Analysis of Drug Transport Kinetics in Multidrug-Resistant Cells Using a Novel Coumarin–Vinblastine Compound†

William G. Bornmann‡ and Paul D. Roepe*,‡,§

Program in Molecular Pharmacology & Therapeutics, Memorial Sloan-Kettering Cancer Center, and Graduate School of Medical Sciences, Cornell University, 1275 York Avenue, New York, New York 10021

Received May 24, 1994; Revised Manuscript Received August 9, 1994®

ABSTRACT: We have synthesized an analogue of vinblastine wherein a coumarin molecule is attached to the C17 of the vindoline moiety via a succinimide bridge (cou-VBL). cou-VBL exhibits fluorescence similar to that exhibited by coumarin. Chinese hamster ovary fibroblasts (LR73 cells) that exhibit an IC_{50} for vinblastine (VBL) of about 100 nM in growth inhibition assays are similarly sensitive to the cou-VBL compound. LR73 cells transfected with the mu MDR 3 gene that were subsequently selected on vinblastine (MDR35 cells) exhibit resistance to cou-VBL that is similar to their VBL resistance. A large change in the quantum efficiency of cou-VBL fluorescence accompanies efflux from intact cells, and comparison between cou-VBL and [^{3}H]VBL efflux from the MDR35 cells reveals that the transport kinetics of the fluorescent analogue is very similar (if not identical) to that exhibited by [^{3}H]VBL. Thus, similar to continuous monitoring of fluorescence (CMF) studies performed with the naturally fluorescent chemotherapeutic doxorubicin (Roepe, 1992), cou-VBL may be used in CMF studies aimed at rigorously defining the kinetics of VBL efflux from multidrug-resistant (MDR) cells. Initial data suggest the following: (1) A single exponential term approximates efflux from sensitive cells, whereas two exponentials are required to fit efflux from MDR35 cells. (2) The faster MDR35 term is virtually identical to the single term for the sensitive cells, whereas the other defines a process that is 5–20 times slower than passive diffusion, depending on the drug concentration. (3) The slower component has a much steeper and nearly linear dependence on drug concentration, whereas the fast passive diffusion component becomes asymptotic near 0.3 pM exchangeable drug/μg of cell protein.

Multidrug resistance (MDR[1]) hampers the treatment of cancer. Upon exposure to a single chemotherapeutic (*e.g.*, doxorubicin), many tumor cells will become resistant to the drug and concomitantly develop resistance to other chemotherapeutics that are structurally and pharmacologically distinct [*e.g.*, vinblastine, colchicine, and actinomycin D; see Biedler and Riehm (1970), Dalton *et al.* (1989), Beck (1987), and Gottesman and Pastan (1993)]. In these MDR cells, overexpression of p-glycoprotein (also called MDR protein) is frequently [but not always, see Cole *et al.* (1992)] related to some extent to the degree of resistance [see Dalton *et al.* (1989) and Roepe *et al.* (1993)]. On the basis of drug retention studies, homology between MDR protein and other transporters (Ames, 1986; Higgins *et al.*, 1990), and photolabeling studies (Cornwell *et al.*, 1986; Safa, 1988), it has been proposed that MDR protein confers the MDR phenotype by actively exporting the different chemotherapeutics out of tumor cells (Gerlach *et al.*, 1986; Gros *et al.*, 1986; Higgins & Gottesman, 1990), that is, MDR protein is believed to be a multisubstrate active drug transporter.

Recent transport studies with inside-out plasma membrane vesicles or secretory vesicles prepared from *Saccharomyces cerevisiae* (Reutz *et al.*, 1993; Reutz & Gros, 1994) appear to support the drug transporter hypothesis. Other studies (Roepe, 1992) that show that anthracycline efflux rates for cells expressing variable MDR protein are identical over a wide range of drug concentrations are inconsistent with the drug transporter hypothesis, since more active transporter in the membrane should heighten the rate of the transport reaction it presumably catalyzes. Along with observed decreased rates of drug influx (Beck *et al.*, 1983; Sirotnak *et al.*, 1986; Ramu *et al.*, 1989), these data support an alternative model wherein altered retention and distribution of drugs are the consequence of altered character and/or magnitude of plasma membrane electrochemical potential ($\Delta\mu_{H^+}$) and perhaps other phenomena that affect their passive distribution [Roepe *et al.*, 1993; see also Beck (1987), Siegfried *et al.* (1985), and Wadkins and Houghton (1993)]. Consistent with the alternative model are many documented pH_i and $\Delta\Psi$ alterations in MDR cells (Keizer & Joenje, 1989; Boscoboinik *et al.*, 1990; Thiebaut *et al.*, 1990; Roepe, 1992; Roepe *et al.*, 1993; Simon *et al.*, 1994; Vayuvegula *et al.*, 1988; Hasmann *et al.*, 1989; Gollapudi & Gupta, 1993; Wei & Roepe, 1994; Luz *et al.*, 1994) and data that suggest MDR protein modulates Cl^- conductance, perhaps as a Cl^- channel (Valverde *et al.*, 1992; Gill *et al.*, 1992; Altenberg *et al.*, 1994; Bear, 1994).[2]

In addition, model studies [see Mayer *et al.* (1985, 1986), Bally *et al.* (1985), and Praet *et al.* (1993)] clearly show that altering the magnitude of $\Delta\Psi$ or ΔpH can have dramatic and profound effects on the intravesicular concentration of

† This research was supported by grants from the Raymond & Beverly Sackler Foundation, the Wendy Will Case Fund, and a Cancer Center Support Grant (NCI-P30-CA-08748). P.D.R. is a Sackler Scholar at MSKCC.

* Address correspondence to this author at the Memorial Sloan-Kettering Cancer Center.

‡ Memorial Sloan-Kettering Cancer Center.

§ Graduate School of Medical Sciences.

® Abstract published in *Advance ACS Abstracts,* September 15, 1994.

[1] Abbreviations: coumarin, 2*H*-1-benzopyran-2-one (1,2-benzopyrone); cou-VBL, coumarin–vinblastine; VBL, vinblastine; CMF, continuous monitoring of fluorescence; MDR, multidrug resistance; $\Delta\mu_{H^+}$, plasma membrane electrochemical potential; LUV, large unilamellar vesicles; ΔpH, transmembrane H^+ gradient; pH_i, intracellular pH; $\Delta\Psi$, plasma membrane electrical potential; HBSS, Hank's balanced salt solution; BSA, bovine serum albumin; D_T, total intracellular drug concentration; D_{ex}, intracellular exchangeable drug concentration; $^{fast}D_{ex}$, fast exchangeable D_{ex} (cytoplasmic D_{ex}).

0006-2960/94/0433-12665$04.50/0

vinblastine, doxorubicin, and other hydrophobic cations/weak bases,[3] as predicted. Little experimental evidence exists to suggest that the partitioning of the neutral hydrophobic drug colchicine is directly affected by $\Delta\Psi$, but since intracellular binding of this compound, as well as the availability of the drug's target (monomeric tubulin), is highly pH dependent (Mukhopadhyay *et al.*, 1990), and perhaps even $\Delta\Psi$ dependent (Aszalos *et al.*, 1986), alterations in the character of $\Delta\mu_{H^+}$ could conceivably affect the retention of colchicine as well.

A third model (Gill *et al.*, 1992) proposes that the MDR protein functions as *both* a Cl^- channel and an active drug transporter. Thus, perhaps both active drug transport and indirect effects of other bioenergetic phenomena play a role in altering the cellular distribution of chemotherapeutic drugs. If so, it is clear that careful dissection of altered drug partitioning indirectly due to bioenergetic effects will be required to fully understand any carrier-mediated transport that may exist. Thus, from several perspectives it is clear that additional detailed drug transport studies, particularly kinetic studies of drug translocation, are extremely important.

However, the kinetic character of transmembranous translocation of hydrophobic drugs is difficult to measure. Painstaking distinction between bound and transported (*i.e.*, osmotically sensitive) drug [see, in particular, Sirotnak *et al.* (1986)] must be made. Also, since hydrophobic drugs exhibit complex behavior within the cell and the membrane bilayer (Wadkins & Houghton, 1993), distinguishing between chemically distinct populations is difficult. Most chemotherapeutic drug transport studies have relied on isotopically labeled drug analogues and fast filtration techniques (Beck *et al.*, 1983; Sirotnak *et al.*, 1986; Hammond *et al.*, 1989; Kamimoto *et al.*, 1989; Ruetz *et al.*, 1993). Because of the many controls required due to the inate hydrophobicity of the translocated compounds, the need for repetitive filtration of multiple samples, and the cost associated with isotopically labeled reagents, these methods are time consuming and expensive.

Another avenue that has the benefit of being temporally well resolved is continuous monitoring of fluorescence [CMF; see Eidelman and Cabantchik (1989)]. The method recently has been exploited in the analysis of the transport kinetics of doxorubicin for a series of MDR myeloma cells (Roepe, 1992), as well as for human lymphocytes, model DNA-containing liposomes, drug-resistant K562 cells (Tarasiuk *et al.*, 1989; Frezard & Garnier-Suillerot, 1991a,b), and MDR P388 cells (Ramu *et al.*, 1989). CMF is applicable in these cases due to (1) the intrinsic fluorescence of doxorubicin and (2) the fact that the quantum efficiency of fluorescence is quenched by nearly 6-fold [see Roepe (1992)] for intracellular drug. If fluorometric equipment is available, CMF investigations can be substantially less expensive and time consuming. With regard to kinetic analyses, the collection of thousands of data points by CMF is superior to fast filtration methods that provide few data points.

