Bicinchoninic Acid Protein Assay in the Determination of Adriamycin Cytotoxicity Modulated by the MDR Glycoprotein

Andrea M. Hall, V. Croy, Thomas Chan, Dustin Ruff, Thomas Kuczek, and Ching-jer Chang*

Department of Medicinal Chemistry and Pharmacognosy, Department of Veterinary Physiology and Pharmacology, and Department of Statistics, Purdue University, West Lafayette, Indiana 47907

Received June 29, 1994[⊗]

The development of simultaneous resistance to structurally unrelated drugs in cancer cells is a major obstacle to effective cancer chemotherapy. This multidrug-resistance (MDR) phenomenon is largely attributed to overexpression of a 170 kD glycoprotein, which serves as a transmembrane efflux pump in extruding a variety of natural anticancer drugs such as vinblastine, doxorubicin, and taxol from cancer cells. It is desirable, therefore, to discover compounds that can block the efflux mechanism and thus reverse drug resistance. The bicinchoninic acid protein assay has been adapted for use in a microtiter plate, into an easy, indirect method for screening MDR efflux blockers in plant extracts. This spectrophotometric assay is used to determine the enhancement of adriamycin cytotoxicity against resistant cancer cells by plant extracts or pure compounds indirectly. We have shown that the optical density measured (amount of cellular protein present) correlates with the number of viable cells and that fluorescence of Adriamycin associated with the cell correlates with the concentrations of Adriamycin added to the media. In addition, the relative efficacy of MDR reversal by various alkaloids has been determined.

The development of simultaneous resistance to multiple structurally unrelated compounds is considered to be one of the major clinical problems in the chemotherapy of various types of cancer. This multidrug resistance (MDR) occurs primarily with therapeutic regimens that involve chemotherapeutic agents derived from natural sources such as vinblastine, Adriamycin (doxorubicin), and taxol and other natural products such as colchicine.[1–3] This resistance has been shown *in vitro* to be related to a decrease in cellular drug accumulation.[4] P-Glycoprotein, a 170 kD transmembrane protein encoded by the *mdr* 1 gene, has been found to export drugs and chemicals from cellular cytosol in an energy-dependent manner,[5,6] and tissues of different tumor samples and normal cells have been shown to be positive for the expression of P-glycoprotein.[7,8] Many agents have been studied for their relative potency to decrease the efflux of anticancer drugs by MDR tumor cells.[9] One of these agents, verapamil, a calcium-channel blocker, is effective in overcoming vincristine resistance in P-388 leukemia *in vivo* and *in vitro*.[10] In recent clinical studies, high doses of verapamil have been administered with Adriamycin for the treatment of malignant lymphomas expressing P-glycoprotein.[11,12]

Currently, there are various methods used to screen compounds for their ability to reverse MDR in cancer cells in vitro. Some methods measure proliferation of cells by a Coulter counter, or measure MDR activity by fluorescence, while others measure intracellular radioactivity of a radioisotope-labeled compound or inhibition of radiolabeled-compound binding to vesicles containing P-glycoprotein.[13–16] Although effective, these methods are time consuming and labor intensive and are therefore not optimal for screening large numbers of compounds or extracts. We have developed an alternative, a microtiter bioassay that measures cell density using the bicinchoninic acid (BCA) protein assay and provides an easy, indirect method with which to screen large numbers of crude fractions or pure compounds for reversal of MDR. This assay allows the MDR-reversing effectiveness of compounds and extracts to be assessed independent of their cytotoxicity.

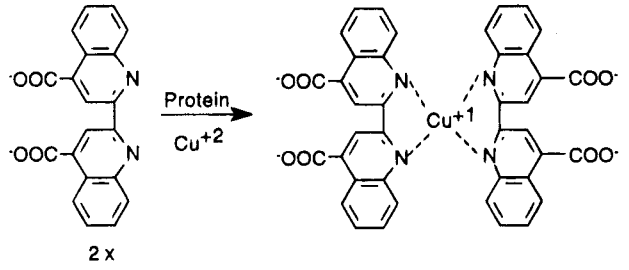


Figure 1. Formation of a complex with bicinchoninic acid and generated cuprous ion.

