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Markedly Decreased Binding of Vincristine to Tubulin in Vinca Alkaloid-Resistant Chinese Hamster Cells Is Associated with Selective Overexpression of α and β Tubulin Isoforms[1]

F. M. Sirotnak,*,†,[2] Kathleen D. Danenberg,‡ Jing Chen,*
Frederike Fritz,* and Peter V. Danenberg‡

**Program of Molecular Pharmacology and Experimental Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021; †Graduate School of Medical Sciences, Cornell University, New York, New York 10021; and ‡Department of Biochemistry and Molecular Biology, School of Medicine, University of Southern California, Los Angeles, California 90033*

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Vinca alkaloids are among a number of cytotoxic agents which target tumor cell microtubules. Studies described herein document the basis for one form of acquired resistance to these plant alkaloids involving an alteration of tubulin in a variant (DC-3F/VCRd-5L) of DC-3F Chinese hamster cells. Our results revealed a markedly decreased binding of [^{3}H]vincristine (VCR) to tubulin extracted from this variant compared to tubulin extracted from wild-type DC-3F cells. This was quantitated as a 10- to 15-fold decrease in on-rate in the presence of GTP for the [^{3}H]VCR associating with tubulin in cell-free cytosol and a 10-fold increase in off-rate for GTP-dependent dissociation of the [^{3}H]VCR–tubulin complex. Quantitative RT-PCR and nucleotide sequencing of poly(A)$^+$ RNA also carried out with variant and wild-type DC-3F cells documented a different pattern of relative expression, but no base pair differences in the open reading frame of the three α and β tubulin isoforms detected in each cell type. This was accounted for by selective overexpression of one α tubulin (αII) and two β tubulin (βI and βIV) isoforms in the variant cells. These results would appear to provide an underlying basis for the large decrease in [^{3}H]VCR binding by tubulin in these variant Chinese hamster cells and a major component of their acquired resistance to this vinca alkaloid.

Vinca alkaloids are useful cytotoxic agents (1) in the treatment of several human neoplastic disorders. As mitotic inhibitors, these agents are effectively targeted (2) to microtubules which results in their destabilization and disaggregation. In tumor cells naturally refractive or made resistant to vinca alkaloids, intracellular binding of vinca alkaloids to tubulin is often markedly decreased (3, 4). In our own studies, we have described (4, 5) this type of alteration as one basis for acquired resistance to vinca alkaloids. In this case, the resistant variant cells (DC-3F/VCRd-5L) also exhibited β tubulin isoforms with electrophoretic mobility that was different from that found (5) in DC-3F wild-type cells. This was documented by Western blotting of cell-free extract from each cell type with a monoclonal antibody that reacted with, but did not discriminate among, the various β tubulin isoforms.

As many as six α and seven β isoforms have been identified (6–8) in mammalian cells and shown to be expressed in a tissue specific manner. However, Chinese hamster cells have been found (6–10) to express only three isoforms within each category. Using automated RT-PCR, we now show in the present studies that one of the α isoforms and two of the β isoforms were significantly overexpressed in DC-3F/VCRd-5L cells when compared to wild-type DC-3F cells. We also show that these differences in this resistant variant markedly affected the ability of isolated tubulin from these cells to bind [^{3}H]vincristine (VCR) in a GTP-dependent manner.

Abbreviations used: VCR, vincristine; ORF, open reading frame; MAPs, microtubule-associated proteins; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride.

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[2] To whom correspondence should be addressed at Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Fax: (212) 794-4342. E-mail: sirotnaf@mskcc.org.

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MATERIALS AND METHODS

Cell culture methodology. Procedures employed for harvesting of cells and maintenance of parental (DC-3F) and variant (DC-3F/VeRd-5L) Chinese hamster lung tumor cells in culture have been described (4, 5). These variant cells are 2,280 fold resistant to VCR as shown (11) in an earlier report. Cells were maintained in 1:1 mixture of Eagles minimum essential medium and Hams F12 medium supplemented with 5% fetal calf serum (DC-3F) or this medium with 50 μg/ml vincristine (DC-3F/VCRd-5L).

