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Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling

(KB cells/vinblastine/verapamil)

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ABSTRACT **Multiple drug resistance of tumor cells is a common problem in cancer therapy. We have demonstrated that membrane vesicles from highly multidrug-resistant human KB carcinoma cell lines exhibit increased specific and saturable binding of vinblastine. To identify the molecules that bind vinblastine, membrane vesicles from multidrug-resistant cells were exposed to two analogs of vinblastine, *N*-(*p*-azido-[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine and *N*-(*p*-azido-[3-^{125}I]salicyl)-*N'*-(β-aminoethyl)vindesine, that could be photoactivated. Our studies show the specific labeling of a 150- to 170-kDa protein in membrane vesicles from two independently selected multidrug-resistant KB cell lines, which was not seen in drug-sensitive parental or revertant cell lines. The labeling of the high molecular weight protein was inhibited in a dose-dependent manner by vinblastine with half-maximal inhibition at about 1 μM. Photolabeling was also inhibited by 100 μM vincristine or 100 μM verapamil but not by 100 μM colchicine or 100 μM dexamethasone. The data suggest that the 150- to 170-kDa protein may play an important role in the multidrug-resistance phenotype.**

The emergence of multidrug-resistant (MDR) tumor cell populations is a common impediment to cancer therapy. Simultaneous resistance to structurally unrelated chemotherapeutic agents in cultured multidrug-resistant mutants correlates with reduced accumulation of these agents (1–5). To study this problem we isolated MDR human KB carcinoma cell lines by initially selecting with either colchicine (KB-C4) (6) or vinblastine (VBL) (KB-V1) (7). These MDR cell lines are resistant to colchicine, VBL, vincristine, adriamycin, and actinomycin D (6, 7) and accumulate these drugs (5) at reduced levels. Both the decreased drug accumulation and the multidrug resistance are reversed by exposure of the cell lines to verapamil (5, 6). We have investigated the biochemical basis of reduced drug accumulation using membrane vesicles from the drug-sensitive parental KB-3-1 cell line, a highly MDR cell line (KB-C4), and a drug-sensitive revertant (KB-R1) and demonstrated that membrane vesicles from MDR cells bound more VBL than vesicles from the parental or revertant cell lines (8). The specificity, saturability, trypsin sensitivity, and temperature dependence of the binding process, as well as the inhibition of [^{3}H]VBL binding by some drugs to which the cells are cross-resistant (8), suggested that a specific molecule might be responsible for increased drug binding to vesicles from MDR cells.

To identify the molecule(s) responsible for increased VBL binding, we examined the labeling of membrane vesicle proteins by two VBL analogs, *N*-(*p*-azido-[3,5-^{3}H]benzoyl)-*N'*-(β-aminoethyl)vindesine ([^{3}H]NABV) and *N*-(*p*-azido-[3-^{125}I]salicyl)-*N'*-(β-aminoethyl)vindesine (^{125}I-labeled NASV), that could be photoactivated. The synthesis and characterization of these tritiated and iodinated VBL analogs will be reported elsewhere (A.R.S. and R.L.F., unpublished results). Both analogs have been shown to have similar pharmacologic properties to VBL (A.R.S., C. J. Glover, and R.L.F., unpublished data) and to label cell-free tubulin with specificity (A.R.S., E. Hammel, and R.L.F., unpublished data). We report that both [^{3}H]NABV and ^{125}I-labeled NASV specifically label a 150- to 170-kDa protein that is overexpressed in membrane vesicles from drug resistant cells, KB-C4 and KB-V1. No labeling was found in vesicles from the drug-sensitive parental cell line KB-3-1 or the revertant KB-V1-R2. The 150- to 170-kDa protein is photolabeled with the same characteristics as shown for [^{3}H]VBL binding (8).

MATERIALS AND METHODS

Materials. [^{3}H]VBL (10.7 Ci/mmol, 1 Ci = 3.7 GBq) was obtained from Amersham. Vinblastine sulfate, vincristine sulfate, daunomycin hydrochloride, colchicine, actinomycin D-mannitol, and (±)-verapamil hydrochloride were purchased from Sigma. [^{3}H]NABV and ^{125}I-labeled NASV were synthesized and characterized (A.R.S. and R.L.F., unpublished results) using the methods of Conrad *et al.* (9) and Ji and Ji (10). Tissue culture media and supplies were obtained from GIBCO. All other chemicals were of reagent grade.