The BCA protein assay uses two reagents, a BCA detection reagent and a 4% copper sulfate solution, which are mixed together prior to use in a 50:1 ratio. BCA is a reagent specific for the cuprous ion (Cu^{1+}). It is thought that Cu^{2+}, existing in the alkaline medium, is reduced by protein when the peptide bonds of a protein forms a complex with the copper atoms. The Cu^{1+} then forms a 1:2 complex with BCA (Figure 1), giving an intense purple color. This product absorbs at a wavelength of 562 nm and has a linear relationship with protein concentration, allowing quantitation of protein in solution by spectrophotometry.[17] Absorption at this wavelength decreases potential interference from the compound being tested, particularly important when this assay is used to screen crude extracts from natural sources.

In order to validate the BCA assay, parallel experiments were conducted determining optical density by the BCA assay and cell number by Coulter counter over a range of Adriamycin concentrations. The correlation between optical density and cell number was then determined. In addition, fluorometry was used to show

[⊗] Abstract published in *Advance ACS Abstracts*, December 1, 1995.

0163-3864/96/3259-0035$12.00/0

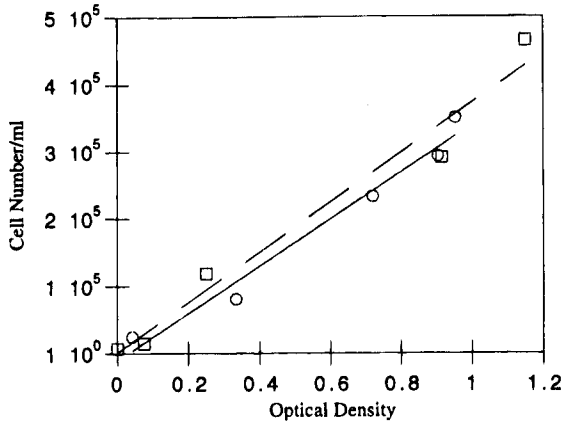


Figure 2. Correlation between cell number and optical density. Adriamycin was added to 24-well plates of MCF-7/ADR cells (solid line) and MCF-7/WT cells (dashed line) at concentrations of 10^{-3} to 10^{1} μg/mL and 10^{-4} to 10^{0} μg/mL, respectively. Incubation conditions and determination of the number of cells present for each concentration of Adriamycin were performed as described in the Experimental Section, Coulter Counted Assay. A 96-well plate was run simultaneously with the same concentrations of Adriamycin for both MCF-7/ADR and MCF-7/WT. After a 6-day incubation period at 37 °C, the plates were developed with bicinchoninic acid reagent and optical density determined for each concentration of adriamycin as described in the Experimental Section, BCA Assay. Each point on the graph represents the average of four values.

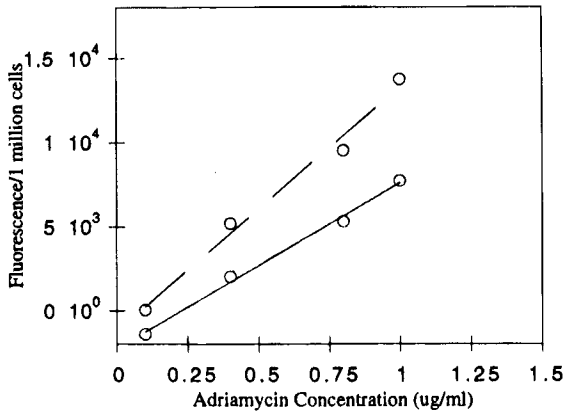


Figure 3. Difference between Adriamycin associated with MCF-7/ADR cells (solid line) and MCF-7/WT cells (dashed line) detected by fluorescence of Adriamycin. Adriamycin concentrations of 0.1, 0.4, 0.8, 1.0, and 2.0 μg/mL were added to 75 cc flasks of both MCF-7/ADR and MCF-7/WT cells. Incubation conditions and detection of fluorescence were performed as outlined in the Experimental Section, Fluorometry Measurements. Each point on the graph represents an average of four values.

that the degree of resistance correlates with Adriamycin concentrations associated with cells exposed to Adriamycin. To demonstrate the use of the BCA assay, degree of resistance reversal relative to verapamil was then carried out on four compounds.

