Reciprocal Cross-Resistance in Human Rhabdomyosarcomas Selected in Vivo for Primary Resistance to Vincristine and L-Phenylalanine Mustard

Julie K. Horton, Peter J. Houghton, and Janet A. Houghton

ABSTRACT

Primary resistance to vincristine (VCR) has been selected in rhabdomyosarcoma xenograft HxRh12 by sequential administration of VCR at 1.5 and subsequently 3 mg/kg/passage. The resistant tumor (HxRh12/VCR-3) was approximately 4-fold resistant to VCR and resistance was stable in the absence of selecting pressure (>2 yr). HxRh12/VCR-3 was 2- to 3-fold cross-resistant to l-phenylalanine mustard (L-PAM) but only slightly cross-resistant to ifosfamide. To determine whether selection for primary resistance to L-PAM conferred cross-resistance to VCR we selected an L-PAM-resistant subline of rhabdomyosarcoma xenograft HxRh28 (HxRh28/L-PAM-3). This tumor was 2- to 3-fold resistant to L-PAM and 3-(p-fluorophenyl)-l-alaanyl-3-(l-m-bis(2-chloroethyl)-amino phenyl)-l-alaanyl-l-methionine ethyloxyhydrochloride, cross-resistant to cyclophosphamide and ifosfamide, and completely resistant to VCR under in vivo conditions. Pharmacokinetic studies in HxRh12/VCR-3 showed decreased retention of [3H]VCR but not alteration in metabolism. Expression of mdr1, a gene that encodes P-glycoprotein, associated with the multiple drug resistance phenotype, was examined. Expression of mdr1 was detected in both HxRh12 and HxRh28 tumors, sensitive to VCR, but there was no increase in expression in tumors selected for primary resistance to VCR or L-PAM. Data suggest that mechanisms other than those associated with "classical" multiple drug resistance confer resistance in these tumors.

In clinical evaluation against childhood rhabdomyosarcomas, L-PAM has demonstrated only slight activity in patients relapsing on conventional therapy (including VCR) but demonstrated marked activity in patients with advanced previously untreated disease. It appears likely, therefore, that cross-resistance between VCR and L-PAM as demonstrated in this model may have clinical significance.

INTRODUCTION

In the treatment of soft tissue sarcomas of children, of which RMS is the most common (1), combinations of alkylating agents and natural products have proven curative in early stage disease. In advanced disease multimodality therapy may induce prolonged remission, with subsequent relapse. In most instances at relapse, RMS is resistant to agents used for induction and other agents. Early studies on cross-resistance in animal models indicated universal resistance to bifunctional alkylating agents (2-4), but more recent reports showed clearly that cross-resistance among alkylating agents is not universal (5-7). However, certain data indicate that cells selected for primary resistance to an alkylating agent may exhibit cross-resistance to natural products such as doxorubicin (8). Also, primary resistance to doxorubicin has been associated with cross-resistance to L-PAM and cis dichlorodiaminoplatinum II (8). In contrast, Teicher et al. (9) did not select cells cross-resistant to DOX or VCR when primary resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea, Nor nitrogen mustard, or cis dichlorodiaminoplatinum II was obtained. In CHO cells, which exhibit a MDR phenotype and in which primary resistance was selected to colchicine, cross-resistance to L-PAM was observed (10). Further, in a revertant line, sensitivity to L-PAM was regained, suggesting that these phenomena were related (11). However, in a line of CHO cells selected for primary resistance to L-PAM, the MDR phenotype was not determined (11). Thus, the relationship between resistance to natural products (DOX, VCR, CLC, Act-D, etc.), the MDR phenotype, and alkylating agent resistance is complex. Presumably, factors that influence cross-resistance are the cell line, selection procedure, degree of resistance, and perhaps the initial definition of the sensitivity of the wild-type cell.