Cell Culture. Human KB carcinoma cells were obtained from the American Type Culture Collection. The parent cell line KB-3-1 was obtained by subcloning the original cell line KB-3-1. The MDR mutants were selected and maintained as described by Akiyama *et al.* (6) and by Shen *et al.* (7). The relative resistances of the cell lines used in these studies are shown in Table 1.

Membrane Vesicle Preparation. Membrane vesicles from drug-sensitive and -resistant cells were prepared essentially as described (8, 11) from cells grown in 24 × 24 cm dishes (GIBCO) under standard growth conditions (6, 7). Protein was determined by the method of Bradford (12).

Drug Binding. [^{3}H]VBL binding to membrane vesicles was assayed essentially as described by Lever (11). To minimize nonspecific filter binding, GF/C filters were preincubated at least 4 hr in 10% (vol/vol) fetal bovine serum. For binding experiments, 40 μg of membrane vesicle protein was incubated with 0.16 μM [^{3}H]VBL in 0.01 M Tris·HCl (pH 7.5),

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Abbreviations: VBL, vinblastine; NABV, *N*-(*p*-azidobenzoyl)-*N'*-(β-aminoethyl)vindesine; NASV, *N*-(*p*-azidosalicyl-*N'*-(β-aminoethyl)vindesine; MDR, multidrug resistant.

Table 1. Drug resistance of KB cell lines and binding of [^{3}H]VBL to membrane vesicles

Cell line	Relative resistance			[^{3}H]VBL bound, pmol/mg of protein		
	VBL	Colch	ADR	Total	Non-specific	Specific
KB-3-1	1	1	1	4.2	1.7	2.5
KB-C4	254	1750	159	21.8	5.4	16.4
KB-R1	3	6	4	2.2	0.8	1.4
KB-V1	213	170	458	16.8	3.5	13.3
KB-V1-R2	1.1	1.1	0.6	ND	ND	ND

KB cell lines were selected by stepwise selection in colchicine (6) or VBL (7). Relative resistance was determined from dose–response curves (6, 7). KB-3-1 D_{10} values were as follows: colchicine (Colch), 2.5 ng/ml; adriamycin (ADR), 6.8 ng/ml; VBL, 0.6 ng/ml. Binding of 0.2 μM [^{3}H]VBL to membrane vesicles was measured by filtration (8). The amount of [^{3}H]VBL bound was measured with (nonspecific) or without (total) 100 μM VBL at 23°C. Data are expressed as the mean of duplicate determinations. KB-3-1, 80 μg of protein per assay; KB-C4; 40 μg per assay; KB-R1, 92 μg per assay; KB-V1, 73 μg per assay; KB-V1-R2, 83 μg per assay; ND, not determined.

0.25 M sucrose (binding buffer) in the presence or absence of other drugs in a final volume of 100 μl for 20 min at 23°C. Samples were diluted with 4 ml of ice-cold binding buffer and collected by filtration on GF/C filters that were washed once more with cold binding buffer. Nonspecific binding was measured in the presence of excess VBL (100 μM). At the K_d concentration of ligand (1 μM) (8) specific binding represented at least 50% of total ligand bound.

Photoaffinity Labeling. Membrane vesicles were incubated with 5 nM [^{3}H]NABV (50.6 Ci/mmol) or 50 nM ^{125}I-labeled NASV (50–60 Ci/mmol) for 15 min at 25°C in the presence or absence of a 200-fold molar excess of VBL (A.R.S. and R.L.F., unpublished data). After continuous irradiation of samples at 302 nm (NABV) or 366 nm (NASV) for 10 min at 25°C or 20 min at 25°C, respectively, samples were solubilized in $NaDodSO_4$ sample buffer. Two different sample preparations were used. The [^{3}H]NABV-labeled samples were solubilized in 4% (wt/vol) $NaDodSO_4$, 0.08 M Tris·HCl (pH 6.8), 20% (vol/vol) glycerol, 4.5 M urea, 0.1 M dithiothreitol, and 0.04% bromphenol blue (A.R.S. and R.L.F., unpublished results). The samples labeled with ^{125}I-labeled NABV were solubilized according to the method described by Debenham *et al.* (13).