Results and Discussion

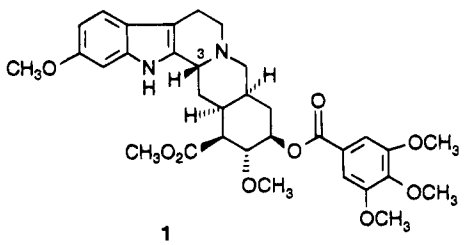
Microculture Growth Evaluation. To evaluate whether the absorbance resulting from the formation of the BCA–Cu^{1+} complex in microculture correlates with total viable cell numbers per well, parallel experiments were performed, determining cell count using a Coulter counted assay and optical density using the BCA assay. Correlated results of the assays for MCF-7/ADR and MCF-7/WT cells are shown in Figure 2. Regression analysis shows a good correlation for both MCF-7/ADR (R = 0.986) and MCF-7/WT (R = 0.985) cells. It can therefore be assumed that the ED_{50} value calculated with the BCA spectrophotometric assay is a good indication of the actual ED_{50} of Adriamycin for both cell types. The average ED_{50} of Adriamycin for MCF-7/WT cells is 1.8×10^{-3} μg/mL (SD, $\pm 2.1 \times 10^{-3}$) for the BCA assay and 2.1×10^{-3} μg/mL (SD, $\pm 2.5 \times 10^{-3}$) for the Coulter counted assay. For MCF-7/ADR the average ED_{50} of Adriamycin is 4.7×10^{-1} μg/mL (SD, $\pm 1.7 \times 10^{-1}$) using the BCA assay and 2.7 μg/mL (SD, ±1.9) using the Coulter counted assay. A *t*-test showed significant differences at *p*-values <0.05 between the ED_{50} of Adriamycin on the wild-type cells and the resistant cells when either the BCA assay or the Coulter counted assay was used. The *p*-values calculated were 0.0001 for the BCA assay and 0.0027 for the Coulter counted assay.

Cellular Concentration of Adriamycin. The differential sensitivity to Adriamycin for multidrug resistant cells is presumably attributed to the overexpression of a transmembrane P-glycoprotein, which acts as an efflux pump, extruding Adriamycin from tumor cells. It is therefore essential to determine the cellular concentration of Adriamycin in the MCF-7/ADR and MCF-7/WT cells to confirm the proposed mechanism. We have directly measured the cellular concentration of Adriamycin using a fluorometric method. Initial concentrations of Adriamycin in the media between 0.1 and 1.0 μg/mL were chosen because ED_{50} values of Adriamycin on the MCF-7/ADR cell lines lie typically between 0.1 and 1.0 μg/mL. We found that cellular concentrations of Adriamycin lower than 0.1 μg/mL were difficult to measure accurately using fluorometry. Furthermore, differences between Adriamycin-resistant and wild-type cells were less prominent at extracellular Adriamycin concentrations lower than 0.1 μg/mL.

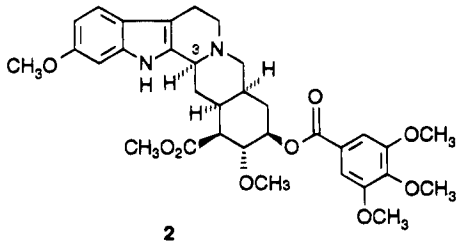
Fluorometry results are shown in Figure 3. Concentrations of Adriamycin ranging from 0.1 to 1.0 μg/mL show a good linear relationship between Adriamycin concentration in the media and Adriamycin associated with the cells as detected by fluorescence of Adriamycin. Indeed, the cellular concentration of Adriamycin in the resistant cells is significantly higher than that in the wild-type cells as determined by an analysis of variance using the Statistical Analysis Systems (SAS) program at a *p*-value < 0.10 ($p = 0.0547$). This result is consistent with the previous data reported by Inaba *et al.*[20] in an experiment in which extracellular ^{14}C-labeled Adriamycin concentration was varied between 0.1 μg/mL and 300 μg/mL, and the intracellular concentration after a 1 h incubation period was determined by scintillation counter. In their study, the greatest difference in intracellular concentration was seen at low extracellular concentrations, between 0.1 and 10 μg/mL.