Thus, we have endeavored to synthesize an analogue of the chemotherapeutic vinblastine that (1) is highly fluorescent yet exhibits different fluorescence characteristic(s) inside *vs* outside cells or vesicles (*i.e.*, a compound that is ammenable to CMF), (2) retains pharmacologic activity and is pharmacologically similar to native vinblastine, and (3) exhibits identical transport kinetics relative to [^{3}H]vinblastine. In this paper, we report on the synthesis and characterization of a coumarin analogue of vinblastine that meets these criteria. In the course of our initial characterization of cou-VBL, we provide evidence that one kinetic component of drug efflux exists for VBL-sensitive LR73 cells, whereas two distinctly different components (fast and slow) exist for MDR35 cells (Gros *et al.*, 1991). These data are interpreted with respect to models for MDR protein function.

MATERIALS AND METHODS

Materials. Vinblastine sulfate was the kind gift of Lilly Research Laboratories (Indianapolis, IN). Vindoline (free base) and additional vinblastine sulfate was supplied by Dr. A. J. Hannart of OMNICHEM (Belgium). The vinblastine sulfate was converted to the free base by standard methods. [^{3}H]Vinblastine sulfate (16 Ci/mmol) was purchased from Amersham International. The purity of the [^{3}H]vinblastine was >95%, as assayed by HPLC with a Hypersil MOS column. 7-Amino-4-methylcoumarin was from Sigma. All other chemicals were reagent grade or better and used without further purification.

Synthesis and Chemical Characterization of Coumarin–Vindoline and cou-VBL. The synthesis of a vinblastine–coumarin conjugate (cou-VBL) and a vindoline–coumarin conjugate (cou-vin) was accomplished in a three-step sequence in which the 17-acetate was first converted into a hydroxyl that can accept a succinic bridge. This bridge can be easily conjugated to an amine using the appropriate chemistry. The first step consisted of converting vinblastine and vindoline to 17-deacetylvinblastine and 17-deacetylvindoline, respectively, using the method of Thompson (1979). Vinblastine (0.1051 g) or vindoline (0.0769 g) was refluxed in 7.5 mL of methanol containing 5 equiv of sodium carbonate under a nitrogen atmosphere. This reaction was continued until complete conversion of the starting material was observed by TLC. Once completed, the compounds were isolated and purified by centrifugal planar chromatography (Chromatotoron Chromatography) to give pure products in quantitative yields in both cases.

In the second step, 0.0965 mmol of 17-deacetylvinblastine or 0.2413 mmol of 17-deacetylvindoline was condensed with 5 equiv of succinic anhydride using pyridine and a catalytic amount (0.2 equiv) of 4-(dimethylamino)pyridine in 5 mL of dichloromethane at 0 °C. Centrifugal planar chromatography

[2] Rasola and colleagues (Rasola *et al.*, 1994) have suggested that the anomalous Cl^- conductance found in MDR cells may be the *indirect* result of MDR protein expression. If the MDR protein is a channel, it is unclear what gates its conductance. In some cells, hypotonic swelling stimulates the anomalous conductance, but in others it apparently does not (Cahalan, 1994). It is important to realize, when examining these data, that there is great variability in the methods used to generate various MDR cell lines that overexpress MDR protein, that a variety of mechanisms likely contribute to MDR, and that overexpression of MDR protein in transfectants can be unstable [see Luz *et al.* (1994)]. In any case, since the distribution and membrane permeability of Cl^- impact greatly on the maintenance of $\Delta\mu_{H^+}$, these observations of anomalous Cl^- translocation in MDR cells strongly support the alternative model.

[3] Cullis and colleagues have shown that the imposition of a −100 to −180 mV $\Delta\Psi$ concentrates doxorubicin by at least 10–20-fold and vinblastine by 5–10-fold within 20–30 min, relative to LUVs with no electrical potential (Mayer *et al.*, 1985; Bally *et al.*, 1985). Praet *et al.* (1993) obtained even larger effects (30–40-fold accumulation) when cardiolipin was included in the lipid mixture used to form the vesicles. Imposition of ΔpH = 2.9 units concentrates doxorubicin by nearly 500-fold for some vesicle preparations (Mayer *et al.*, 1986). Thus, the recent interpretation of some data (Ruetz & Gros, 1994) that strongly concludes that $\Delta\Psi$ does not affect the distribution of vinblastine at all in some systems is puzzling. Continued investigation of the role of these parameters in altered chemotherapeutic drug distribution will be informative. As demonstrated in the LUV studies, as well as in work by Slayman and colleagues (Ballarin-Denti *et al.*, 1984), the effects of protonophores, ionophores, and ion composition on various potentials, and hence these partitioning phenomena, are complex and need to be carefully calibrated in different systems.

(3:1 ethanol/ethyl acetate) gave the corresponding hemisuccinate derivatives in 91% yield for the vinblastine derivative and 94% yield for the vindoline derivative. The third step of the syntheses consisted of condensation of the hemisuccinate derivatives with 4 equiv of 7-amino-4-methylcoumarin. This was accomplished by conversion of the hemisuccinate to the isobutyl anhydride by treatment with 4 equiv of isobutyl chloroformate and 6.5 equiv of *N*-methylmorpholine in 8.0 mL of dichloromethane at 0 °C. Once the addition was complete as monitored by TLC, the dichloromethane was removed under reduced pressure at 20 °C, and the product was redissolved in 2 mL of anhydrous DMF, to which was added the aminocoumarin. This was allowed to stir at room temperature for 24 h, after which time the product was isolated by centrifugal planar chromatography (10% methanol/dichloromethane) to give the corresponding vinblastine–coumarin conjugate (cou-VBL) in 33% yield and the vindoline–coumarin conjugate (cou-vin) in 51% yield. The structures of these conjugates were verified by H NMR (see Results and Table 1), ^{13}C NMR, IR, UV, and high-resolution mass spectroscopy (data available from the authors). NMR spectra were obtained on a Brucker 400 MHz machine in $CDCl_3$, and IR spectra of KBr pellets were obtained on a Perkin-Elmer 1600 spectrometer. UV spectra were obtained in 100% EtOH using an IBM spectrometer. Analysis of these data and comparison to model vindoline, vinblastine, and 7-amino-4-methylcoumarin spectra revealed characteristic resonances, vibrations, peaks, and calculated masses that verified the anticipated structure.

Tissue Culture. Construction of the MDR cell line MDR35, which overexpresses the mu MDR 3 protein, has been described (Gros *et al.*, 1991). Northern and Western blots verified the overexpression of mu MDR 3 mRNA and protein for cells used in these experiments [data not shown; see Luz *et al.* (1994)]. The cell lines LR73 and MDR 35 were grown at 37 °C in a 5% CO_2 atmosphere in DME medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. MDR35 was maintained in the presence of 100 ng/mL vinblastine, but grown in the absence of drug for 7–10 days before transport measurements or resistance assays (see below). This did not lead to significant changes in phenotype. For transport measurements (see below), cells were harvested by trypsinization, washed, and gently resuspended in fresh medium. For Northern and Western blot analyses, the cells were harvested by either trypsinization or scraping with a sterile rubber policeman.

Fluorescence Spectroscopy. Fluorescence spectra were obtained with a Photon Technologies Inc. (PTI, New Brunswick, NJ) fluorometer interfaced to a Dell 433/L computer. Sample cuvettes were jacketed within an aluminum holder, and temperature was controlled by a circulating water bath. Cell suspensions were mixed rapidly with a magnetic stirrer situated beneath the cuvette. Excitation/emission wavelengths and the other parameters of various experiments may be found in the individual figure captions.

Drug Transport Assays. Relative retention of [^{3}H]VBL was performed essentially as described by Sirotnak and colleagues (Sirotnak *et al.*, 1986). Cells were cultured on 100 cm^2 planes, harvested by trypsinization, and washed in DME medium preequilibrated at 5% CO_2 before performing the assay. Aliquots of cells were incubated with [^{3}H]-vinblastine (200 mCi/mM) at 37 °C and 5% CO_2 (incubating [^{3}H]VBL varied from 0.5 to 10 μM; see Results). The cells were then washed with cold medium to remove external vinblastine and incubated in medium or HBSS (pH 7.3 and 37 °C) with intermittent gently swirling. The medium and cells were then collected after different incubation times, and the radioactivity in the cells, incubation (transport) medium, and wash medium was assayed by liquid scintillation spectrometry. Total cell – associated protein was quantitated by an amido black assay performed with an aliquot of the cell pellet, with BSA used as a standard. We verified that the average protein content per cell was similar for the different cell lines by counting cell suspensions.

Transport of cou-VBL was performed via CMF essentially as described previously (Roepe, 1992). Excitation was at 330 nm, emission was monitored at 395 nm, and excitation and emission slit widths were 6 and 4 nm, respectively. Additional details may be found in the appropriate figure captions. Cells for transport measurements were grown and harvested as described earlier and incubated with cou-VBL, as performed for [^{3}H]VBL (see Results for incubation concentrations). They were then washed as before and fast-injected into a 300-fold volumetric excess of buffer that was rapidly mixed and temperature controlled (Roepe, 1992).

In these measurements, it was important to have knowledge of the relative total intracellular and exchangeable intracellular drug concentrations (D_T and D_{ex}, respectively) since we ultimately wished to compare the kinetics of efflux for [^{3}H]-VBL and cou-VBL. After the average cell volume was determined (see below) and the total cou-VBL was extracted (Roepe, 1992), the total intracellular cou-VBL concentration (D_T) could be calculated. Exchangeable cou-VBL was determined by allowing the efflux to reach a steady state (30–90 min, depending on D_{ex}, see Figure 6A) and then measuring the fluorescence of the collected supernatant and comparing it to a fluorescence *vs* concentration calibration curve obtained in the same solvent. Emission intensity was a linear function of cou-VBL concentration (0–1 μM). Fast exchangeable (cytoplasmic) cou-VBL, $^{fast}D_{ex}$, was determined as described in Results.