Preparation of cytosols and analysis of [^{3}H]VCR–tubulin complexes. Cells were prepared as described earlier (4, 5) and resuspended in phosphate-$MgCl_2$ buffer (10 mM potassium phosphate plus 10 mM $MgCl_2$, pH 6.8) prior to disruption by sonication using intermittent pulses of 30 sec each with a Heat Systems ultrasonicator, Plainview, NJ) while held at 0–4°C. The sonicate was centrifuged at 100,000*g* for 60 min and the supernatant collected and held on ice. For removal of GTP cytosol was passed through two successive G25 columns (PD10 columns purchased from Pharmacia) and protein within the void volume collected. HPLC analysis of [^{3}H]VCR-tubulin complex formation was carried out by adding [^{3}H]VCR to GTP repleted cytosol in 200 mM sodium phosphate, 100 mM NaCl, 5% glycerol and 0.1 mM PMSF (pH 6.8) and fractionated (5) on a Protein Pak SW300 column (Waters, Milford, MA) during elution with the same buffer. Standard proteins (Sigma, St. Louis, MO) also eluted (0.4 ml/min at room temperature) on the same column were β-amylase (200K), aldolase, 151K, bovine serum albumen (67K), carbonic anhydrase (29K) and cytochrome *c* (12.4K).

RT-PCR and DNA sequencing. Total RNA was prepared using RNA Trizol according to the manufacturers (Gibco BRL) instructions. A 5 μg aliquot was used to synthesize cDNA using 17-38 mer oligo-DT as primers. PCR was performed with Amplitag DNA polymerase (Perkin–Elmer, Foster City, CA) using the recommended buffer, 2 pmol of the appropriate primers, 5 μg of cDNA and 200 μ mole dNTP in a total volume of 50 μl. After the initial denaturation step of 2 min at 94°C, 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C was used and the reaction extended for 10 min at 72°C. Double-stranded DNA or restriction enzyme fragments subcloned in pBluescript II SK$^+$ were sequenced in both directions according to the dideoxy method of Sanger *et al.* (12).

Measurement of [^{3}H]VCR–tubulin complex formation and dissociation. In accordance with a previously described procedure (3), 50–80 μg cytosolic protein in 100 μl phosphate-$MgCl_2$ buffer with 1 mM GTP was incubated with 50 nM [^{3}H]VCR for varying periods of time and filtered through a Whatman DE-81 filter. The filter was washed with phosphate–$MgCl_2$ and subsequently examined for radioactivity by scintillation counting. Alternatively, cells incubated with [^{3}H]VCR were disrupted and a 7- to 8-mg aliquot of the cell-free cytosol after dilution in phosphate–$MgCl_2$ buffer collected on Whatman DE- 81 filters. Since tubulin is the only known intracellular target of vinca alkaloids and binding of [^{3}H]VCR to cytosolic protein is GTP dependent, we concluded that the [^{3}H]VCR-protein interactions measured in these experiments involve tubulin. Further evidence in support of this conclusion is provided below.

Quantitative RT-PCR analysis. The quantitation of α and β tubulin isoform mRNA levels was carried out with the aid of the AB1 Prism 7700 Sequence detection system (TagMan, Perkin–Elmer, Foster City, CA). A detailed description of this methodology has been previously provided (13). Poly(A)$^+$ RNA was isolated by a published procedure (14). The tubulin isoform cDNA prepared from this poly(A)$^+$ RNA along with a reference human β-actin cDNA were PCR-amplified separately with the TagMan using an oligonucleotide probe with a 5′ fluorescent reporter dye (6FAM) and a 3′ quencher dye (TAMRA, Ref. 15). The primer and probe sequences are as follows: (a) β actin, ATCCTGTGGCATCCACGAA, 6FAM5′-TCCATCATGAAGTGTGACGTCGACATCC-3′TAMRA, CACTGTGTTGGCATAGAGGTCTTT; (b) α1, TCATTGCGTTACTTACCTCGACTCT, 6FAM5′-ACGGTAACCGTGACCCGCTGTCTG-3′TAMRA, TAGCTACGGGTTAGAAGGCGAA; α2, CCTAAGAGTCGCGCTGTAAGAAG, 6FAM5′-TCCGCCAACCTCCGGGCAG-3′TAMRA, CATGGTTGCTGCTTTGCG; (c) α4, CCCGGGACTCCTTGGTAGTC, 6FAM5′-TCGTCACCCTCTCTTAACTCAGACCTCGC −3′TAMRA, CACTCACGCATGTTGTCTAGGAA; (d) β1, GGGAAATCGTGCACATCCA, 6FAM5′-CCGGCCAATGTGGCAACCAGA-3′TAMRA, TGATCACCTCCCAGAACTTAGCA; (e) β4, GACCTGAGAAAGCTGGCTGTAAAT, 6FAM5′-TGGTGCCCTTCCCTCGCTTGC-3′TAMRA, GCAAAGCCAGGCATGAAGAA, (f) βV, GAGCAGTTCTCGGCCATGTT, 6FAM5′-CGCCGCAACGCTTTTCTGCA-3′TAMRA, CCTCGCCGGTGAACCA.