$NaDodSO_4$ Gel Electrophoresis. [^{3}H]NABV-labeled samples were electrophoresed on a $NaDodSO_4$ linear polyacrylamide gradient gel of 9.7% acrylamide [10% (vol/vol) glycerol] to 17% acrylamide [17% (vol/vol) glycerol] (A.R.S. and R.L.F., unpublished data). Samples labeled with ^{125}I-labeled NASV were electrophoresed on an $NaDodSO_4$/polyacrylamide/urea gel using a modification of the system described by Debenham *et al.* (13). The system was modified by

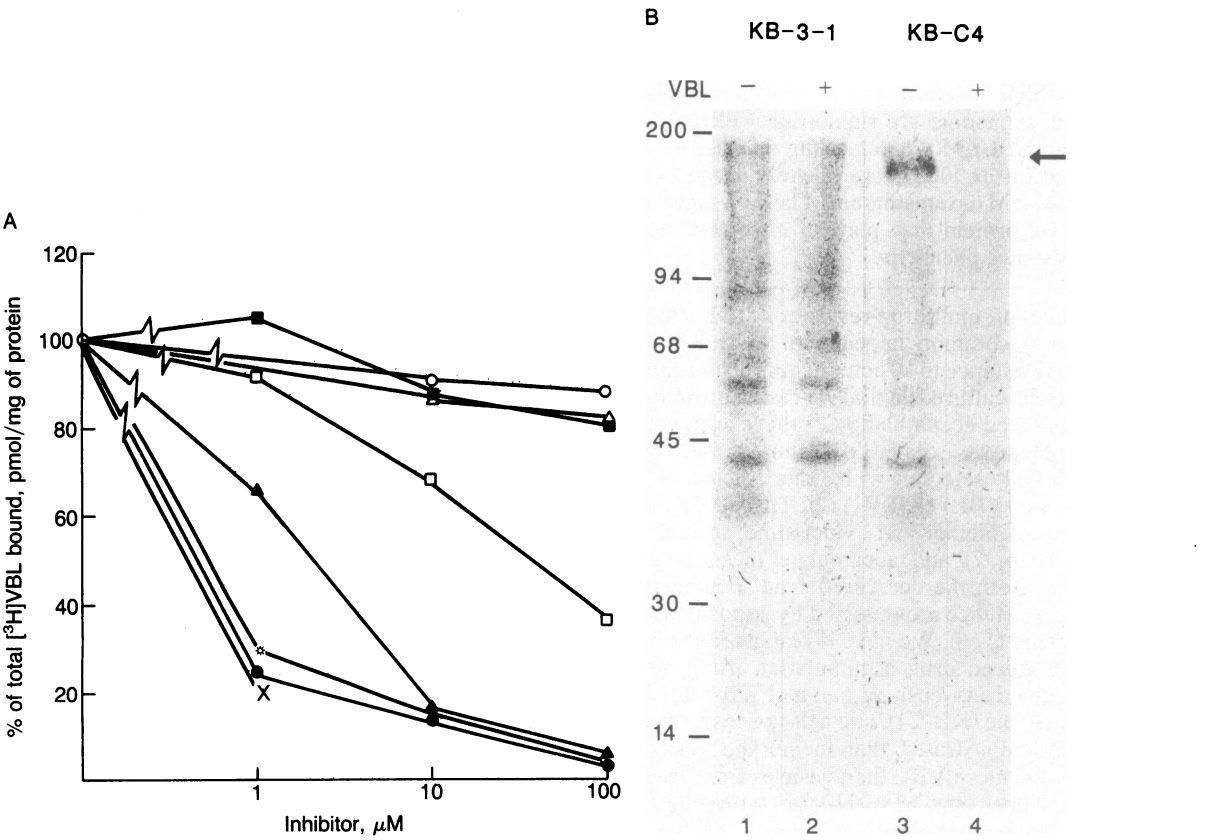

FIG. 1. Drug binding to KB-C4 membrane vesicles. (*A*) The specificity of [^{3}H]VBL binding to KB-C4 vesicles was measured using the equilibrium binding assay. KB-C4 vesicles (40 μg per assay) were incubated for 20 min at 23°C in 0.01 M Tris·HCl (pH 7.5), 0.25 M sucrose containing 0.16 μM [^{3}H]VBL and the indicated concentration of each drug in a total volume of 100 μl. Drug binding to vesicles was measured by filtration. Data are expressed as the percent of the total specific pmol of [^{3}H]VBL bound per mg of vesicle protein (total minus nonspecific binding) measured in the absence of other drugs. ●, VBL; ▲, vincristine; ☆, NABV; □, daunomycin; ■, actinomycin D; △, colchicine; ○, dexamethasone; X, verapamil. (*B*) Photoaffinity labeling of membrane vesicles. KB-3-1 (lanes 1 and 2) and KB-C4 (lanes 3 and 4) vesicles (100 μg of protein per lane) were incubated with 50 nM [^{3}H]NABV (50.6 Ci/mmol) in the presence (+) or absence (−) of 1 μM VBL for 15 min at 25°C. Samples were irradiated at 302 nm for 10 min, 25°C. Each sample was solubilized in $NaDodSO_4$ gel sample buffer, boiled for 5 min, sonicated briefly, and then electrophoresed on a 10–17% linear $NaDodSO_4$/polyacrylamide gradient gel. Molecular size markers were myosin heavy chain, 200 kDa; phosphorylase b, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; ribonuclease, 14 kDa. The position of a 150- to 170-kDa band specifically labeled by [^{3}H]NABV is indicated by an arrow.