Drug Resistance Assays and Cell Survivability. For growth inhibition assays, cells were subcultured in 96-well plates and incubated overnight at 5% CO_2. Drug at various concentrations was added the following day, and cells were grown for 3 more days at 5% CO_2. Cell growth was assayed by crystal violet staining as described by Prochaska and Santamaria (1988). We also verified that staining was a linear function of both cell number and total cell-associated protein (S. Basu and P.D.R., unpublished) for these cell lines grown under different conditions.

Measurements of Intracellular Volume (V_i). Total cell volume was calculated after determining the mean particle size of cell suspensions by the single threshold Coulter method (Kachel, 1990), performed with a ZM Coulter counter (Coulter Scientific Instruments, Hialeah, FL) as described by Roepe *et al.* (1993).

Intracellular water volume was estimated by ratioing [^{14}C]-inulin *vs* ^3H_2O dpm as described by Rottenberg (1979). Radioactivity in the supernatant and the cell pellet was quantitated with a dual-channel $^{14}C/^3H$ dpm Beckman LS 5801 program, using the autoquench-correcting capabilities of the LS 5801, which maximized counting efficiency and decreased channel spillover [see Roepe *et al.* (1993)].

Kinetic Analysis of Transport Data. CMF transport curves were collected as described above and initially stored as PTI data files on our Dell/433 PC. They were converted to ASCii files, translated by Apple file exchanger, and stored on a Macintosh IIci. These files were then imported as Sigma plot files (Jandel Scientific) for further analysis. This program

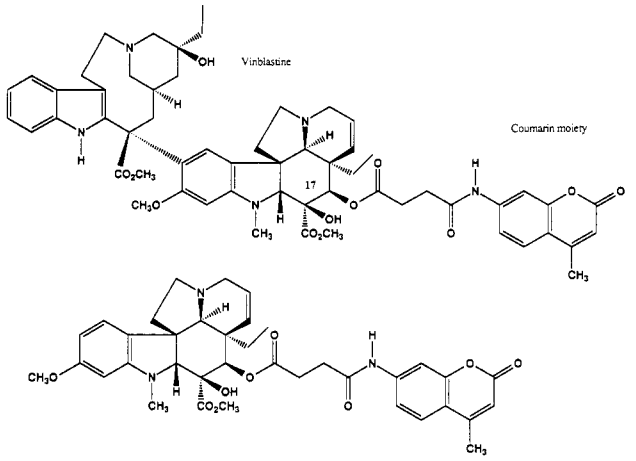

FIGURE 1: Chemical structures of cou-vin (bottom) and cou-VBL (top). Note the attachment of the coumarin moiety to the C17 of vindoline via a succinamide bridge using the 7-amino group of the modified coumarin.

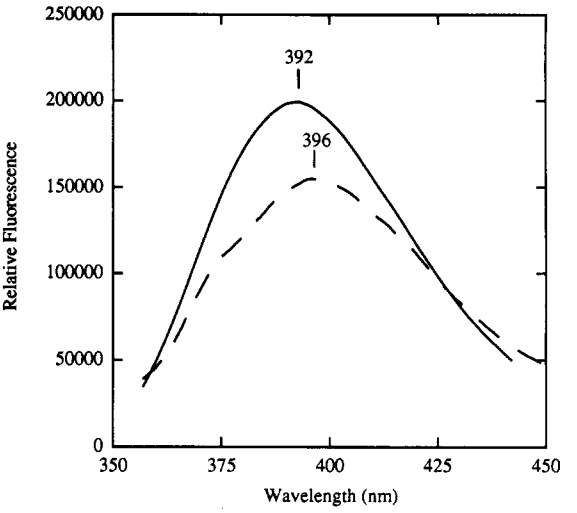

FIGURE 2: Steady state fluorescence spectra of 20 μM cou-vin (—) and cou-VBL (– –) in HBSS/10 mM glucose (pH 7.3, 37 °C). Excitation was at 330 nm (4 nm slit width).

uses an iterative least-squares procedure (the Marquardt–Levenberg algorithm) to minimize differences between the data and the calculated fit. Tolerance was initially set to 0.000 01, with even higher constraints imposed in more refined fitting; however, in most cases this tolerance provided satisfactory fits with a reasonable convergence time. Raw cou-VBL fluorescence counts were converted to a more convenient form via the equation $Y = C - [(-1)(\Delta F)]/100000$, where C is a constant, and ΔF is the change in fluorescence units. Before we assumed anything about the nature of drug translocation, initially an attempt was made (see Results) to fit each transport curve to a single-exponential function of the form $y = c - a\exp(-b/x)$, where c is a constant, a and b are variables, and x and y represent time and cou-VBL, respectively. For the sensitive cells, this fit was satisfactory, converging with tolerance satisfied in <20 iterations, with reasonable variable dependencies (see Results). For the MDR cells, this clearly was not the case (see Results); thus, a second exponential term was added, and this proved sufficient to accurately model the data. Rate constants from these exponential fits immediately provided important kinetic information (see Results).

Having information pertaining to the mono- or biexponential character of drug translocation, we could also reliably estimate rates with a linear fit to the first 60 s of the efflux curve (IR60), which was found in general to be nearly linear (see Results). In previous work in other laboratories, this was typically performed "by eye" for transport curves constructed using [^{3}H]drug analogues where the number of data points is limited. However, because our CMF data typically represents 5000–10 000 pairs of numbers, analytical curve fitting, although certainly more time consuming and tedious, was possible and more quantitative.

RESULTS

Due to the expense inherent in the chemical modification of vinblastine, we initially synthesized a 7-amino-4-methylcoumarin adduct of vindoline (Figure 1) and tested whether attachment of the fluorophore to vindoline affected its fluorescence properties (Figure 2). Fortuitously, excitation and emission maxima were not significantly affected by the addition to vindoline, although a slight decrease (of about 15%) in the quantum yield of emission was noted. This provided the impetus for further synthesis of cou-VBL (see Materials and Methods for details of the syntheses). Again, as shown in Figure 2, the general fluorescence characteristics of cou-VBL are not significantly different from those of cou-vin, except for an additional 25% decrease in quantum efficiency and about a 4 nm red shift in emission. Due to the intense native fluorescence of 7-amino-4-methylcoumarin, the cou-VBL adduct was found to exhibit sufficient fluorescence to merit continued use in CMF studies (see below).

We performed detailed UV, IR, HPLC, and NMR characterization of cou-VBL to verify its chemical integrity (see Materials and Methods). Table 1 lists the proton resonances from an NMR spectrum of our purified product, obtained in $CDCl_3$ using a Brucker 400 MHz spectrometer. Key assignments support the structure presented in Figure 1 [see Bhushana Rao *et al.* (1990)]. In high-resolution mass spectrometry, a mass of 1026.4810 Da was obtained (calculated mass of the $C_{58}H_{68}N_5O_{12}$ compound is 1026.4860 Da). Finally, HPLC on a C18 column (1 mL/min, 90:10:1 MeOH/H_2O/Et_3N) gave a single peak (R_f = 4.3 min). These data in their entirety verify the integrity of our cou-VBL.

We next tested whether cou-VBL retained reasonable pharmacological activity, relative to vinblastine (Figure 3). Although more detailed work remains to be done with respect to analysis of the subcellular distribution of cou-VBL, we can conclude from these initial data that cou-VBL is likely to have similar tubulin binding characteristics, since LR73 cells exhibit a similar IC_{50} for cou-VBL, relative to VBL, and since the MDR cell line MDR35, previously shown to be resistant to VBL (Gros *et al.*, 1991), exhibits resistance to cou-VBL. These observations are consistent with the design of the fluorescent compound, since the integrity of the pharmacologically active portion of VBL remains intact and unmodified (see Figure 1).

To determine whether cou-VBL was suitable for CMF-based measurements of VBL drug efflux, we first compared [^{3}H]VBL and cou-VBL efflux from MDR35 cells, which express high levels of the putative drug transporter mu MDR 3 protein (Figure 4). [^{3}H]VBL efflux measurements were performed with aliquots of preloaded cells using standard techniques (see Materials and Methods), and the CMF curve was produced by rapid injection of cells preloaded with cou-VBL, as previously described for doxorubicin CMF measurements (Roepe, 1992; see below). Control experiments (see Materials and Methods) verified that the levels of exchangeable intracellular drug (D_{ex}) were similar in the two prepara-

Table 1: ^{1}H NMR Data for cou-VBL and cou-vin in $CDCl_3$[a]

