes using a motor-driven Potter–Elvehjem homogenizer) and centrifuged at 9000 *g* (30 min, 2°). The supernatant fraction was retained on ice. For metabolic studies, reactions (300 μl) contained 200 μl tumor extract, 4 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 10 mM NADP, 30 mM $MgCl_2$, 40 mM potassium phosphate buffer (pH 7.4) and 0.5 μM [G-^{3}H]VCR (sp. act. 12 Ci/mmol). Reactions were incubated for up to 60 min at 37°. To terminate the reaction, 50-μl aliquots were removed and added to 50 μl ice-cold acidified ethanol (95% ethanol, pH adjusted to 4.9 using H_3PO_4). The mixture was retained on ice for 3 min, centrifuged at 13,500 *g* (2°, 5 min) and the pellet reextracted as before. Supernatant fractions were combined, mixed with 10 μl authentic VCR (1 mg/ml), and stored at −20° until analysis. Samples were analyzed by reverse-phase HPLC using an RAC ODS 3 (Whatman, Clifton, NJ) analytical column as described previously [12]. Briefly, samples were eluted using a gradient from 20% methanol (pH 3.9, containing 10 mM KH_2PO_4) to 70% methanol (pH 3.9 adjusted with H_3PO_4) over 60 min; VCR eluted at 26 min.

Mitotic accumulation. Tumor-bearing mice received a single i.p. injection of VCR (3 mg/kg). Tumors were excised 24–72 hr later and placed in 10% formalin for 24 hr. Tissue sections were prepared and stained with hematoxylin and eosin. The proportion of cells arrested in mitosis was determined by light microscopy.

RESULTS

The sensitivities of the xenografts to VCR (3 mg/kg) are shown in Fig. 2. RMS derived from previously untreated patients (Rh12, Rh28, Rh18) were considerably more sensitive to the drug than RMS selected for resistance to VCR (Rh12/VCR-3, Rh18/VCR-3). The KB subline selected for resistance *in vitro* to colchicine was also resistant to VCR. The colon adenocarcinoma GC_3 showed little response to VCR and was, therefore, intrinsically resistant to this drug. Tumor responses have been quantitated in Table 1; Rh12 and Rh28 tumors were the most sensitive to VCR and had very low repopulating fractions (<5%), whereas in the moderately sensitive Rh18 the repopulating fraction was calculated to be 12.5%. For Rh18/VCR-3, GC_3 and KB-Ch^{R}8-5 there was little inhibition of growth with repopulating fractions being between 71 and 92% (Table 1).

The relationship between tumor VCR pharmacokinetics and VCR sensitivity was evaluated by determining tumor drug levels 1–72 hr after administration. In each tumor line, VCR accumulated rapidly, reaching maximal concentrations

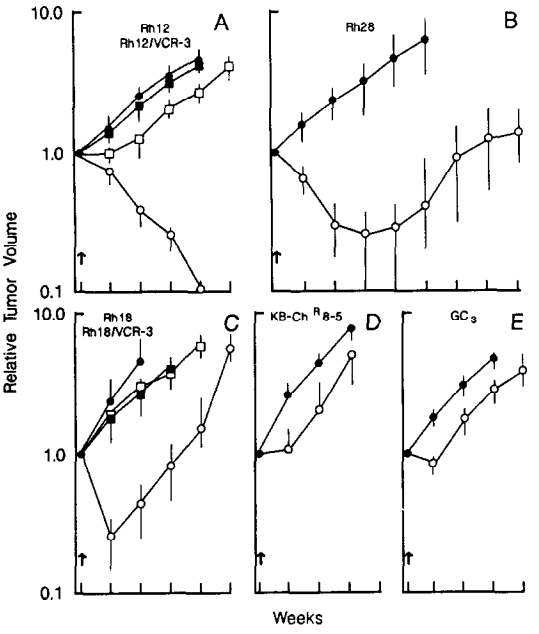

Fig. 2. Growth response of (A) Rh12 (●, ○) and Rh12/VCR-3 (■, □), (B) Rh28, (C) Rh18 (●, ○) and Rh18/VCR-3 (■, □), (D) KB-Ch^{R}8-5, and (E) GC_3. (●, ■) controls, (○, □) VCR, 3 mg/kg. Each curve represents the mean ± SD of 10–14 tumors. Relative tumor volume = V_t/V_0 where V_t is volume on day t, and V_0 is tumor volume at time of treatment (arrows).

between 4 and 8 hr after treatment. Accumulation and retention of VCR in Rh12 and the resistant variant Rh12/VCR-3 are shown in Fig. 3A. As reported previously for Rh18 and its resistant subline Rh18/VCR-3, prolonged retention of VCR was observed in the sensitive RMS, with more rapid elimination from the line with acquired VCR resistance [9]. Drug elimination from the KB-Ch^{R}8-5 xenograft was very rapid (Fig. 3B). VCR was also not retained in the intrinsically resistant tumor GC_3, and its elimination followed a pattern similar to that demonstrated in the RMS with acquired VCR resistance (Fig. 3C).