electrophoresing proteins on a 10% polyacrylamide/4.5 M urea gel, pH 8.9, with a 4% polyacrylamide/4.5 M urea stacking gel, pH 6.8. Proteins were stained with Coomassie blue [0.25% in 50% (wt/vol) trichloroacetic acid].

RESULTS

In previous studies we have shown increased binding of [^{3}H]VBL to membrane vesicles from MDR cells (8). Binding of [^{3}H]VBL was found to be a specific and saturable process ($K_d \approx 1$ μM) that showed temperature dependence and trypsin sensitivity. As shown in Fig. 1, [^{3}H]VBL binding to KB-C4 vesicles is inhibited in a dose-dependent manner by VBL and vincristine (Fig. 1*A*). In addition, NABV, the nonradioactive photoaffinity analog of VBL, was similar to VBL in its ability to inhibit [^{3}H]VBL binding to KB-C4 vesicles. NABV, VBL, and verapamil were the most effective inhibitors of [^{3}H]VBL binding, followed by vincristine and daunomycin. Colchicine, actinomycin D, and dexamethasone did not inhibit binding in agreement with previous data (8).

For photoaffinity labeling, membrane vesicles from drug-sensitive KB-3-1, MDR KB-C4, and the drug-sensitive revertant KB-R1 cell lines were incubated with [^{3}H]NABV (50.6 Ci/mmol) with or without a 200-fold molar excess of VBL (Fig. 1*B*; A.R.S. and R.L.F., unpublished data). The time course of labeling by [^{3}H]NABV indicated that by 20 min maximum labeling had occurred (A.R.S. and R.L.F., unpublished data). After UV exposure, the samples were solubilized and electrophoresed on 10–17% gradient NaDodSO$_4$/polyacrylamide gels. As shown in Fig. 1*B*, very few protein bands were labeled in KB-3-1 vesicles (lane 1), and the labeling of those bands was not inhibited by the addition of excess VBL (lane 2). In KB-C4 vesicles a prominent band of 150–170 kDa (lane 3) was labeled with [^{3}H]NABV, and the labeling was inhibited by added VBL (lane 4).