cou-VBL	cou-vin
δ 0.792, t, J = 7.3 Hz, 3H	δ 0.453, t, J = 7.3 Hz, 3H
δ 0.891, t, J = 7.4 Hz, 3H	δ 1.136, sextet, J = 7.2 Hz, 1H
δ 1.255–1.461 complex overlapping multiplets	δ 1.606, sextet, J = 7.3 Hz, 1H
δ 1.600 broad multiplet, 1H	δ 2.040, sharp singlet, 3H
δ 1.722–1.863 complex overlapping multiplets	δ 2.135–2.354 unresolved multiplet
δ 2.164, pentet, J = 5.9 Hz	δ 2.490, broad quartet, J = 8.4 Hz, 1H
δ 2.285, d, J = 13.4 Hz	δ 2.622, broad doublet, J = 16.5 Hz, 1H
δ 2.412, sharp singlet 3H	δ 2.623, sharp singlet, 1H
δ 2.651, sharp singlet 1H	δ 2.670, sharp singlet, 3H
δ 2.714, sharp singlet 3H	δ 2.719, broad doublet, J = 16.6 Hz, 1H
δ 2.696–2.961, complex overlapping multiplets	δ 2.804–2.816, complex multiplet
δ 3.146–3.222, complex overlapping multiplets	δ 3.195, dd, J = 4.2, 16.5 Hz, 1H
δ 3.267–3.376, complex overlapping multiplets	δ 3.387–3.397, complex multiplet, 1H
δ 3.600, sharp singlet, 3H	δ 3.754, sharp singlet, 1H
δ 3.733, sharp singlet, 1H	δ 3.775, sharp singlet, 3H
δ 3.7861, sharp singlet, 6H	δ 5.183, d, J = 10.2 Hz, 1H
δ 3.958, broad triplet, J = 13.7 Hz, 1H	δ 5.484, sharp singlet, 1H
δ 5.279, d, J = 10 Hz, 1H	δ 5.679, dd, J = 3.9, 10.2 Hz, 1H
δ 5.505, s, 1H	δ 6.078, d, J = 1.9 Hz, 1H
δ 5.693, dd, J = 3.9, 10 Hz, 1H	δ 6.185, sharp singlet, 1H
δ 6.130, s, 1H	δ 6.297, dd, J = 2, 8.15 Hz, 1H
δ 6.193, s, 1H	δ 6.883, d, J = 7.2 Hz, 1H
δ 6.625, broad singlet, 1H	δ 7.488, d, J = 8.6 Hz, 1H
δ 7.076–7.172, m, 3H	δ 7.590, d, J = 8.7 Hz, 1H
δ 7.264, sharp singlet, 1H	δ 7.617, singlet
δ 7.511, d, J = 7.4 Hz	δ 8.440, broad singlet, 1H
δ 7.522, sharp singlet, 1H	δ 9.752, broad singlet, 1H
δ 7.6212–7.652, m, 2 1H	
δ 8.021, gs, 1H	
δ 8.396, gs, 1H	
δ 10.119, gs, 1H	

[a] Resonances of catharanthine protons (*e.g.*, H-9′ at 7.522 and NH at 8.021) are evident, as are those of the vindoline moiety [*e.g.*, H-9 at 6.625, H-21 at 2.651, and CH_3-18 at 0.891; see Bhushana Rao *et al.* (1990)]. Supplementary ^{13}C NMR and IR data are available from the authors.

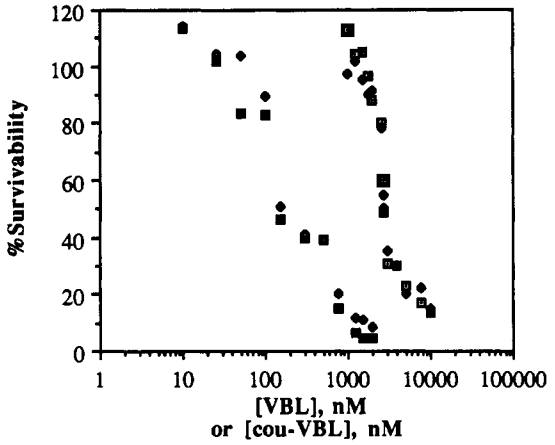

FIGURE 3: Growth inhibition in the presence of VBL and cou-VBL for LR73 (■ and ◇, respectively) and MDR35 cells (□ and ◆, respectively). Each point is the average of three determinations at a single drug concentration. VBL sulfate stock solutions were made in water; cou-VBL was initially dissolved in anhydrous DMSO before addition to culture medium. See Materials and Methods for additional details.

tions. Figure 4 shows that, at similar levels of D_{ex}, the overall kinetics of [^{3}H]VBL and cou-VBL efflux are very similar (initial rate of about 0.03 pM/μg of protein/min, as calculated

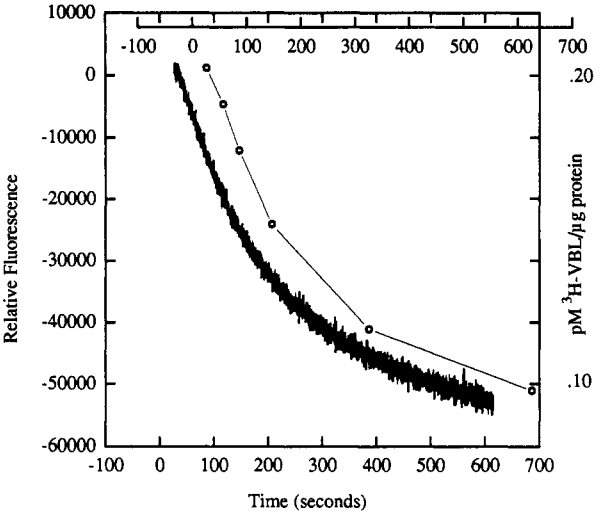

FIGURE 4: Comparison of [^{3}H]VBL efflux (○) obtained via standard fast filtration techniques (see Materials and Methods) to cou-VBL efflux obtained via a CMF experiment (solid trace). In both cases, MDR35 cells were preloaded with 10 μM of the vinca compounds for about 45 min, and aliquots of the loaded cells were assayed to determine that the different preparations harbored equal D_{ex} (using known specific activity and a cou-VBL fluorescence calibration curve; see Materials and Methods). The preloaded cells were then washed, and aliquots were fast-diluted into a 300-fold volumetric excess of buffer. Actual CMF data collected after the first dilution of cells (see Figure 6) were inverted for comparison purposes. In the case of the [^{3}H]VBL experiments, cells were separated from the transport medium at different times, and radioactivity in the supernatant and cell pellet was measured by liquid scintillation photometry. CMF data represent one trace (data shown are representative of three experiments), and each point in the [^{3}H]VBL efflux curve is the average of three determinations (SE (standard error) < 6%).

from a straight line fit to the first 60 s of the transport curves). Note efflux of cou-VBL produces an *increase* in fluorescence over time (see Figure 6); these CMF data are inverted for comparison purposes. Importantly, since these cells were washed before rapid dilution into transport medium (see Materials and Methods), these rates correct for nonspecific binding of the two compounds [see Sirotnak *et al.* (1986)]. Similar very good agreement is observed at other values of D_{ex} (not shown; see below). These data highlight the very similar kinetic behavior of [^{3}H]VBL and cou-VBL transport from these cells, thus providing the impetus for further applications of CMF.

In order to use the CMF method for analyzing drug efflux, the monitored change in fluorescence of the compound associated with exit from cells or vesicles must be shown to be due to transport of the compound and not to other events associated with membrane binding, intracellular partitioning, etc.[4] The data in Figure 4 clearly support the contention that the change in cou-VBL fluorescence we are measuring is due to efflux. In previous work (Roepe, 1992), a complementary approach was followed for doxorubicin by performing the simple experiment that we now also show for cou-VBL (Figure 5). Aliquots of cells were preloaded with cou-VBL as described, and efflux was initiated by dilution into drug-free medium. After different incubation times, efflux medium and cells were separated by fast centrifugation (see Materials and Methods), similar to the procedure for [^{3}H]VBL efflux assays. Fluorescence in the transport medium and cell pellet (*i.e.*, retained drug) was then measured and plotted *vs* time of incubation (Figure 5). The sum of these two curves is also shown in Figure 5. In addition, an aliquot of the cells preloaded with cou-VBL under identical conditions was fast diluted into a cuvette that was mixed rapidly, and fluorescence was

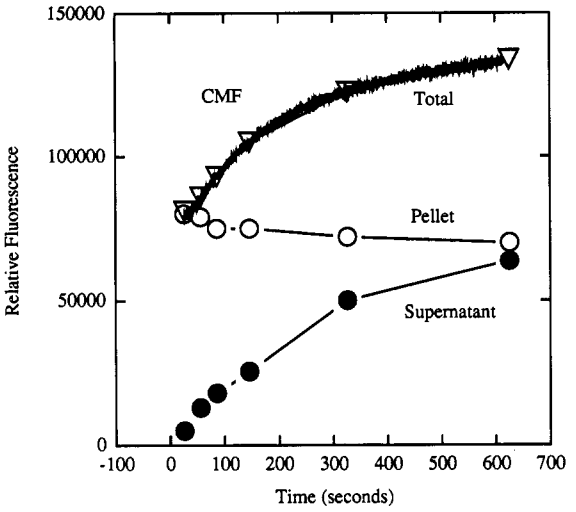

FIGURE 5: Demonstration that the increase in cou-VBL fluorescence during the CMF experiment is due to efflux of the fluorescent analogue and not to other effects. Aliquots of 1×10^6 cells were preloaded with 10 μM cou-VBL for 45 min and then fast-diluted into a 300-fold volumetric excess of buffer. One aliquot was fast-diluted into the CMF chamber, and cou-VBL emission was continuously monitored (top solid trace; the instantaneous departure from the baseline upon fast dilution of the preloaded cells (see Figure 6) has been removed for clarity). Six other aliquots were diluted into centrifuge tubes, cells were separated from the transport medium at different times, and the fluorescence in the supernatant (●) and cell pellet (○) was measured. Fluorescence of the cell pellets was obtained in rapidly mixed buffer at 4 °C to prevent additional efflux. Note that the sum of the supernatant and pellet curves (▽) exhibits similar magnitude and curvature relative to the CMF curve, proving that the signal from the CMF experiment represents efflux. Thus, if the pellet data are expressed as $y = c - ae^{xt}$, and the supernatant data as $y' = be^{x't}$, then the CMF curve (equal to the sum) can be verified to be equal to $y + y' = c + (b - a)e^{xt}$ ($x = x'$ since the rate of disappearance from the cells is the same as the rate of appearance in the supernatant; see Roepe (1992), for a more detailed discussion). Note that scatter from the cells (about 10^4 relative fluorescence units) needs to be subtracted from the pellet data before estimating intracellular cou-VBL.

monitored over time (Figure 5, top). As can be seen, both the height and curvature of the CMF curve are very similar to those of the curve produced by the addition of pointwise fluorescence data collected by separation of the cells and efflux medium at different incubation times. As described in detail previously for doxorubicin (Roepe, 1992), these data clearly indicate that the rather large change in cou-VBL fluorescence intensity observed in the CMF experiment upon the fast dilution of cells preloaded with the compound is essentially entirely due to efflux from the cell. We can calculate, by comparing the pellet data to the supernatant data, that the