The question thus arises as to whether such studies in vitro are of value in predicting for resistance patterns in tumors selected under conditions in vivo. For example, both P388/ADR (selected in vivo for resistance to DOX) (12) and P388/VCR (13) show the MDR phenotype but are not cross-resistant to L-PAM. In contrast, P388/L-PAM is cross-resistant to VCR, partially cross-resistant to Act-D, and not cross-resistant to DOX (13).

Thus, whereas cross-resistance to L-PAM appears in some instances associated with the MDR phenotype (10), there appears a more specific relationship between resistance to L-PAM and cross-resistance to VCR. Our interest in this problem was stimulated because of data obtained using human rhabdomyosarcomas maintained in mice. Using these models (derived from surgical specimens prior to chemotherapy), L-PAM was identified as demonstrating considerable efficacy (14). In a subsequent Phase II evaluation in patients with refractory RMS, L-PAM showed only slight activity, whereas in previously untreated patients it was clearly a highly active agent (15). Thus, resistance to induction and maintenance therapy (VCR, DOX, Act-D, cyclophosphamide) appeared to confer resistance to L-PAM. Clearly, which agents were responsible for such clinical cross-resistance is difficult to elucidate, although cyclophosphamide would appear a good candidate because of its mechanism of action. In this article we have selected for resistance to VCR or L-PAM in two human RMS xenografts in vivo. Under these conditions of selection there is reciprocal resistance independent of the agent used for primary selection.

MATERIALS AND METHODS

Immune Deprivation of Mice. Female CBA/CaJ mice (The Jackson Laboratory, Bar Harbor, ME), 4 wk old, were immune deprived by thymectomy, followed at 3 wk by i.p. administration of 1-β-D-arabino-furanosylcytosine (200 mg/kg) 48 h prior to receiving whole-body irradiation (950 rads at 170 rads/min, using a 137Cs source) (16).

Tumor Lines and Selection of Resistance. Tumors HxRh12 and HxRh28 have been described previously (14, 17). Briefly, HxRh12, a moderately differentiated embryonal RMS, was established as a xenograft from an untreated tumor specimen. The HxRh28 xenograft was...
Vincristine-L-PAM Cross-Resistance in Vivo

Selection of VCR Resistance in HxRh12 Xenografts. The responses for groups of HxRh12 tumors following a single administration of VCR are presented in Fig. 1. For each curve, the mean volume relative to that volume at the time of treatment is shown for groups of 12 to 14 tumors. At the highest dose level (3 mg/kg) tumors rarely regrew during the observation period (≥10 weeks). At 1.5 mg/kg VCR, tumors regressed completely and subsequently regrew. The tumor demonstrating the poorest response was transplanted into recipient mice and the process repeated. Data in Fig. 1 demonstrate also the dose-response relationship for HxRh12. Thus, relatively low levels of resistance would be reflected in a considerable difference in tumor response (c.f., 3 mg/kg and 0.75 mg/kg). Growth curves for groups of HxRh12/VCR-3 tumors (after 6 exposures to 3 mg/kg) are presented in Fig. 2A. Although there is still some response, tumors are approximately 4-fold resistant to VCR (i.e., the response is equivalent to that obtained in the parent line following 0.75 mg/kg VCR). This level of resistance has been maintained in the absence of selection pressure for over 2 yr (data not shown). Further, this tumor response does not

RESULTS

Isolation of mRNA. Tumors were rapidly excised, weighed, and homogenized (Polytron) in 4 m guanidine isothiocyanate (4 ml/g tissue). Extraction was performed as described by Feramisco et al. (19). RNA was precipitated by addition of sodium acetate to 600 mM and 3 volumes of 95% ethanol. After centrifuging the pellet was resuspended (0.1 M Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.2% SDS) and digested with proteinase K (200 μg/ml) for 2 h at 37°C. Protein was extracted twice with phenol/chloroform and a further 2 times with chloroform at room temperature and RNA was precipitated as before. The pellet was washed extensively with 70% ethanol. For oligodeoxythymidylate-cellulose affinity chromatography, RNA was dissolved in loading buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% SDS, 0.5 M LiCl), heated at 65°C for 5 min, cooled, and applied to an oligo-dT column (Collaborative Research). The eluent was collected, heated at 65°C, and again applied to the column an additional 2 times. After washing with loading buffer (≥20 column volumes) polyadenylate-containing RNA was eluted in 3 ml elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS). Aliquots of mRNA were precipitated, washed twice with 70% ethanol, and stored under ethanol at −70°C. RNA was quantitated by determining the absorbance at A260/280.