To increase our ability to detect the labeled protein, the iodinated analog of VBL, ^{125}I-labeled NASV, was synthesized (refs. 9 and 10; A.R.S. and R.L.F., unpublished data). We examined the specific labeling by ^{125}I-labeled NASV of membrane vesicles made from MDR KB-V1 cells (7) and a drug-sensitive revertant KB-V1-R2 isolated by cloning in nonselective medium (Table 1). Membrane vesicles from KB-V1 cells bind about the same amounts of [^{3}H]VBL as vesicles from the KB-C4 cell line (Table 1). As shown in Fig. 2*A*, a protein of about 150–170 kDa (indicated by the arrow) was found in much higher amounts in KB-V1 vesicles (Fig. 2*A*, lane 2) than in the parental KB-3-1 (Fig. 2*A*, lane 1) or revertant KB-V1-R2 vesicles (Fig. 2*A*, lane 3). As shown in Fig. 2*B*, membrane vesicles from KB-V1 cells contain a protein of 150–170 kDa that was photolabeled with the iodinated analog (lane 4). ^{125}I-labeled NASV labeling was inhibited by the addition of excess unlabeled VBL (lane 3). The labeled band was not detectable in vesicles from the parental KB-3-1 (lanes 1 and 2) or in the revertant KB-V1-R2 cell lines (lanes 5 and 6) under these conditions. These results indicate that MDR cells express a protein of 150–170 kDa that specifically binds VBL.

To characterize further the labeling of the 150- to 170-kDa protein by ^{125}I-labeled NASV, KB-V1 vesicles were incubated with increasing concentrations of VBL for 20 min prior to UV exposure (Fig. 3*A*). As shown in Fig. 3*A*, 0.1 μM VBL inhibits labeling very little. At 1 μM VBL the amount of ^{125}I-labeled NASV labeling decreased by about 50% (Fig. 3*A*, lane 4) and at 10–100 μM VBL labeling was almost completely inhibited (Fig. 3*A*, lanes 5 and 6). The decrease in labeling was not due to a filtering effect of high concentrations of VBL since under standard assay conditions total conversion of the analog to product has been reported in the presence of 20 mM VBL (A.R.S. and R.L.F., unpublished data). The data were quantitated by cutting out gel slices corresponding to the bands on the autoradiogram and determining their radioactivity. Half-maximal inhibition of labeling occurred at about 1 μM VBL, which is similar to that determined for [^{3}H]VBL binding to KB-C4 vesicles (8). These data indicate that the binding of VBL to the 150- to 170-kDa protein is specific and saturable. Furthermore, these data suggest that the binding of VBL to this protein accounts for the increased level of VBL observed in vesicles from two independently selected MDR cell lines.