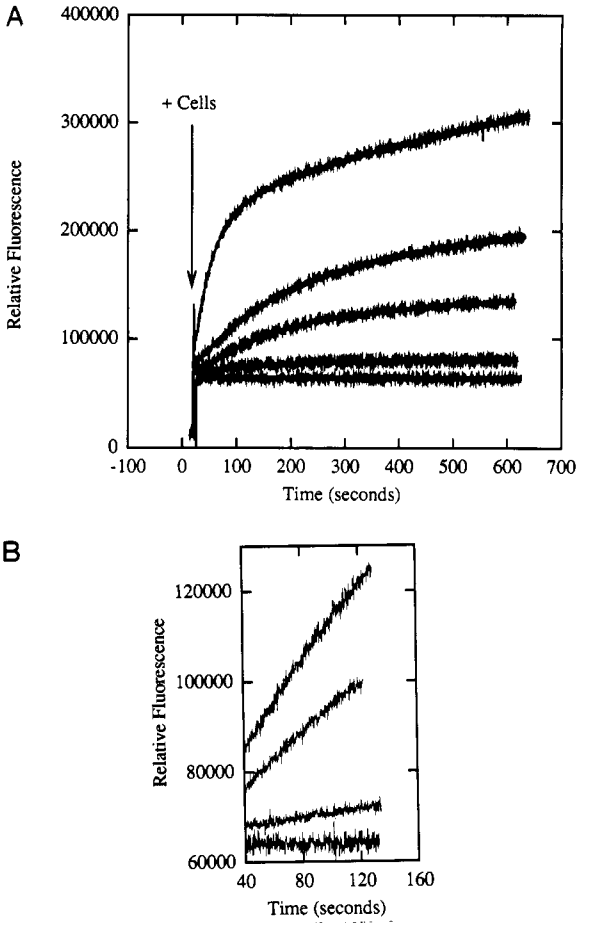

FIGURE 6: CMF curves for MDR35 cells loaded to different levels of D_{ex}; 20 points/s were collected. Cells were loaded in the presence of (from top) 80, 20, 10, 1, and 0.2 μM cou-VBL for 45 min. We estimate (see Materials and Methods) that D_{ex} for these samples corresponds to about 0.302, 0.138, 0.063, 0.022, and 0.0013 mM, respectively. Thus, our measurements span the range of attainable D_{ex} for whole cells and the concentrations of VBL previously examined in efflux experiments (see also Figure 10B). In part A, instantaneous departure from the baseline near 0 time indicates rapid injection of the preloaded cells. Most transport curves achieved steady state (plateaued) within 30–40 min, although the top trace took longer due to a higher portion of reversibly bound cou-VBL (see Figure 9). Although very shallow, the bottom curve does not indeed exhibit a positive slope. In part B, we expand the bottom four traces shown in part A to demonstrate the linear apparent initial rates of efflux. Note that the sample preloaded in 0.2 μM cou-VBL has measureable, but relatively little, exchangeable drug, whereas the sample loaded in the presence of 1 μM has an easily measurable exchangeable portion. The measurement of low levels of exchangeable drug can be enhanced by increasing slit widths (not shown; the data presented were all obtained at the same settings, as were all data used in the kinetic analyses). Samples were injected at time = 35 s; the initial instantaneous departure from the baseline (see part A) is not shown.

fluorescence of extracellular cou-VBL is about 7 times more efficient than that of intracellular cou-VBL. Similar to previous work with doxorubicin (Roepe, 1992), model studies (data not shown) indicate mild differences in pH_i and interactions with lipid could not conceivably account for the huge change in quantum efficiency apparent in this experiment, which clearly is due to efflux.

Since one ultimate application of cou-VBL drug efflux studies will involve detailed kinetic comparison of efflux from MDR cells overexpressing the putative drug pump p-glycoprotein to efflux from drug-sensitive cells not expressing the protein, we performed several initial experiments toward this end (Figure 6A,B). As shown in Figure 6A, we are able to acquire transport curves for the efflux of cou-VBL from

[4] It is important to recall that the *time* any given hydrophobic drug molecule will spend in a membrane during a transport assay is limited and governed more by any existing drug concentration gradient than by relative hydrophobicity, which merely ensures that a greater number of net displacements in the direction of the concentration gradient will occur during random diffusion. Also, since the relative *volume* of the cell membrane is 10^{-3}, relatively little drug resides within the membrane at equilibrium (10^{-3}–10^{-7} of that within the cell), regardless of hydrophobicity (*e.g.*, with a partitioning coefficient for vinblastine near 2, it is predicted that at most 0.2% of total cell-associated vinca resides within the membrane at equilibrium; when one considers affinities for tubulin and other intracellular compartments, it is reasonable to assume that this is an overestimate). See Coley *et al.* (1993) to verify that this is indeed observed to be the case for anthracyclines, and note that our preliminary microscopy data with cou-VBL (not shown) supports this for the vinca compound as well. These concepts may appear anti-intuitive, but they are fundamental and support the observation that contributions to the CMF curve due to cou-VBL/membrane interactions are minimal.

MDR 35 cells at a variety of concentrations that span the practical range of D_{ex} used previously in [^{3}H]VBL measurements (Beck *et al.*, 1983; Sirotnak *et al.*, 1986; Hammond *et al.*, 1989; Yang *et al.*, 1990) and that straddle a previously calculated K_m for vinblastine transport hypothesized to be mediated by MDR protein (Horio *et al.*, 1988; see also Figure 10B below). As found previously for efflux of the related vinca alkaloid vincristine (Sirotnak *et al.*, 1986), efflux of cou-VBL at relatively high levels of D_{ex} (top curve, Figure 6A) is easily seen to be multicomponent in character, whereas drug efflux from the MDR cells at lower values of D_{ex} (at least by visual inspection) appears to be better fit by a single exponential term (see below). As suggested previously (Sirotnak *et al.*, 1986), the slow component seen most clearly in the upper CMF trace is likely due to the slow release of reversibly bound drug, whereas the faster initial component is likely due to release of the immediate osmotically sensitive portion (what we define as $^{fast}D_{ex}$; see below). In Figure 6B, we show the apparently linear initial rates of cou-VBL efflux from the MDR cells at several low values of D_{ex}. Analysis of these represents one way to compare efflux kinetics for MDR and sensitive cells.

Before attempting this, however, we more quantitatively compared the kinetics of cou-VBL efflux from the MDR and sensitive cells by performing iterative least-squares curve fitting of our data using the Marquardt–Levenberg algorithm (see Materials and Methods). As shown in Figure 7A, efflux from LR73 cells is nicely fit by a single exponential term with a rate constant of −52.1 (see Figure 7 caption; note that residuals from the fit lie within the noise of the original data; note also increasing ΔF *vs* time has been converted to a more convenient form; see Materials and Methods). In contrast, two exponentials are required to fit the data for the MDR35 cells (Figure 7B); one exponential is clearly insufficient since it is accompanied by residuals with clearly defined shape (bottom window, Figure 7B). In Figure 7, we show data obtained for LR73 and MDR 35 cells predicted to have the same initial concentration of freely exchangeable (cytoplasmic) drug at time = 0.[5] One exponential term for the fit to the MDR35 data has a rate constant of −57.0, which is very close to the rate constant from the single-exponential fit to LR73, whereas

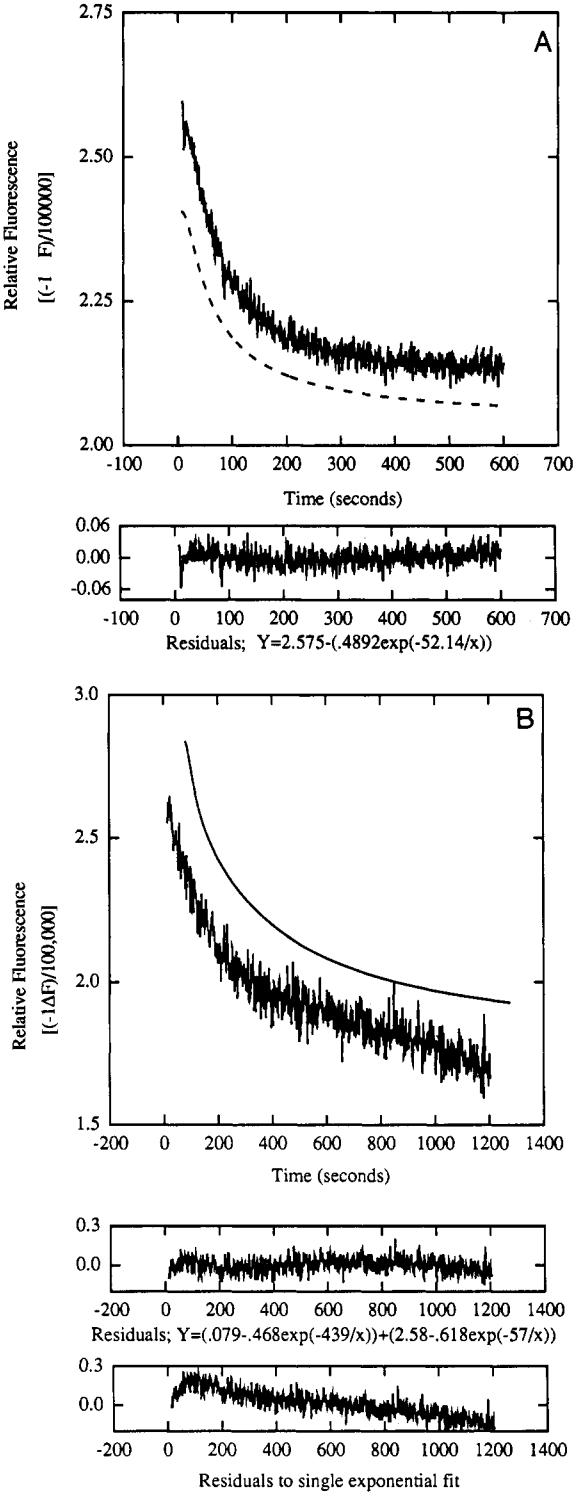

FIGURE 7: Exponential fits of cou-VBL efflux CMF data for (A) LR73 or (B) MDR35 cells. Cells were loaded in the presence of 10 μM (A) or 20 μM cou-VBL (B) (see footnote 4) to produce nearly equal $^{fast}D_{ex}$ values, as measured by fast dilution and separation of the supernatant at 3 min. Raw data are shown, as is the computed fit for LR73 (dashed, A) and MDR35 (solid, B). Equations describing the data are below the residual windows. Also shown are the residuals from these fits; note that these lie within the noise level of the original data. The LR73 trace is nicely fit by a single exponential, but the MDR35 trace clearly requires two terms (see Materials and Methods) since residuals from a single-exponential fit using the same initial parameters leave a residual with a clearly defined shape (bottom window, B).