Hybridization Studies. pMDR-1, containing a genomic segment of the mdr1 gene which encodes P-glycoprotein (20, 21), was obtained from Dr. I. B. Roninson, Genetics Institute, Chicago, IL. The insert was cleaved from this plasmid using SacI and BamHI restriction enzymes, and the digested fragments were separated on a low melting point agarose gel. The MDR-1 fragment was extracted, precipitated using conventional methods, and denatured by boiling. The resulting single stranded DNA was radiolabeled using 32P-dATP (New England Nuclear) by the primer extension procedure of Feinberg and Vogelstein (22). The probe was purified by Sephadex G-50 chromatography. Plasmid pA1, containing a CDNA insert complementary to β-actin (23), was obtained from Dr. D. W. Cleveland, Johns Hopkins University, Baltimore, MD. This was radiolabeled by nick translation (24). mRNA was bound to nitrocellulose filters using standard techniques. Filters were hybridized at 41°C for a minimum of 2 h in 50% formamide, 5 × SSC, 1 × Denhardt's solution (1% Ficoll, 1% polyvinylpyridolone, 1% bovine serum albumin, Pentax Fraction V), 0.25 mg/ml denatured salmon sperm DNA, 0.1% SDS, and 0.05 M sodium phosphate, pH 6.5. Prehybridization solution was replaced with fresh solution containing in addition 1 × 10^6 cpm/ml 32P-labeled MDR-1 probe. Hybridization was for a minimum of 16 h at 41°C. Subsequently, filters were washed 4 times for 5 min each in 2 × SSC, 0.1% SDS at room temperature, then 4 times for 15 min in 0.1 × SSC, 0.1% SDS at 50°C. Filters were dried and exposed to preflushed Kodak X-Omat AR film using intensifying screens for 24 to 48 h. Filters were dehybridized by boiling in water for 5 min. Complete dehybridization was confirmed by exposing the filter to film. Filters were rehybridized using 32P-labeled pA1 as described for pMDR-1 above.

Cytotoxic Agents. L-PAM and CLC were obtained from Sigma Chemical Co. (St. Louis, MO), VCR (Oncovin) from the Eli Lilly Co. (Indianapolis, IN), cyclophosphamide, ifosfamide, and maytansine from the Drug Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, and PT119 was a gift from Dr. J. G. Bekesi, Mt. Sinai Hospital, New York, NY. All agents were administered by i.p. injection (0.1 ml/10 g body weight).

![Fig. 1. Responses of HxRh12 xenografts to administration of single dose of VCR.](image-url)
tumors treated at 450 and 300 mg/kg or HxRhl2/VCR-3
whether cross-resistance to L-PAM was a phenomenon general
of L-PAM against the parent tumor and HxRhl2/VCR-3 se
pair feeding experiments did not demonstrate any inhibition of
appear to be a consequence of drug-induced host toxicity, as
represents the responses of 14 tumors (mean ± SE).
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data shown depict mean responses for groups of HxRhl2
parental or resistant tumor (data not shown). To determine
ments, the parental line was considerably more sensitive to I
flank of the same mice. Clearly, in such double-flank experi
lected for resistance to VCR. Results of a representative exper
Fig. 2. A, response of HxRhl2/VCR-3 xenografts to vincristine. Mice bearing
HxRhl2/VCR-3-3 xenografts (selected at 3 mg/kg for 6 passages) were treated
with VCR 1.5 mg/kg (△); 3 mg/kg (●), or saline (○). Each curve represents the
responses of 12 or 14 tumors (mean ± SE), subsequent to a single administration
of drug. B, responses to L-PAM of HxRhl2 and HxRhl2/VCR-3 xenografts
growing in opposite flanks of recipient hosts. Mice received s.c. grafts of HxRhl2
(left flank) and HxRhl2/VCR-3 (right flank). Mice received a single administration
(once) of L-PAM (7 mg/kg) or vehicle when tumors reached 1 cm diameter. Each curve represents the responses of 7 tumors (mean ± SE). HxRhl2: ▲
control; ○, L-PAM. HxRhl2/VCR-3: □, control; ○, L-PAM.