Multidrug Resistance Reversal (Blocking of Adriamycin Efflux). We have also applied this newly developed microculture spectrophotometric method to test the efflux-blocking efficacy of several pure alkaloids: reserpine (**1**), isoreserpine (**2**), and hydroquinidine 4-methyl-2-quinolyl ether (**3**) (Chart 1). Verapamil (**4**) was studied as a reference compound for evaluating the relative efficacy. The quantitative results of compounds screened for effectiveness in reversing MDR are depicted in Table 1 and Figure 4. Each point on the graph represents the resistance-reversing effectiveness of one concentration chosen for a compound, and a greater

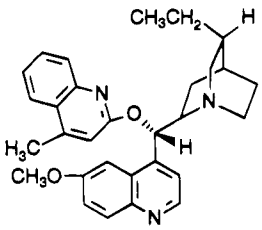
Chart 1



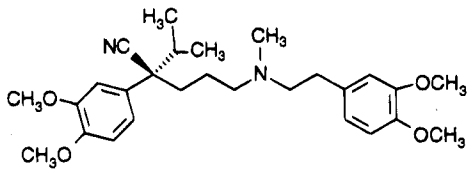
1



2



3



4

Table 1. Bicinchoninic Acid (BCA) Assay Data[a]

compound	concentration (μM)	ED_{50} (μM) of Adriamycin	cytotoxicity factor	cytotoxicity enhancement factor
verapamil	1.10	5.3×10^{-1}	0.009	3.0
	2.20	2.8×10^{-1}	0.02	6.0
	5.50	9.0×10^{-2}	0.05	18.0
	11.0	6.1×10^{-2}	0.09	26.0
	15.4	5.0×10^{-2}	0.13	32.0
	22.0	3.1×10^{-2}	0.19	50.0
reserpine	0.15	7.0×10^{-1}	0.005	2.0
	0.30	3.7×10^{-1}	0.009	4.0
	0.62	2.0×10^{-1}	0.02	8.0
	1.23	9.6×10^{-2}	0.04	17.0
	2.46	6.4×10^{-2}	0.08	25.0
isoreserpine	0.82	2.9×10^{-1}	0.04	5.5
	1.64	1.0×10^{-2}	0.09	16.0
	3.29	5.7×10^{-2}	0.17	28.0
hydroquinidine 4-methyl-2-quinolyl ether	0.28	8.3×10^{-1}	0.03	3.0
	0.53	5.0×10^{-1}	0.06	6.0
	1.07	2.0×10^{-1}	0.11	18.0
Adriamycin		1.6		

[a] Column one lists names of compounds tested for their ability to reverse Adriamycin resistance. Column two lists the concentrations used for each compound tested, and column three the ED_{50} value of Adriamycin alone and the ED_{50} values of Adriamycin obtained for each of these concentrations using the BCA assay as described in the Experimental Section. Columns four and five show the results of the calculations performed on the data listed in the previous two columns as described in the Calculations. These data are depicted graphically in Figure 4.

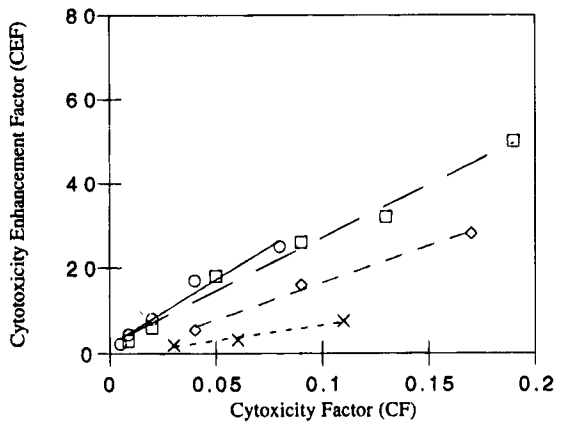


Figure 4. Bicinchoninic acid assay results of reserpine (**1**) (○), isoreserpine (**2**) (◇), hydroquinidine 4-methyl-2-quinolyl ether (**3**) (×), and verapamil (**4**) (□). The graph is derived from data presented in Table 1 using methods described in the Calculations.

slope means a greater ability of the compound within a given cytotoxicity range to reverse drug resistance.

We have determined (data not shown) that there is less than a 10-fold enhancement in the cytotoxicity of Adriamycin on wild-type cells using the standard MDR-reversing compound, verapamil, as compared with a compound that has not been shown to reverse resistance in either MCF-7/WT or MCF-7/ADR cell lines, *N*-benzylquininium chloride. Because this enhancement is minimal, it will not significantly interfere with the interpretation of our data.