the other rate constant reveals a much slower transport process (rate constant of −439). Figure 8 plots these two computed components separately and compares them to the computed

[5] We wished to compare the trace in Figure 7A to a trace obtained for MDR35 cells harboring the same initial cytoplasmic concentration of cou-VBL. This is because the rate of passive diffusion is highly dependent on the cytoplasmic free concentration, and we wished to separate the passive diffusion that exists in the MDR cells from any transport mediated (directly or indirectly) by MDR protein. Since the efflux of exchangeable drug from LR73 cells begins to plateau sharply in about 3 min (see Figure 7A), and since this is likely due to the fast diffusion of cytoplasmic drug (Sirotnak *et al.*, 1986), we preloaded MDR35 cells in different concentrations of cou-VBL near those used for the LR73 cells for different periods of time (see the caption to Figure 7B) and then measured D_{ex} at 3 min by fast diluting cells into buffer with no drug, separating the cells from transport medium, and quantitating cou-VBL fluorescence in the supernatant (linear with concentration between 0.1 and 1000 nM; not shown). Knowing that the relative volume for the LR73 and MDR35 cells is similar (see Materials and Methods), we could determine loading conditions that give similar $^{fast}D_{ex}$ for the MDR cells, relative to the LR73 cells. This procedure was necessary since the MDR35 cells appear to harbor at least two populations of cou-VBL that exhibit different transport kinetics (see below). We did not want to over- or underestimate $^{fast}D_{ex}$ for MDR35, and we did not want the $^{slow}D_{ex}$ population from the MDR35 cells to interfere with our analysis of the kinetics of transport for $^{fast}D_{ex}$. As shown below, since the efflux of $^{slow}D_{ex}$ from MDR35 is minimal for the first several minutes, the estimate of $^{fast}D_{ex}$ provided by the separation of cells and supernatant at 3 min after dilution is reasonable. Another complementary approach, that of back-extrapolation of different transport curves obtained under different loading conditions, will be reported elsewhere.

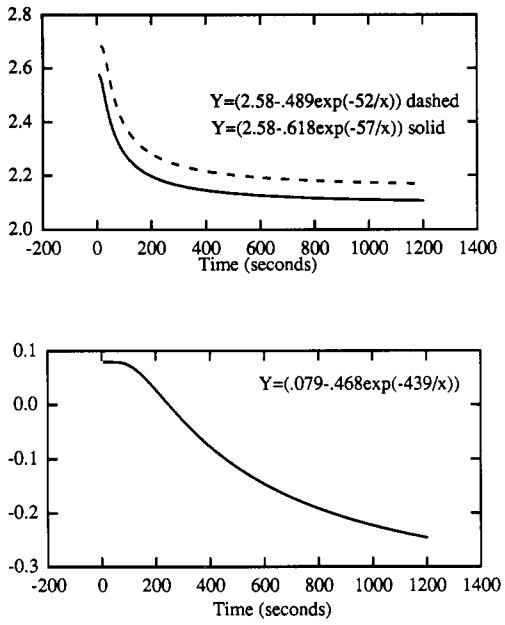

FIGURE 8: Plots of the individual components of the two-exponential-term curve fit for MDR35 (top solid line is the fast component, bottom is the slow), as well as the single-term fit for LR73 (dashed line in top panel). Note the very good agreement between the single term for LR73 and the fast term for MDR35.

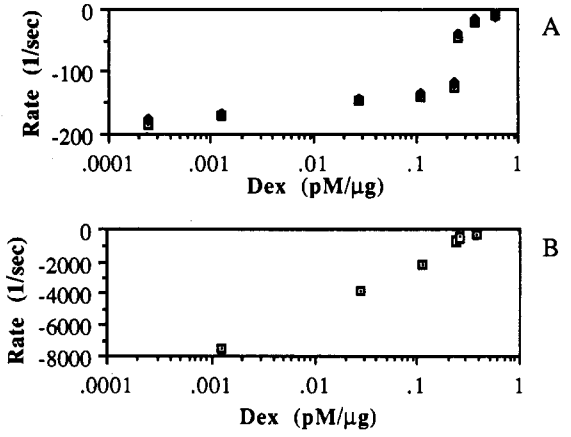

FIGURE 9: Concentration dependence of the rate constants from the single-term fit for LR73 (solid diamonds) and the two terms for MDR35 (fast term in A, open squares; slow term in B). Note the stronger concentration dependence for the slow term, which essentially disappears at lower concentrations of D_{ex}. Each point is the average of three calculated rates at a given D_{ex}; SE ≤ 4%. Conversion of the slow rate constants to half-times followed by plotting *vs* D_{ex} reveals that the slow component does not follow Michaelis–Menten behavior; a biphasic curve that is linear over two broad concentration ranges is obtained (not shown).

single-exponential fit to the LR73 trace obtained at the same $^{fast}D_{ex}$. Thus, this analysis reveals two kinetically distinct processes for the MDR cells, whereas only one simple decay process is evident for the sensitive cells.

Figure 9A,B plots the rate constants obtained from fits to LR73 and MDR35 data obtained for cells harboring a variety of similar $^{fast}D_{ex}$ values. Note the very different concentration dependencies for the two components for the MDR35 cells, as well as the similarity between the concentration dependencies for the LR73 and fast MDR35 rate constants. Interestingly, Figure 9A reveals a sigmoidal shape for the rate *vs* [drug] plot for both LR73 and MDR35 cells (see Discussion).

We thus find that MDR35 cells harbor either two distinct populations of cou-VBL that exhibit different efflux kinetics

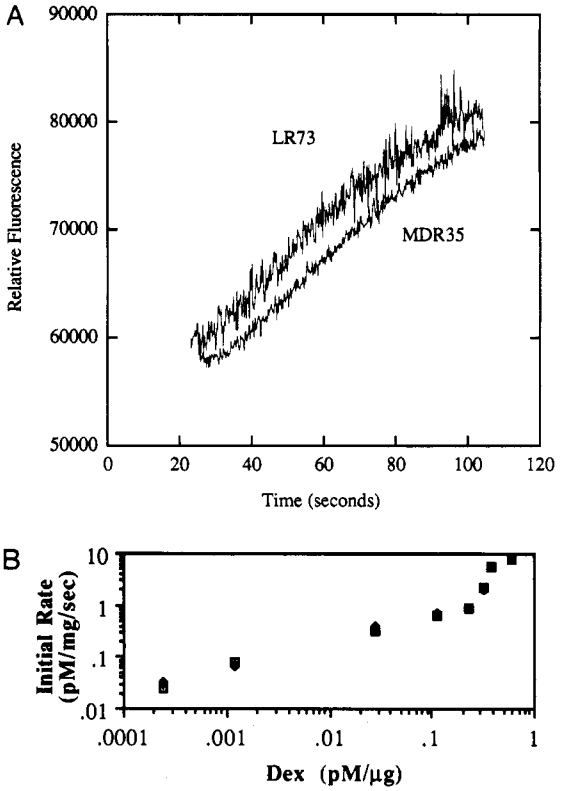

FIGURE 10: (A) Comparison of the linear apparent initial rates of cou-VBL efflux for drug-sensitive (LR73, top) and drug-resistant (MDR35, bottom) cells. In this example, both cell samples are estimated to harbor 15 μM $^{fast}D_{ex}$ (see Materials and Methods). (B) Concentration dependence (in pM/μg of cell protein) of the apparent initial rate (IR60) of cou-VBL efflux from MDR35 (open squares) and LR73 (closed diamonds) cells harboring similar $^{fast}D_{ex}$ values. Note the similarity between the cell types and the sigmoidal shape that precludes application of Michaelis–Menten treatments of the data. Each point is the average of three calculated rates; SE ≤ 5%. Note that the data cover the entire range of drug concentrations previously examined in MDR cell drug transport studies (estimated 10^{-7}–10^{-3} M D_{ex}).

or two kinetically distinct efflux processes. The first is clearly very similar, if not identical, to LR73 cou-VBL efflux (Figure 9A). The second represents a population or process that is unique to MDR cells. Two possibilities immediately spring to mind: this component represents either drug transported by the MDR protein or a population of vinca alkaloid with different binding characteristics, as hypothesized previously (Sirotnak *et al.*, 1986). Both possibilities are entertained in the Discussion.