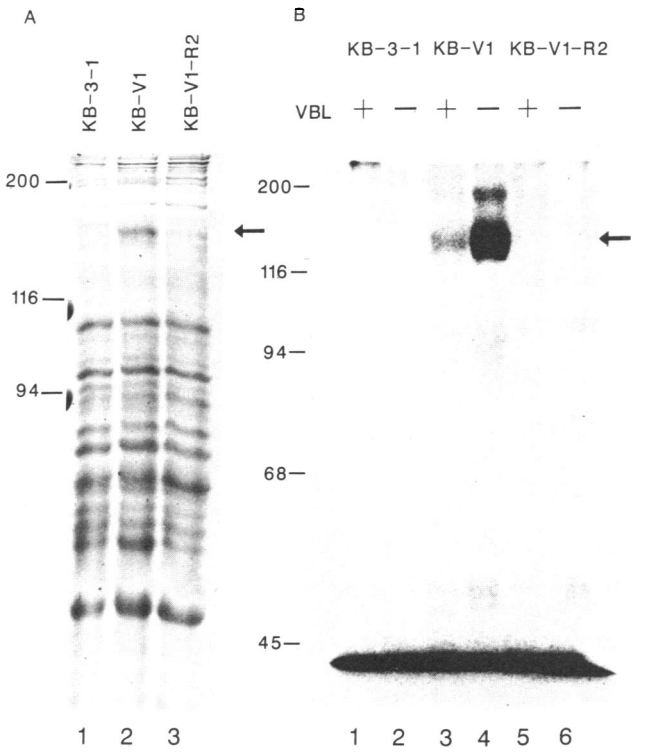

FIG. 2. ^{125}I-labeled NASV labels a 150- to 170-kDa membrane protein that is overexpressed in MDR cells. (*A*) Membrane vesicles (100 μg per lane) were electrophoresed on a NaDodSO$_4$/polyacrylamide/urea gel, using a modification of the system described by Debenham *et al.* (13). Protein was stained with Coomassie blue. The position of the 150- to 170-kDa band is indicated by the arrow. Lane 1, KB-3-1 vesicles; lane 2, KB-V1 vesicles; lane 3 KB-V1-R2 vesicles. (*B*) Membrane vesicles from drug-sensitive and MDR cells were incubated with 50 nM ^{125}I-labeled NASV (50–60 Ci/mmol) in the presence (+) or absence (−) of 1 μM VBL. After continuous photoactivation at 366 nm for 20 min, 25°C, proteins were electrophoresed on a NaDodSO$_4$/polyacrylamide/urea gel, using the system described in *A*. Lanes 1 and 2, KB-3-1 vesicles, 172 μg of protein per lane; lanes 3 and 4, KB-V1 vesicles, 230 μg of protein per lane; lanes 5 and 6, KB-V1-R2 vesicles, 196 μg of protein per lane. The autoradiogram was developed after a 48-hr exposure. The arrow indicates the position of the 150- to 170-kDa band specifically labeled in KB-V1 vesicles (lanes 3 and 4). Molecular size standards are in kDa.

We next examined the ability of other drugs to inhibit ^{125}I-labeled NASV labeling of the 150- to 170-kDa band. We have found that some agents to which KB-C4 cells are cross resistant inhibit the binding of [^{3}H]VBL to membrane vesicles (8). As shown in Fig. 3*B*, 100 μM VBL (Fig. 3*B*, lane 2) and 100 μM vincristine (Fig. 3*B*, lane 3) inhibit the labeling of the 150- to 170-kDa protein. In addition, 100 μM verapamil, which has been shown to reverse the drug-resistance phenotype in intact cells (5, 14) and to inhibit [^{3}H]VBL binding to vesicles from MDR cells, also inhibited photolabeling of the 150- to 170-kDa protein in KB-V1 vesicles (Fig. 3*B*, lane 4). Colchicine at 100 μM did not inhibit ^{125}I-labeled NASV labeling of the band (Fig. 3*B*, lane 5). These results are