As shown in Fig. 4, no correlation existed between tumor response and the peak level of VCR achieved in tumors. These maximal concentrations ranged between 0.45 and 0.89 nmol/g after the same dose of VCR (3 mg/kg), and the highest levels were not always seen in the most sensitive tumors. In contrast, however, there appeared to be a good correlation between tumor sensitivity and the initial half-times ($T_{\frac{1}{2}}$) for VCR retention (Fig. 5), which were calculated for each tumor during the initial elimination phase. Non-responsive tumors demonstrated $T_{\frac{1}{2}}$ values of <70 hr, whereas sensitivity was associated with increasing drug retention. The most responsive tumor, Rh12, had a retention half-time of >1000 hr.

To determine whether failure of resistant lines to respond was due to conversion of VCR to a metabolite that was less tightly bound within the cell, [G-^{3}H]VCR was incubated in 9000 g extracts from resistant tumors (KB-Ch^{R}8-5, GC_3, Rh18/VCR-3, Rh12/VCR-3). Analysis of 60-min reaction products revealed no metabolism of [G-^{3}H]VCR in any of the resistant tumors (data not shown). Analysis of extracts from tumors Rh12, Rh12/VCR-3, Rh18, and Rh18/VCR-3, 24 hr after administration of [G-^{3}H]VCR to mice, also showed no differences between sensitive and resistant tumors in the elution profile of radiolabel by HPLC (data not shown).

To test the hypothesis that a prolonged accumulation of cells arrested in mitosis may correlate with increased drug retention (Fig. 1), the proportion of cells in mitosis after VCR treatment was determined. The proportion of mitotic cells in each tumor line (mitotic index) expressed as a percentage of control value between 24 and 72 hr after administration of VCR (3 mg/kg) to tumor-bearing mice is shown in Fig. 6. Without drug treatment, 1.5 to 2% of tumor cells were undergoing mitosis at any one time. An accumulation of mitoses was seen 24 hr after VCR administration in all tumor lines except KB-Ch^{R}8-5, even in tumors poorly responsive to this agent. At later time points, in the most VCR-sensitive RMS, Rh12 and Rh28, the proportion of cells arrested in mitosis continued to increase. In Rh18, only moderately sensitive to VCR, there was an increasing accumulation of cells in mitosis until 48 hr, but by 72 hr after treatment, the mitotic index decreased. The VCR resistant tumors Rh12/VCR-3, Rh18/VCR-3 and GC_3 showed a decreasing proportion of cells arrested in mitosis at times later than 24 hr. In the KB-Ch^{R}8-5 xenograft, only very small increases

Table 1. Sensitivity of xenografts to vincristine

Tumor line	Mean volume-doubling time (c) (days)	Mean growth delay (t) (days)	Repopulating fraction (P)* (%)
Rh12	11.2	>120	<0.06
Rh12/VCR-3	12.6	9.1	61
Rh18	6.3	18.9	12.5
Rh18/VCR-3	10.5	1.4	91
Rh28	12.6	>52	<5
GC_3	9.1	8.4	92
KB-Ch^{R}8-5	6.3	6.3	71

* $P = (0.5^{t/c}) \times 100$, where t is the mean growth delay in days, and c is the mean tumor-volume doubling time (days).