Finally, Figure 10 shows an alternative method for comparing cou-VBL efflux kinetics for the two cell types. In Figure 10A, we show the apparent initial rates of cou-VBL efflux from drug-sensitive LR73 cells and MDR35 cells that harbor the same estimated $^{fast}D_{ex}$. Although more drug is effluxed from the MDR35 cells when the two cell types are preloaded to the same D_T [data not shown; see Roepe (1992) and Beck *et al.* (1983)], the apparent initial rates of efflux are virtually identical. Apparent initial rates of efflux (IR60 calculated from a straight line fit of the first 60 s of transport) for curves obtained at other values of $^{fast}D_{ex}$ indicate that the similarity in efflux kinetics extends over a wide range of concentrations (Figure 10B). In addition, a curious sigmoidal shape of initial velocity *vs* [S] is again obtained for transport via this complementary analysis (see Discussion) over the concentration range most typically analyzed in previous work.

DISCUSSION

We have synthesized and performed an initial characterization of a fluorescent analogue of vinblastine, cou-VBL. Analysis of cou-VBL transport from drug-sensitive and MDR cells and comparison to [^{3}H]VBL transport reveal that the compound will be useful in detailed kinetic analyses of VBL transport. Unresolved issues in the MDR field include whether MDR protein functions directly as an active drug pump with respect to decreasing the retention of a variety of structurally divergent chemotherapeutics [see Gottesman and Pastan (1993), Roepe *et al.* (1993), Roepe (1992), Simon *et al.* (1994), and Reutz and Gros (1994)]. Kinetic studies of the translocation of a variety of compounds believed by many investigators to be directly transported ("pumped") by MDR protein will likely be helpful in this regard. Alternatively, if the active drug transporter hypothesis is ever proven to be correct, cou-VBL will be helpful in measuring any subtle differences in VBL transport via MDR protein *vs* the transport of other drugs (*i.e.*, anthracyclines). This compound may also be helpful in binding competition studies and in detailed analyses of putative reversal agents and MDR protein inhibitors (Wadkins & Houghton, 1993).

Notably, in this work we examine efflux kinetics over a wide range of intracellular drug concentrations, in contrast to other detailed kinetic measurements of vinca alkaloid efflux (Sirotnak *et al.*, 1986). We also examine an MDR cell line that exhibits a much lower level of resistance; since MDR protein overexpression is more closely correlated with MDR $\leq 10^2$-fold, it is less likely that additional potent resistance mechanisms that might further alter membrane characteristics are induced in these MDR35 cells. We do not expose cells in this study to the deleterious effects of azide, MDR reversal agents, or other treatments as has been the case in most previous studies, and we do not subject these cells to the rigors of vesicle preparation. For these reasons, the present data may better represent the kinetic behavior of vinca alkaloid efflux from MDR cells.

Although more detailed work remains to be performed, initial data indicate that the efflux kinetics of ^{fast}cou-VBL$_{ex}$ for LR73 cells is very similar (if not identical) to that for efflux of a similar drug population from MDR35 cells. These data, in analogy to other data obtained with the naturally fluorescent chemotherapeutic doxorubicin (Roepe, 1992), argue that MDR protein does not lower immediate osmotically sensitive (cytoplasmic) concentrations of drug by direct active transport. If passive transport in the absence of MDR protein has the same rate as transport in the presence of the putative pump, it is extremely difficult to envision how the putative pump would manage to lower steady state cytoplasmic concentrations of drug and, hence, the degree of intracellular retention. Notably, this paper presents the first analysis of chemotherapeutic drug efflux where *all* data is used to derive a rate constant; we do not exclusively assume that rates of transport are best described by a linear fit to an arbitrary choice of data points. The latter is perhaps appropriate for well-described transporters, such as the glucose transporter of erythrocytes (Helgerson & Carruthers, 1989), that transport hydrophilic molecules and yield positive hyperbolic curves in velocity *vs* [S] plots; however, it may be misleading in other examples where copious passive diffusion clearly contributes to translocation of the putative substrate. It is clearly dangerous in this case without additional information, since two distinct processes exist in the MDR cells but only one exists for the LR73 cells. We also do not arbitrarily assign kinetic components to a hypothesized process on the basis of the effects of high levels of putative inhibitors as preferred by others (Spoelstra *et al.*, 1992). Since the pharmacology of putative inhibitors like verapamil is complex, we feel this approach is premature without additional physical data.

The second kinetic component for MDR35 cells is interesting. We assume that it is due to MDR protein overexpression and/or selection on VBL. Thus, we could speculate that it corresponds to the hypothesized MDR protein-pumping component (*i.e.*, the slow component represents drug that is directly transported by MDR protein, whereas the fast component[6] represents passive diffusion common to the two cell types). A previous analysis (Sirotnak *et al.*, 1986) argued differently and suggested that this slower component of vinca efflux represents a more loosely bound portion that is reversibly bound to a modified tubulin molecule, since it was found to be specific to other vinca-selected MDR cells. Subsequent work (Pain *et al.*, 1988) supported this in that altered tubulin that exhibited altered drug binding characteristics was identified in the MDR cells used in that study. Similar experiments for MDR35 cells would thus be valuable. In any case, we favor the interpretation of Sirotnak and colleagues in the present work, since a plot of ln(2)/(slow rate constants) *vs* D_{ex} (*i.e*, $T_{1/2}$ *vs* [S]; not shown) does not reveal typical carrier-mediated behavior [see Helgerson and Carruthers (1989)]. Also, notably, efflux of the anthracycline doxorubicin from MDR myeloma cells that express about as much MDR protein as MDR35 cells (Roepe, 1992) does not exhibit a unique second slow kinetic component in similar analyses. Since active pump models propose that MDR protein pumps both vinca alkaloids and anthracyclines, if the slow component represents pumping by MDR protein, it should have also been present in previous work that examined the kinetics of anthracycline efflux [see also Sirotnak *et al.* (1986)]. Finally, preliminary data suggest that the second slow component is absent from MDR transfectants not previously exposed to VBL (P.D.R. and W.G.B., unpublished).

Regardless, if we ignore this reasoning, it remains difficult to envision how the dramatically slower rate (calculated 5–20-fold slower than the fast component, depending on concentration) could compete with the passive diffusion revealed for LR73 and MDR35 cells, such that this process would be able to lower steady state intracellular concentrations of drug [we estimate that its maximal rate would correspond to transport of about 1 drug molecule per MDR protein molecule per 10 min, which is 100–1000 times slower than what is predicted to be required to explain typical lowered steady state levels of drug for MDR cells; see Demant *et al.* (1990)]. To circumvent this dilemma, we could speculate that at very low concentrations of drug, the slow component might overwhelm

[6] The apparent sigmoidal shape of the rate constant *vs* $^{fast}D_{ex}$ and IR60 *vs* $^{fast}D_{ex}$ plots is curious. Traditionally, this shape begs analysis via the Hill equation ($V = V_{max}[S]^h/(K + [S]^h)$). We calculate apparent positive cooperativity (Hill coefficient > 2), but as pointed out repeatedly in the literature, it is dangerous to overinterpret the significance of a high Hill coefficient. For any system involving sequential binding of substrate molecules (in this case, drug to the membrane), a Hill plot is expected to be linear only over a narrow range of [S]. Although interesting, with regard to understanding the differences in drug translocation for MDR *vs* sensitive cells, analysis of this fast passive diffusion term common to both cell types is less important than analysis of the component specific to MDR 35 cells.

[7] Other recent studies frequently cited as support for active drug transport by MDR protein (Sharom *et al.*, 1993) measure a process with a turnover of about 1 drug molecule "pumped" per MDR protein molecule per *hour*. Also, the stoichiometry is apparently near 36 000 ATP molecules hydrolyzed per drug molecule "pumped", which converts to rather unusual cellular bioenergetics.

the passive and thus compete favorably. However, our determination of concentration dependencies reveal that the slow component decays more dramatically as a function of D_{ex}; thus, at low drug concentrations it is even less able to compete with passive diffusion. Some active transport models suggest that MDR protein removes only membrane-associated drugs. If this component represents that process, again, it is very difficult to envision how it would significantly lower the steady state retention of drug (see also footnote 4).

Similar to others (Beck *et al.*, 1983; Sirotnak *et al.*, 1986; Ramu *et al.*, 1989), we find a slower rate of drug *influx* for the MDR35 cells, relative to LR73 (data not shown; manuscript in preparation). It is possible that decreased influx along with altered intracellular binding (revealed in the present work as the slow efflux component) explains the VBL resistance exhibited by MDR35 cells. One explanation for a substantially reduced rate of influx that is independent of an outward-directed active transport process is that lowered electrical membrane potential for these cells [P.D.R., unpublished; see Luz *et al.* (1994)], perhaps caused by MDR protein over-expression (Roepe *et al.*, 1993), in combination with altered lipid properties (Ramu *et al.*, 1984; Escriba *et al.*, 1990; Wadkins & Houghton, 1993), perhaps caused by VBL selection, reduces the rate of intracellular partitioning of the hydrophobic cation [see also Bally *et al.* (1985)]. The reduced influx, coupled with decreased efficiency of intracellular binding [see Beck *et al.* (1983)] perhaps due to altered pH_i and other factors (Roepe, 1992; Roepe *et al.*, 1993), is predicted to lead to substantially reduced intracellular retention over time, relative to the sensitive cells.

On the other hand, recent vesicle studies appear to support the possibility of active drug transport by MDR protein (Ruetz *et al.*, 1993; Ruetz & Gros, 1994), although the calculated concentrative effects are small and depend on assumptions and kinetic analyses in these systems are yet to be performed. Also, as pointed out in a recent review (Simon & Schindler, 1994), hypothetical turnover for MDR protein, calculated on the basis of previously published transport data, appears to make little physical sense (again, the turnover calculated from these data is orders of magnitude slower than what is putatively required). How the putative active drug transport could possibly compete against the copious passive diffusion of these drugs to lower the retention of these drugs as observed is a mystery.[7] Models that envision some type of transport of exclusively membrane-associated drug by the MDR protein might perhaps, in theory, resolve some of the apparent controversies, but the thermodynamic and kinetic arguments that need to be invoked in this case are very unusual and are not consistent with the data presented in this paper. A fundamental difficulty with any permutation of an active drug efflux pump model that needs to be addressed (along with the difficulty in rationalizing the lack of specificity) is why no significant increase in the rate of drug efflux from MDR cells, relative to the sensitive parent, has ever been found (where rate clearly is measured under conditions where $^{fast}D_{ex}$ is the same). The significant differences in influx rates for MDR *vs* sensitive cells that have been observed (Beck *et al.*, 1983; Sirotnak *et al.*, 1986; Ramu *et al.*, 1989; P.D.R., unpublished) suggest that consideration of other possibilities (*i.e.*, decreased uptake due to electrical potential perturbations and membrane fluidity changes) is worthwhile.