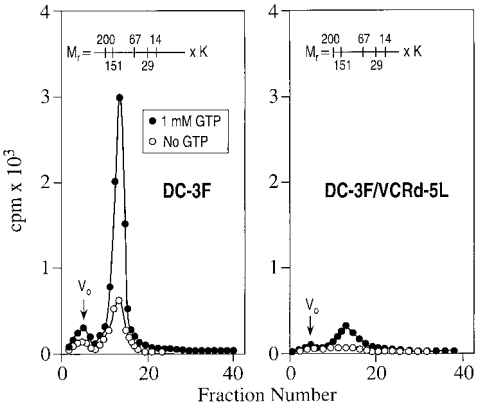

FIG. 1. Molecular- sieve HPLC of [^{3}H]VCR–tubulin complexes. Aliquots of cytosol (6–8 mg in 700 μl) in HPLC buffer (see Materials and Methods) were incubated for 10 min at 37°C with 28 nM [^{3}H]VCR for 10 min with and without GTP. The data shown are for a typical run. Additional details are provided in the text.

RESULTS AND DISCUSSION

Our earlier studies (4) with intact cells showed that there was reduced intracellular binding of [^{3}H]VCR in DC-3F/VCRd-5L cells. We now describe two additional experiments in which we were able to quantitate the large decrease in binding to tubulin extracted from these variant cells compared to binding to similarly extracted wild-type (DC-3F) tubulin. An on-rate for [^{3}H]VCR binding to tubulin was obtained by incubating cytosolic extract at 37°C with 50 nM [^{3}H]VCR in the presence of 1 mM GTP. Prior to carrying out this time-course, we showed that binding of [^{3}H]VCR by these cell extracts was, in fact, to tubulin. This was done by demonstrating (Fig. 1) that binding was GTP dependent and that this bound component migrated during HPLC molecular-sieve chromatography with an apparent kDa of 110–115, characteristic of dimeric tubulin. Tubulin–[^{3}H]VCR complex formation during the time-course experiment was measured by collecting the complex on a DE-81 filter and sampled. The resulting data showed (Fig. 2), that in the presence of GTP, there was a rapid rate of binding of [^{3}H]VCR by wild-type tubulin but the rate of binding by variant

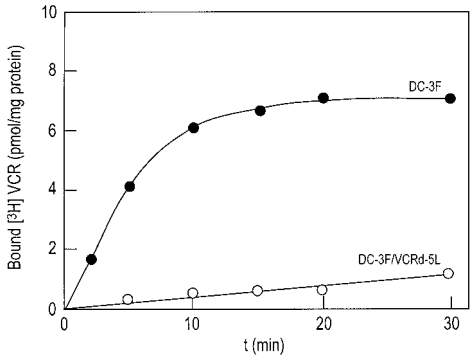

FIG. 2. Association of [^{3}H]VCR with cytosolic tubulin derived from DC-3F and DC-3F/VCRd-5L cells. Aliquots of 50–80 μg of cytosolic protein were incubated at room temp., and collected on Whatman DU-81 filters with 50 mM [^{3}H]VCR in 1 mM GTP for varying periods of time. Additional experimental details are provided in the text. The data represent averages of three experiments with standard error of the mean of $<\pm 14\%$.