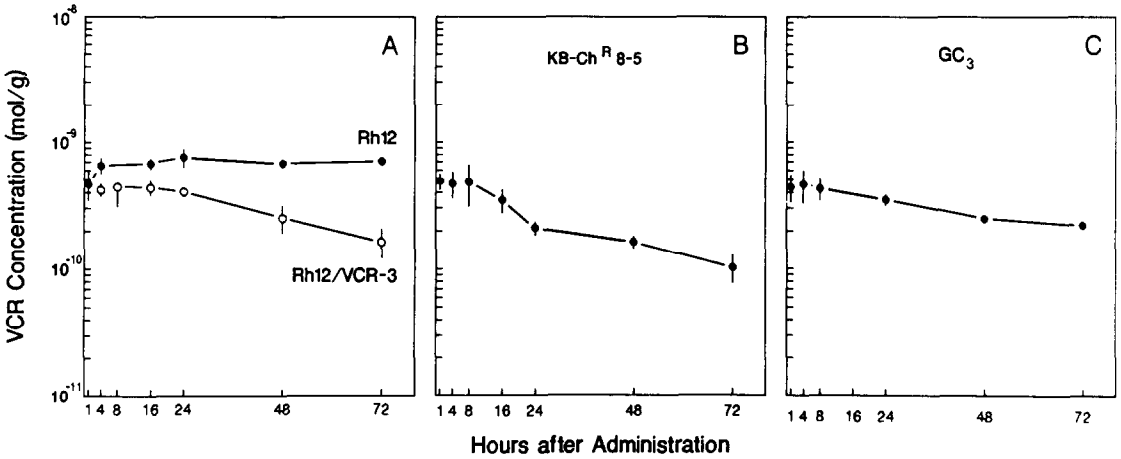

Fig. 3. Retention of VCR in xenografts after a single i.p. administration of [^{3}H]VCR (3 mg/kg) to tumor bearing mice (A) Rh12 (●), Rh12/VCR-3 (○), (B) KB-Ch^{R}8-5, and (C) GC_3. Results represent the mean ± SD of pooled data from 4 to 6 tumors at each time point.

in mitotic accumulation (200% of control) were observed at each of the three time points.

The relationship between the concentration of VCR in tumors at 72 hr after treatment and the increase in the mitotic index is shown in Fig. 7. The higher the drug concentration in tumors at 72 hr, the greater the degree of mitotic arrest ($r^2 = 0.74$) and the greater the degree of VCR sensitivity.

DISCUSSION

Previous studies from this laboratory [12] and others [10, 19] have suggested that the therapeutic selectivity of VCR is dependent upon tumor-specific retention of drug. However, alternative possibilities could be proposed. For example, the threshold concentration of drug required to inhibit mitosis may vary between tumor lines. To support the model proposed in Fig. 1 it would be anticipated that in sensitive tumors, where VCR is retained for prolonged periods, cells would continue to arrest in mitosis, whereas in resistant cells accumulation of mitotic cells would be relatively transient. There should also be a correlation between drug concentration and mitotic accumulation independent of tumor type. To test this possibility, the relationships between drug concentration, mitotic accumulation, and tumor sensitivity to VCR was examined. The tumor types comprised VCR-sensitive RMS, RMS with acquired resistance to VCR, a KB line selected for resistance to colchicine and also showing resistance to VCR, and a colon adenocarcinoma intrinsically VCR resistant. The relatively high dose of VCR used in these studies (3 mg/kg; LD_{10}) was selected, as at lower dose levels the growth of KB-Ch^{R}8-5, GC_3, Rh18/VCR-3, Rh18 and Rh12/VCR-3 was

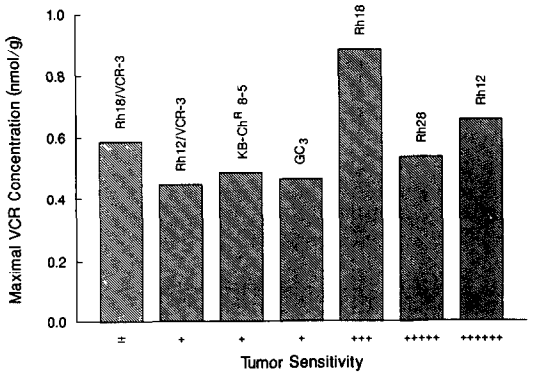

Fig. 4. Relationship between the sensitivity of tumors to VCR (3 mg/kg) and the peak drug concentration achieved between 4 and 8 hr after a single i.p. administration of [^{3}H]VCR (3 mg/kg). The grading system for tumor sensitivity: (++++++) complete regression with no regrowth; (+++++) complete regression with subsequent regrowths; (+++) growth inhibition ≥3 Td_2 (where Td_2 is the mean volume doubling time); (+) growth inhibition ≥Td_2; and (±) transient response, inhibition <Td_2.