ACKNOWLEDGMENT

The authors thank Dr. Phillipe Gros, McGill University, for generously providing the cell lines used in this work and Mr. Subham Basu for help with tissue culture. They also thank Drs. Patrick Ahl, the Liposome Company, Princeton, NJ, and Francis M. Sirotnak, Sloan-Kettering Institute, for helpful discussions. The stimulus for much of this research was provided by careful reading of several papers written by Dr. William T. Beck, St. Jude Children's Research Hospital. This research was performed in the Sackler Laboratory of Membrane Biophysics at the Sloan-Kettering Institute.

REFERENCES

Altenberg, G. A., Young, G., Horton, J. K., Glass, D., Belli, J. A., & Reuss, L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9735–9738.

Ames, G. F.-L. (1986) *Annu. Rev. Biochem. 55*, 397–425.

Aszalos, A., Damjanovich, S., & Gottesman, M. M. (1986) *Biochemistry 25*, 5804–5809.

Azzaria, M., Schurr, E., & Gros, P. (1989) *Mol. Cell. Biol. 9*, 5289–5297.

Ballarin-Denti, A., Den Hollander, J. A., Sanders, D., Slayman, C. W., & Slayman, C. L. (1984) *Biochim. Biophys. Acta 778*, 1–16.

Bally, M. B., Hope, M. J., Van Echteld, C. J. A., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 66–76.

Bear, C. E. (1994) *Biochem. Biophys. Res. Commun. 200*, 513–521.

Beck, W. T. (1987) *Biochem. Pharmacol. 36*, 2879–2887.

Beck, W. T., Cirtain, M. C., & Lefko, J. L. (1983) *Mol. Pharmacol. 24*, 485–492.

Bhushana Rao, K. S. P., Cornet, J., & De Bruyn, A. (1990) *Bull. Soc. Chim. Belg. 99*, 187–193.

Biedler, J. L., & Riehm, H. (1970) *Cancer Res. 30*, 1174–1184.

Boscoboinik, D., Gupta, R. S., & Epand, R. M. (1990) *Br. J. Cancer 61*, 568–572.

Cahalan, M. D. (1994) *Biophys. J. 66*, A3.

Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., & Deeley, R. G. (1992) *Science 258*, 1650–1654.

Coley, H. M., Amos, W. B., Twentyman, P. R., & Workman, P. (1993) *Br. J. Cancer 67*, 1316–1323.

Cornwell, M. M., Safa, A. R., Felsted, R. L., Gottesman, M. M., & Pastan, I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3847–3850.

Dalton, W. S., Grogan, T. M., Rybski, J. A., Scheper, R. J., Richter, L., Kailey, J., Broxterman, H. J., Pinedo, H. M., & Salmon, S. E. (1989) *Blood 73*, 747–752.

Demant, E. J. F., Sehested, M., & Jensen, P. B. (1990) *Biochim. Biophys. Acta 1055*, 117–125.

Devault, A., & Gros, P. (1990) *Mol. Cell. Biol. 10*, 1652–1663.

Eidelman, O., & Cabantchik, Z. I. (1989) *Biochim. Biophys. Acta 988*, 319–334.

Endicott, J. A., & Ling, V. (1989) *Annu. Rev. Biochem. 58*, 137–171.

Escriba, P. V., Ferrer-Montiel, A. V., Ferragut, J. A., & Gonzalez-Ros, J. M. (1990) *Biochemistry 29*, 7275–7282.

Frezard, F., & Garneir-Suillerot, A. (1991a) *Eur. J. Biochem. 196*, 483–491.

Frezard, F., & Garneir-Suillerot, A. (1991b) *Biochemistry 30*, 5038–5043.

Gerlach, J. H., Endicott, J. A., Juranka, P. F., Henderson, G., Sarangi, F., Deuchars, K. L., & Ling, V. (1986) *Nature (London) 324*, 485–489.

Gill, D. R., Hyde, S., Higgins, C. F., Valverde, M. A., Mintenig, G. M., & Sepúlveda, F. V. (1992) *Cell 71*, 23–32.

Gollapudi, S., & Gupta, S. (1993) *Cell. Pharmacol. 1*, 3–7.

Gottesman, M. M., & Pastan, I. (1993) *Annu. Rev. Biochem. 62*, 385–427.

Gros, P., Croop, J., & Housman, D. (1986) *Cell 47*, 371–380.

Gros, P., Raymond, M., Bell, J., & Housman, D. (1988) *Mol. Cell. Biol. 8*, 2770–2778.

Gros, P., Dhir, R., Croop, J., & Talbot, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7289–7293.
Hammond, J. R., Johnstone, R. M., & Gros, P. (1989) *Cancer Res. 49*, 3867–3871.
Hasmann, M., Valet, G. K., Tapiero, H., Trevorrow, K., & Lampidis, T. (1989) *Biochem. Pharmacol. 38*, 305–312.
Helgerson, A. L., & Carruthers, A. (1989) *Biochemistry 28*, 4580–4594.
Higgins, C. F., & Gottesman, M. M. (1992) *Trends Biochem. Sci. 17*, 18–21.
Higgins, C. F., Gallagher, M. P., Hyde, S. C., Mimmack, M. L., & Pearce, S. R. (1990) *Philos. Trans. R. Soc. London 326*, 353–365.
Horio, M., Gottesman, M. M., & Pastan, I. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3580–3584.
Kamimoto, Y., Gatmaitan, Z., Hsu, J., & Arias, I. M. (1989) *J. Biol. Chem. 264*, 11693–11698.
Keizer, H. G., & Joenje, H. (1989) *J. Natl. Cancer Inst. 81*, 706–709.
Luz, J. G., Wei, L. Y., Basu, S., & Roepe, P. D. (1994) *Biochemistry 33*, 7239–7249.
Mayer, L. D., Bally, M. B., Hope, M. J., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 816*, 294–302.
Mayer, L. D., Bally, M. B., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 857*, 123.
Mukhopadhyay, K., Parrack, P. K., & Battacharyya, B. (1990) *Biochemistry 29*, 6845–6852.
Pain, J., Sirotnak, F. S., Barrueco, J. R., Yang, C.-H., & Biedler, J. L. (1988) *J. Cell. Physiol. 136*, 341–347.
Praet, M., Defrise-Quertain, F., & Ruysschaert, J. M. (1993) *Biochim. Biophys. Acta 1148*, 342–350.
Prochaska, H. J., & Santamaria, A. B. (1988) *Anal. Biochem. 169*, 328–336.
Ramu, A., Glaubinger, D., & Weintraub, H. (1984) *Cancer Treat. Rep. 68*, 637–641.
Ramu, A., Pollard, H. B., & Rosario, L. M. (1989) *Int. J. Cancer 44*, 539–547.
Rasola, A., Galietta, L. J. V., Gruenert, D. C., & Romeo, G. (1994) *J. Biol. Chem. 269*, 1432–1436.
Roepe, P. D. (1992) *Biochemistry 31*, 12555–12564.
Roepe, P. D., Wei, L.-Y., Cruz, J., & Carlson, D. (1993) *Biochemistry 32*, 11042–11056.
Ruetz, S., & Gros, P. (1994) *J. Biol. Chem. 269*, 12277–12284.
Ruetz, S., Raymond, M., & Gros, P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11588–11592.
Safa, A. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7187–7191.
Sharom, F. J., Yu, X., & Doige, C. A. (1993) *J. Biol. Chem. 268*, 24197–24202.
Siegfried, J. M., Burke, T. G., & Tritton, T. R. (1985) *Biochem. Pharmacol. 34*, 593–598.
Sikic, B. I. (1993) *J. Clin. Oncol. 11*, 1629–1635.
Simon, S. M., & Schindler, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3497–3504.
Simon, S. M., Roy, D., & Schindler, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1128–1132.
Sirotnak, F. M., Yang, C.-H., Mines, L. S., Oribe, E., & Biedler, J. L. (1986) *J. Cell. Physiol. 126*, 266–274.
Spoelstra, E. C., Westerhoff, H. V., Dekker, H., & Lankelma, J. (1992) *Eur. J. Biochem. 207*, 567–579.
Tarasiuk, J., Frezard, F., Garnier-Suillerot, A., & Gattegno, L. (1989) *Biochim. Biophys. Acta 1013*, 109–117.
Thiebaut, F., Currier, S. J., Whitaker, J., Haugland, R. P., Gottesman, M. M., Pastan, I., & Willingham, M. C. (1990) *J. Histochem. Cytochem. 38*, 685–690.
Thompson, G. L. (1979) U.S. Patent 4,143,041.
Valverde, M., Diaz, M., Sepulveda, F. V., Gill, D. R., Hyde, S. C., & Higgins, C. F. (1992) *Nature 355*, 830–833.
Vayuvegula, B., Slater, L., Meador, J., & Gupta, S. (1988) *Cancer Chemother. Pharmacol. 22*, 163–168.
Wadkins, R. M., & Houghton, P. J. (1993) *Biochim. Biophys. Acta 1153*, 225–236.
Wei, L. Y., & Roepe, P. D. (1994) *Biochemistry 33*, 7229–7238.