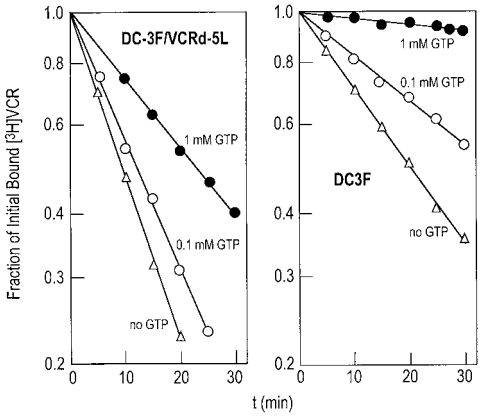

FIG. 3. Dissociation of [^{3}H]VCR–tubulin complexes in cytosol derived from DC-3F and DC-3F/VCRd-5L cells. Cells were incubated with 50 μM [^{3}H]VCR for 30 min at 37°C. Cell-free extracts were prepared and incubated at 37°C for varying periods of time with and without 0.1 or 1 mM GTP and collected on Whatman DU-81 filter. Additional experimental details are provided in the text. The data represent on average of three experiments with Standard error of the mean of $<\pm 12\%$.

tubulin by comparison was severely decreased. Also, in the absence of GTP the rate of [^{3}H]VCR binding to tubulin from DC-3F cells was severely reduced but in DC-3F/Vcrd-5L cells it was undetectable (data not shown). The results shown in Fig. 2 are quantitated in Table 1 as a 16-fold difference in on-rate for [^{3}H]VCR–tubulin complex formation (DC-3F/VCRd-5L versus DC-3F).

In the second experiment, we measured a difference in off-rate for [^{3}H]VCR–tubulin complex formation in each cell type. DC-3F and DC-3F/VCRd-5L cells were incubated with 50 μM [^{3}H]VCR for 60 min in culture medium. Cytosolic extracts were then prepared and incubated following dilution in phosphate-$MgCl_2$ buffer for 30 min at 37°C in the presence and absence of 0.1 or 1 mM GTP. At zero time and at various times thereafter, the [^{3}H]VCR–tubulin complexes were collected on DE-81 filters and sampled. The results in Fig. 3 and Table 1 show that the rate of dissociation of [^{3}H]VCR from variant tubulin was substantially greater than from wild-type tubulin. In both cases, the off-rate was greater in the absence of GTP than in its presence. However, the influence of GTP was much greater in the case of dissociation from wild-type tubulin compared to variant tubulin. Thus, while the off-rates for variant compared to wild-type tubulin differed by only 2-fold in the absence of GTP, they differed 3- and 8-fold, respectively, in the presence of 0.1 and 1 mM GTP.

Using quantitative RT-PCR, we were able to examine DC-3F and DC-3F/VCRd-5L cells for their expression of the various tubulin isoforms at the level of their mRNA. The data in Table 2 show that DC-3F cells express three α and three β isoforms to varying degree. Overall, the α-isoforms were expressed at a higher level than the β-isoforms in the wild-type cells. Among

TABLE 1

Rates of Association of [^{3}H]VCR with Wild-Type and Variant-Derived Tubulin and Dissociation of [^{3}H]VCR–Tubulin Complexes

	On-rate		Off-rate	
GTP (mM)	DC-3F (pmol/min/mg protein)	DC-3F/VCR-5L (pmol/min/mg protein)	DC-3F $t_{1/2}$ (min)	DC-3F/VCR-5L $t_{1/2}$ (min)
0	0.071 ± 0.002	0	17 ± 3	9 ± 1
0.1	ND[a]	ND[a]	35 ± 5	12 ± 2
1.0	0.82 ± 0.1	0.051 ± 1	180 ± 28	22 ± 3

Note. For on-rates, tubulin was obtained from DC-3F and CD-3F/VCR-5L cells as a cell-free extract and 50–80 μg of protein incubated with 50 nM [^{3}H]VCR in phosphate–$MgCl_2$ buffer with 1 mM GTP. For off-rates, cell-free extracts were obtained after incubation of these same cells with 50 μM [^{3}H]VCR and incubated in phosphate–$MgCl_2$ buffer with and without 0.1 or 1 mM GTP. Tubulin–[^{3}H]VCR complex formation was measured with time by filtration on DE-81 filters. Additional details are given in the text. Average of three experiments ± SEM.