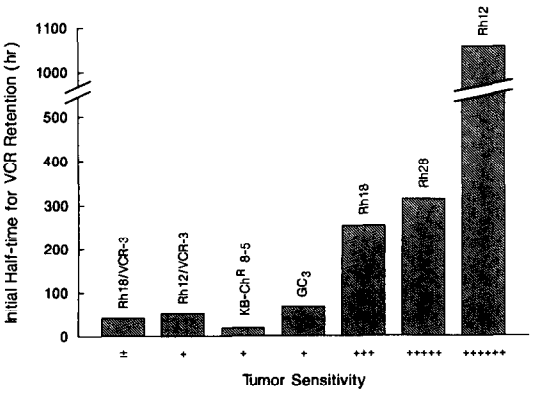

Fig. 5. Relationship between responsiveness of tumors to VCR (3 mg/kg) and the initial half-time (T_i) for drug retention after a single i.p. administration of [^{3}H]VCR (3 mg/kg) to tumor-bearing mice. The grading system for tumor sensitivity is described in the legend to Fig. 4. T_i values were calculated by regression analysis during the initial elimination phase.

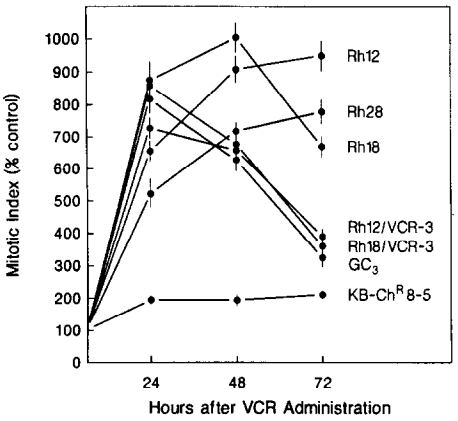

Fig. 6. Mitotic index (expressed as a percentage of the control value) in xenografts 24–72 hr after a single i.p. administration of VCR (3 mg/kg) to tumor-bearing mice. Results represent the mean ± SE of 28–50 determinations using a minimum of 5000 cells.

not inhibited significantly. Thus, correlations between mitotic accumulation and tumor inhibition could not be established at lower dose levels for the majority of tumors.

Although there was no correlation between response to VCR and the maximal tumor drug level (Fig. 4), there did appear to be a correlation between response and the half-time for drug retention (Fig. 5). Maximal drug levels were similar in each of the tumors, but VCR was not retained in tumors with either acquired or intrinsic resistance to VCR. These data suggest that the duration of exposure to VCR over some threshold intra-tumor concentration may be critical in achieving tumor response. These data support previous conclusions from studies with 4'-deoxyepiVCR [20].

Studies *in vitro* using other VCR-resistant tumor cell lines have frequently utilized selection under conditions of continuous drug exposure. The mechanism of VCR resistance has been found to be related to reduced uptake and accumulation in L5178Y cells [21], multidrug resistant Chinese hamster lung cells [22], P388 murine leukemia cells [23] and, possibly, in Ehrlich ascites tumor cells as a result of an energy-dependent efflux system in the resistant variant [24]. Fojo *et al.* [25] found a qualitative inverse correlation between drug resistance and drug accumulation in KB cells. It is probable that, in these cell culture studies, VCR achieved levels above that required for cytotoxicity only in the sensitive cell lines. In the current study, however, mitotic arrest was observed at 24 hr in all but the KB-Ch^{R}8-5 tumor irrespective of its sensitivity (Fig. 6). These data indicate that, at least initially, cytotoxic concentrations were achieved in all but KB-Ch^{R}8-5, and that resistance was not a consequence of reduced accumulation of VCR.

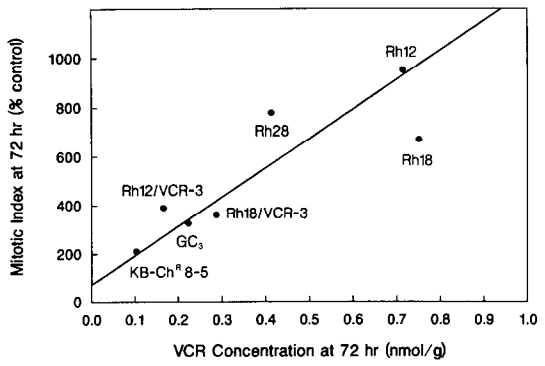

Fig. 7. Relationship between tumor VCR concentration and the mitotic index (expressed as a percentage of the control value) 72 hr after a single i.p. administration of [^{3}H]VCR or VCR (3 mg/kg).