appear to be a consequence of drug-induced host toxicity, as
pair feeding experiments did not demonstrate any inhibition of
tumor growth.

HxRhl2 xenografts have been shown to be very sensitive to
L-PAM (14). It was of interest therefore to compare the efficacy
of L-PAM against the parent tumor and HxRhl2/VCR-3 se
lected for resistance to VCR. Results of a representative exper
iment are shown in Fig. 2B in which HxRhl2 tumors were
implanted into the left flank and HxRhl2/VCR-3 into the right
flank of the same mice. Clearly, in such double-flank exper
iments, the parental line was considerably more sensitive to L
PAM than was HxRhl2/VCR-3. Similar results were obtained
in experiments in which mice were transplanted with only
parental or resistant tumor (data not shown). To determine
whether cross-resistance to L-PAM was a phenomenon general
to other bifunctional alkylating agents or more specific for L
PAM, ifosfamide was examined in both tumor models (Fig. 3).

Data shown depict mean responses for groups of HxRhl2
tumors treated at 450 and 300 mg/kg or HxRhl2/VCR-3
treated with 450 mg/kg ifosfamide. In the VCR-resistant tumor
this dose of ifosfamide inhibited growth by 39 days, whereas
growth inhibition for HxRhl2 tumors at 300 mg/kg tumors
was 47 days. However, with respect to the growth inhibition in
terms of the growth rate of controls (HxRhl2 doubled in 7.5
days compared to 5.5 days for HxRhl2/VCR-3), treatment
resulted in inhibition of 7.1 and 6.3 volume doublings for VCR-
resistant and parent, respectively. Thus, for ifosfamide, there
appeared to be only slight cross-resistance (<1.5-fold) in
HxRhl2/VCR-3 tumors.

The responses of HxRh28 and HxRh28/L-PAM tumors
(second exposure to 13 mg/kg) to L-PAM and VCR are shown in
Fig. 4A. In HxRh28 tumors L-PAM (13 mg/kg) caused
complete regression, with no subsequent regrowths. In contrast,
in HxRh28/L-PAM the same treatment resulted in a growth
delay of only 12 days. A similar result was obtained in "double-
flank" experiments (data not shown). This represents a 2- to 3-
fold increase in resistance (14). Also presented is response of
HxRh28 tumors in mice receiving a single administration of
VCR (3 mg/kg). At this dose approximately half of the tumors
regrew subsequent to complete regression. In contrast HxRh28/
L-PAM tumors are quite unresponsive to treatment with VCR
(Fig. 4B). At 1.5 and 3 mg/kg there was no significant growth
inhibition. Further studies indicated some cross-resistance to
cyclophosphamide, ifosfamide, and the L-PAM analogue
PT119 in HxRh28/L-PAM (Fig. 5).

Cross Resistance to Natural Products. Acquired resistance to
VCR has been associated with cross-resistance to other natural
products in cells maintained in vitro. Such studies are difficult
to perform in vivo, as agents such as colchicine, maytansine,
and actinomycin D may demonstrate only marginal therapeutic
efficacy. For HxRh28 doxorubicin demonstrates only slight
activity at maximum tolerated dose levels in the mouse, whereas
the other agents do not inhibit tumor growth in either HxRh28
or HxRh28 xenografts.