[a] Not done.

TABLE 2

Relative Expression of α and β Tubulin Isoforms in Variant and Parental DC-3F Cells

Tubulin isoform	DC-3F cells	CD-3F/VCRd-5L cells
α I	26.8 ± 3.1	27.2 ± 3.4
α II	5.6 ± 0.8	20.2 ± 2.7
α VI	21.3 ± 2.6	21.7 ± 3.1
β I	2.54 ± 0.3	7.4 ± 0.9
β IV	1.9 ± 0.2	3.7 ± 0.6
β V	11.7 ± 1.3	11.9 ± 1.4

Note. The level of isoform mRNA relative to β-actin mRNA is given. Methodologic details are provided in the text. The data shown are averages of two determinations with variation between values also given.

the α-isoforms, αI and αVI were 4- to 5-fold more prominent than αII, while among the β isoforms, βII was 4- to 5-fold more prominent than βI or βIV. There were a number of differences in relative expression of these isoforms in variant DC-3F/VCRd-5L cells compared to DC-3F cells. This result reflected changes in expression of some isoforms but not of others. Among the α-isoforms in variant DC-3F cells, there was close to a 4-fold overexpression of αII, while among the β-isoforms there were 3- and 2-fold, respectively, overexpression of βI and βIV. In addition to the above, cDNA sequencing was carried out on all six isoforms in DC-3F and DC-3F/VCRd-5L cells. When compared to the published sequences, there were base-pair differences in wild-type DC-3F involving αII (C $\rightarrow$ T at nucleotide 1001), αIII (C $\rightarrow$ T at nucleotide 564), βI (C $\rightarrow$ G and G $\rightarrow$ C at nucleotides 100 and 101, A $\rightarrow$ G at nucleotide 592, C $\rightarrow$ T, T $\rightarrow$ G, and G $\rightarrow$ C at nucleotides 631, 632, and 633, A $\rightarrow$ C at nucleotide 645, and A $\rightarrow$ C at nucleotide 753) and βIV (C $\rightarrow$ G at nucleotides 236 and 237) and βV (T $\rightarrow$ C at nucleotide 51, T $\rightarrow$ C at nucleotide 496, C $\rightarrow$ G at nucleotide 915, and T $\rightarrow$ C at nucleotide 918). However, this analysis also showed that there were no base pair differences in the ORF of these isoforms between each cell type.

In summary, our results showed that there was a substantial difference in the ability of tubulin in cell-free extract from DC-3F/VCRd-5L cells to bind [^{3}H]VCR when compared to tubulin from DC-3F cells. The results also implicated the selective overexpression of one α tubulin (αII) and two β tubulin (βI and βIV) isoforms alone as the basis for the difference observed in binding of [^{3}H]VCR by tubulin in each cell-type. Sequencing of α and β isoforms cDNAs derived from DC-3F/VCRd-5L cells did not reveal any base-pair alterations in the ORF from that found in DC-3F cells. In contrast, however, it should be noted that base-pair alterations identified at two positions within the ORF of one β isoform appeared to explain Taxol resistance in 2 human ovarian cancer cell lines (16). However, the extent to which these mutational changes observed in these studies might have affected binding of Taxol to tubulin in their variant cell lines was not explored.

Alterations of tubulin in cells resistant to mitotic inhibitors have been described previously (5, 16, 17). These alterations were reported as differences in the electrophoretic mobility of either α or β tubulin isoforms. The studies presented here would appear to be more informative since they directly examined extracted tubulin from parental and variant DC-3F cells for its ability to bind VCR and documented both α and β tubulin isoform overexpression in the absence of nucleotide alterations in their ORFs. The molecular basis for the influence of isoform composition on binding of vinca alkaloids to tubulin remains to be elucidated, but it is clear that it is a factor in the acquired resistance of the variant Chinese hamster cells to this class of mitotic inhibitor.

Note added in proof. The authors wish to note that Haber, M., *et al., J. Biol. Chem.* **270,** 31269–31275, 1995, had reported the overexpression of a class II β-tubulin isoform in a Taxol-resistant murine cell line.

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