The retention profile of VCR in the resistant tumors was similar to that observed for VLB in VCR-sensitive, but VLB-resistant RMS [12], suggesting the inability to retain the drug for a sufficient period above the cytotoxic concentration to allow for a high proportion of cells to be killed. Differential sensitivity to VCR and VLB of HL-60/Cl cells [26], Nb2 rat lymphoma cells [11] and HeLa cells [27] similarly correlated with the selective retention of VCR and the rapid release of VLB. Differences in retention of VCR between parent and VCR-resistant lines appear not to be a consequence of modified drug metabolism. Analysis by HPLC of tumor extracts from Rh18 and Rh18/VCR-3 shows a similar distribution of metabolites, the predominant species being VCR [9]. Similar results were obtained with Rh12 and Rh12/VCR-3, where at 24 hr after drug administration the predominant species (>90%) was confirmed as parent VCR by mass spectrometry (K. N. Thimmaiah and P. Houghton, unpublished data). However, detection of predominantly parent VCR at 24 hr does not address the possibility that within tumor cells VCR is metabolized to species that are not retained. To examine this, metabolism of VCR was examined in 9000 *g* supernatant fractions prepared from each of the resistant tumors (GC_3, KB-Ch^{R}8-5, Rh12/VCR-3, and Rh18/VCR-3). Over 60 min no metabolism of [G-^{3}H]VCR was detected in any tumor extract. It thus appears unlikely that decreased retention of VCR in resistant tumors is due to conversion of VCR to metabolites that are not retained within the tissue.

The binding of Vincas to tubulin results in inhibition of tubulin polymerization [2, 3], preventing formation of the mitotic spindle and therefore mitosis. In tumors with the greatest retention of VCR, there was a higher proportion of cells arrested in mitosis at the longest time point studied, 72 hr (Fig. 7). In many of the cells, arrest was in metaphase with chromosomes assembled in the middle of the cell (ball metaphases) as described previously in a human cell line treated with VCR [28]. Beyond 72 hr, the structure of sensitive tumors began to break down and it was no longer feasible to accurately determine the mitotic index. Tumors with the greatest retention half-times for VCR and the greatest increase in mitotic index at 72 hr were also the most VCR responsive tumors. There appears, therefore, to be reasonable correlations between VCR sensitivity, VCR retention and the proposed mechanism of VCR cytotoxicity, namely mitotic arrest.

The tenacious retention of VCR in sensitive tumors presumably allows drug levels to remain above the threshold for cytotoxicity throughout the cell cycle. Thus, although the majority of cells will be exposed to VCR at phases other than mitosis, the drug will remain at toxic concentrations until virtually all cycling cells reach mitosis. Others have noted that VCR is more effective against a murine mammary carcinoma than would be anticipated for a phase-specific agent [29], presumed to be as a result of the long elimination time for VCR.

In resistant tumors, if the "antimitotic" concentration of drug was exceeded for a comparatively short period of time, cytotoxicity would be limited to only that fraction of cells entering mitosis during this period. It was of interest that the maximal drug concentrations in each of the tumors, whether VCR-sensitive or -resistant, were of the same order, and the percentage increases in mitotic index at the earliest time point of the study (24 hr) other than in the KB line were also very similar. Whereas the accumulation of mitoses continued to increase in sensitive tumors, in resistant tumors drug concentrations were no longer high enough to cause mitotic arrest, and the mitotic index began to return to control levels. The exception was the KB-Ch^{R}8-5 xenograft, where peak drug levels were similar to those in the other tumor types, yet there was not a significant increase in the mitotic index. It is possible that the threshold concentration required for mitotic arrest in the KB tumor is higher than that in the other xenograft lines, or that the very rapid elimination of VCR ($T_{\frac{1}{2}}$ = 18 hr) resulted in an extremely transient anti-mitotic level at a time-point earlier than 24 hr. The relationship between mitotic accumulation and drug concentration in the Rh18 tumor (Fig. 7) was somewhat different from that predicted from regression analysis. In part, this may be explained by rapid lysis of cells arrested in mitosis in this tumor. Reference to Fig. 2 (panel C) shows that the response of Rh18 tumors was characterized by an initial rapid volume regression (>70%) and rapid regrowth. Thus, 72 hr after VCR administration the decrease in mitotic index may reflect selective loss of mitotic cells in this tumor. There still remains some controversy regarding the fate of VCR-arrested cells once drug levels fall, but in many cases the cells do not re-enter the normal proliferative cycle [4]. In resistant tumors, these cells will be a small proportion of the total.

The relationship between tumor responsiveness and an increased mitotic index 72 hr after drug administration suggests that it is the length of time during which cells continue to arrest in mitosis, rather than the maximal level of accumulation that is an important determinant of tumor responsiveness, and this is determined by drug retention (Fig. 7). The relationships between tumor responsiveness, VCR pharmacokinetics and mitotic arrest thus appear to support the model proposed.

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