Biochemical Studies. Accumulation and retention of [G-3H]-
VCR in HxRh28 and HxRh28/VCR-3 and [Q-3H]VLB in
HxRh28 tumors are presented in Fig. 6. Acquired resistance to
VCR in HxRh28/VCR-3 is associated with decreased retention,
such that by 72 h concentrations of VCR and VLB (in the
resistant and parent tumor, respectively) are similar. Analysis
by high-performance liquid chromatography and by mass spec-
situ for low-level resistance to VCR or L-PAM.

DISCUSSION

In this study we have identified cross-resistance between VCR and L-PAM irrespective of the selecting agent in human tumors.

Expression of mdr1. Overexpression of mdr1, a gene that encodes P-glycoprotein associated with MDR phenotype, has been reported to precede amplification (25). To examine whether expression of mdr1 was increased in VCR- and L-PAM-resistant lines, mRNA was examined by dot blot analysis (Fig. 7). Both parental lines have detectable levels of mdr1 mRNA, but this is not increased in tumors selected for resistance to VCR or L-PAM. To ensure similar loading of mRNA onto the filter, blots were dehybridized and rehybridized to pA1, complementary to β-actin. Results indicate similar levels of β-actin mRNA in each lane. Similar results have been obtained using pHDR10, a cDNA complementary to the human mdr1 gene (26). Thus, in HxRh12 and HxRh28, expression of mdr1 is detectable but is not increased in tumors selected in situ for low-level resistance to VCR or L-PAM.

Recent studies have demonstrated that the MDR phenotype may be conferred by transfection of a single functional cDNA (29). This gene (mdr1), which encodes P-glycoprotein (21), is overexpressed in many cell lines and overexpression may precede amplification (25). Overexpression has also been identified in 1 of 4 clinical samples of relapsed RMS (30), and increased P-glycoprotein has been identified in some ovarian tumors (31) selected for resistance in vivo. Cross-resistance to L-PAM has been associated with the MDR phenotype in CHO cells selected for high levels of resistance to colchicin (10). However, in independently derived lines of P388 (P388/ADR; P388/VCR) which show a MDR phenotype, cross-resistance to L-PAM was not found (12, 13). Doxorubicin, an agent associated with the MDR phenotype, may give rise to cross-resistance to L-PAM and other alkylating agents which are not associated with the MDR phenotype (8). Further, resistance was modulated by decreasing reduced glutathione levels using buthionine sulfoximine. The relationship between the MDR phenotype and L-PAM cross-resistance is thus tenuous, particularly where VCR is the primary selecting agent. Of note are the data of Schabel et al. (13) in which P388/L-PAM was significantly cross-resistant to VCR but P388/VCR was not resistant to L-PAM. These workers presented other examples of “unilateral cross-resistance.” Our data suggest that, under the in vivo conditions used, selection with either agent led to cross-resistance to the other agent. However, to determine whether this is unilateral or bilateral must await selection of HxRh12/L-PAM and HxRh28/VCR-3 tumor lines in vivo. These experiments are ongoing. Resistance to VCR in HxRh12 tumors was significant after the third exposure to VCR (1.5 mg/kg) and full resistance was achieved by the second exposure to 3 mg/kg. Comparison of responses of HxRh12 and its VCR-resistant subline (Figs. 1 and 2) indicate that HxRh12/VCR-3 is approximately 4-fold resistant to VCR. Clearly, HxRh12/VCR-3 is cross-resistant to L-PAM, although probably only 3-fold. However, because of the dose-response relationships for L-PAM and VCR under these conditions, small changes in tumor sensitivity (i.e., 2- to 4-fold) have significant impact upon degree of overall responses.