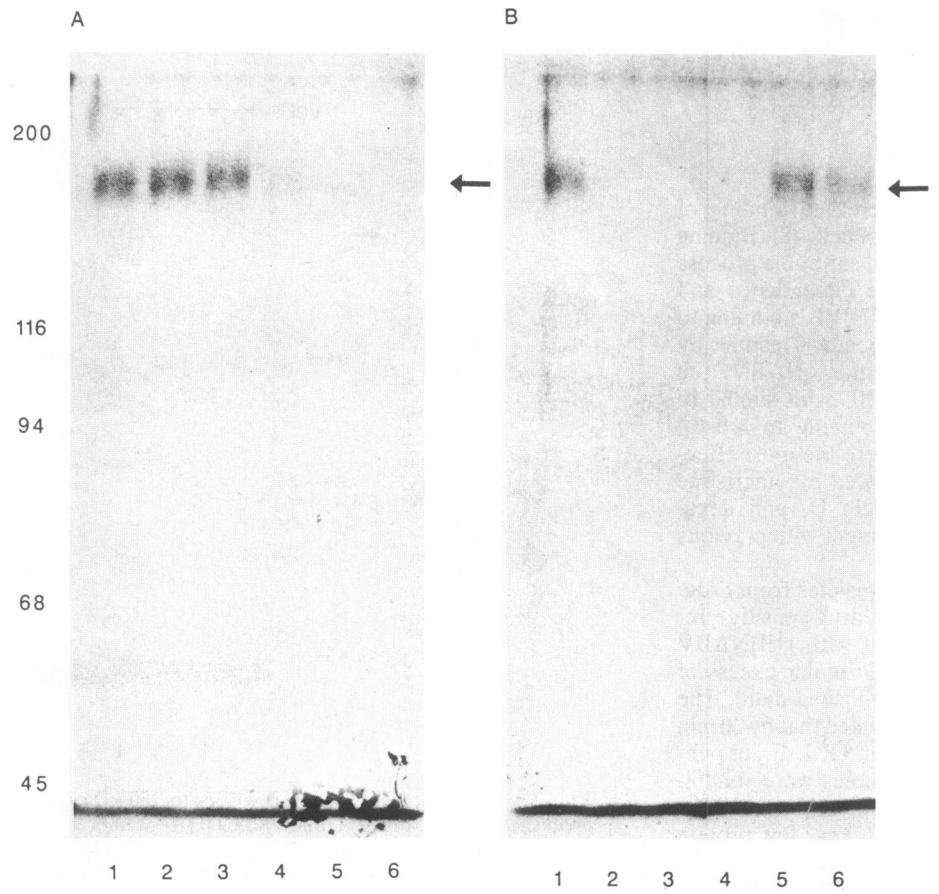

FIG. 3. Specificity of ^{125}I-labeled NASV labeling of the 150- to 170-kDa protein in KB-V1 vesicles. (*A*) KB-V1 vesicles (230 μg of protein per lane) were incubated with 50 nM ^{125}I-labeled NASV (50–60 Ci/mmol) in the absence or presence of various concentrations of VBL for 20 min prior to UV exposure. Lane 1, no added VBL. Lane 2, 50 nM VBL. Lane 3, 100 nM VBL. Lane 4, 1 μM VBL. Lane 5, 10 μM VBL. Lane 6, 100 μM VBL. After photoactivation, as described in Fig. 2, solubilized proteins were electrophoresed on a 10% polyacrylamide/4.5 M urea gels, as described above. (*B*) KB-V1 vesicles (460 μg of protein per lane) were incubated with ^{125}I-labeled NASV (50–60 Ci/mmol) in the absence (lane 1) or presence (lanes 2–6) of 100 μM of the indicated drugs. Autoradiograms were developed after a 48-hr exposure. The arrow indicates the position of a 150- to 170-kDa band seen by Coomassie blue staining. Lane 2, VBL; lane 3, vincristine; lane 4, verapamil; lane 5, colchicine; lane 6, dexamethasone. Molecular size markers at the left are in kDa.

consistent with the results of previous studies showing that colchicine did not inhibit [^{3}H]VBL binding to MDR KB-C4 vesicles (8). Finally, dexamethasone, to which drug-sensitive and -resistant cells are equally sensitive (1, 5) and which does not inhibit [^{3}H]VBL binding to KB-C4 vesicles, did not inhibit labeling of the KB-V1 150- to 170-kDa protein by ^{125}I-labeled NASV (Fig. 3*A*, lane 6).

DISCUSSION

This work demonstrates the specific labeling of a 150- to 170-kDa protein in membrane vesicles from MDR cell lines. We believe these studies are of special significance because they suggest a functional correlation between the increased levels of a membrane protein in MDR human tumor cells (7) and the level of binding of a specific chemotherapeutic agent (ref. 8 and Table 1). We have reported elsewhere (7) that the MDR KB cell lines contain an increased amount of a membrane protein that reacts with a monoclonal antibody to P170 provided by V. Ling ^{125}I-NASV (15). We do not know yet whether P170 and the ^{125}I-NASV-labeled 150- to 170-kDa bands are identical.

A second interesting point is whether verapamil treatment of MDR cells may increase VBL accumulation and reverse the multidrug-resistance phenotype by inhibiting the binding of VBL at specific sites. Such an interaction would be an unusual and not a previously described function for verapamil. Finally, the failure of colchicine to inhibit the binding of [^{3}H]VBL to vesicles or labeling of vesicles by ^{125}I-labeled NASV is still unexplained, since the MDR cell line KB-C4 is very resistant to colchicine and shows an increase in the [^{3}H]NABV-labeled protein. One interpretation of these results is that colchicine has a low affinity for the 150- to 170-kDa protein. Another possibility is that resistance to VBL and resistance to colchicine may occur by somewhat different biochemical mechanisms. Further studies on the nature of the VBL binding protein may help to resolve some of these questions.

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