We were interested to determine whether the VCR-resistant subline of HxRh12 was cross-resistant to other alkylating agents and whether L-PAM resistance in HxRh28 was more general for this class of agent. Cyclophosphamide and its isomer ifosfamide were examined, as they are of interest for treatment of childhood rhabdomyosarcoma and are not transported into cells via amino acid transport systems. Consequently, cross-resistance between L-PAM and these agents is unlikely to be a consequence of altered transport. PT119 was chosen as this tripeptide analogue enters cells by both the L-system and ASC-system for amino acid transport (27), hence it is similar to L-PAM. Of interest is that a slight cross-resistance of ifosfamide was found in HxRh12/VCR-3. HxRh28/L-PAM, a subline selected for resistance to L-PAM, was ≈3- to 4-fold more resistant to both L-PAM and PT119, cross-resistant to ifosfamide and cyclophosphamide (1.5- to 2-fold), and completely cross-resistant to VCR.

Preliminary data indicate that in HxRh12/VCR-3, VCR is retained to a lesser extent and drug is eliminated in a manner similar to that of VLB in the parent line (16). Thus, the t½ for retention in HxRh12 and HxRh12/VCR-3 was 723 and 34 h, respectively, and 48 h for VLB in HxRh12 tumors. This decreased retention is similar to that in HxRh18/VCR-3 reported previously (28) and is not associated with altered metabolism of VCR.

Recent studies have demonstrated that the MDR phenotype may be conferred by transfection of a single functional cDNA (29). This gene (mdr1), which encodes P-glycoprotein (21), is overexpressed in many cell lines and overexpression may precede amplification (25). Overexpression has also been identified in 1 of 4 clinical samples of relapsed RMS (30), and increased P-glycoprotein has been identified in some ovarian tumors (31).
Fig. 7. Dot blot analysis of mdr1 mRNA in sensitive and resistant xenografts. mRNA was isolated and hybridized to probes as described in "Materials and Methods." Left, serial 2-fold dilutions of mRNA from HxR12, HxR12/VCR-3, HxR28, and HxR28/L-PAM were probed with a fragment of pMDR1. Right, after dehybridization the filter was hybridized to 32P-labeled pAl. Yeast tRNA was used as a negative control for hybridization.

in some 25% of adult sarcomas. In our RMS xenografts, HxR12 and HxR28, both sensitive to VCR, there are detectable levels of mdr1 expression, which is of interest. Of note, however, is that there was no apparent increase in expression in either HxR12/VCR-3 or HxR28/L-PAM (Fig. 7). Similar results were obtained using pHDR-10, another probe for the mdr1 gene. Whether this endogenous level of expression accounts for intrinsic resistance to DOX, CLC, or maytansine is unknown. However, the difference in efficacy between VCR and VLB in HxR12 appears to be due to the lower affinity of binding for VLB in membrane-free extracts from tumor (32).

Several of these studies suggest that resistance to VCR in HxR28/L-PAM and HxR12/VCR-3 is not a consequence of increased membrane efflux phenomena. (a) This mechanism would not account for cross-resistance to cyclophosphamide, ifosfamide, and PT119 in HxR28/L-PAM tumors. (b) There is no apparent increased expression of mdr1 in either resistant line. (c) Verapamil, an agent reported to overcome VCR resistance (33) and to reverse the MDR phenotype in vitro (34) and in vivo (35), does not alter the accumulation or retention of VCR in HxR12/VCR-3, even when it is infused at dose levels that exceed 10 μM for 4 days in tumor. Further study of the mechanism of L-PAM selected resistance to VCR (particularly with respect to a role for reduced glutathione) may provide further insight into the determinants of cytotoxicity and therapeutic selectivity for Vinca alkaloids.

The cross-resistance between agents with different chemical structure, different cellular transport mechanisms, and apparently distinct biological targets (36–40) is intriguing. One possibility is that by the nature of selection, from presumably multiclonal tumors, we may have selected cells already resistant to the other agent. This appears less likely, however, since reciprocal cross-resistance was found for both independently selected lines. Further, that this may be a clinically relevant phenomenon is borne out by the clinical evaluation of L-PAM in children with RMS.

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