These results indicate that reserpine and isoreserpine have efficacy comparable to the standard MDR-reversing compound, verapamil. The quinidine derivative has a lower efficacy than verapamil. Previous studies also demonstrated that verapamil, reserpine, and quinidine have similar efficacy in reversing MDR.[20-22] Beck *et al.*[21] investigated the effect of the blocker on the binding of radiolabeled compound, *N*-(*p*-azido-[3-^{125}I]salicyl)-*N*′-β-aminoethylvindesine, to P-glycoprotein. They demonstrated that with 12.4 μM concentrations of either reserpine or verapamil, reserpine inhibited binding by 79.2%, while verapamil inhibited binding by 55.1%. A similar study in which either quinidine or verapamil was added with Adriamycin at concentrations lower

than their ED_{20}s for 24 h showed only a slight difference in the ability of verapamil over quinidine to decrease the ED_{50} of Adriamycin on resistant cells.[22] All three compounds, verapamil, reserpine, and quinidine, were studied for their ability to increase accumulation of a fluorescent dye, rhodamine 6G, in resistant cells[23] as a method for determining the degree of MDR reversal. In this study, the increased fluorescence intensity of the rhodamine 6G varied at most only 3-fold among the three compounds when the compounds were tested at concentrations below their ED_{20} values. These results demonstrate that all three compounds have similar abilities to increase accumulation of rhodamine 6G in resistant cells and therefore similar abilities to reverse MDR. Our data show by the lack of a significant difference between the ability of isoreserpine and reserpine to enhance the cytotoxicity of Adriamycin that the stereochemistry at C-3 in these compounds appears to play only a minor role in their ability to reverse MDR.

As seen in Figure 3, concentrations of the compounds being assayed are chosen that are below the ED_{20} value of the compound so that the cytotoxicity of the compound does not significantly affect the final ED_{50} of Adriamycin. This minimizes the possibility of measuring an enhancement of Adriamycin toxicity on resistant cells resulting from the synergistic cytotoxicity of the compound and Adriamycin, instead of from the blockage of the efflux of Adriamycin. We also assess the enhanced cytotoxicity of Adriamycin based on the cytotoxicity factor (defined in the Experimental Section), which allows us to evaluate the relative potency of various efflux blockers at comparable toxicity levels. This method of evaluation reduces the risk of isolating and testing compounds that may be too toxic in animals to pursue as therapeutic agents for the reversal of multidrug resistance.

Experimental Section

Cells. MCF-7 wild-type (MCF-7/WT) cells, human adenocarcinoma of the breast, were used to isolate an MCF-7 Adriamycin-resistant (MCF-7/ADR) cell strain by Dr. Kenneth Cowan *et al.* at the National Cancer Institute (NCI).[18] Cell stock was received from Dr. Craig Fairchild, NCI. MCF-7 wild-type cells were maintained in RPMI medium with 10% fetal calf serum, heat inactivated (FCSHI) and penicillin-streptomycin (PS) and were transferred twice a week at a 1:3 split. Adriamycin-resistant MCF-7 cells (MCF-7/ADR) were maintained in 10 μM adriamycin for two passages prior to freezing for future use. MCF-7/ADR cells were also maintained in RPMI medium with 10% FCSHI and PS and transferred twice weekly at a 1:6 split. Resistant cells retain resistance for about 6 months in the absence of Adriamycin.

Chemicals. Adriamycin (ADR), verapamil, Nonidet SP-40, poly-D-lysine, reserpine, isoreserpine, and hydroquinidine 4-methyl-2-quinolyl ether were all obtained from Sigma Chemical Co., St. Louis, MO. The bicinchoninic acid protein assay was obtained from Pierce, Rockford, IL.

Preparation of Plates. Because the resistant cells do not adhere tightly to plastic surfaces, they are easiy sheared off the 96-well plates during the washing steps. To circumvent possible cell loss, the microtiter plates were coated with poly-D-lysine. Poly-D-lysine was dissolved in distilled H_2O to a concentration of 100 μg/mL and was filtered through a 0.2 μm filter. Aliquots of 60 μL were added to each well of 96-well microtiter dishes for about 30 min. The dishes were then rinsed twice with sterile H_2O (unbound poly-D-lysine prohibits cell growth) and allowed to dry in a sterile environment.[19] The plates can be stored for up to two weeks at 4 °C before use.

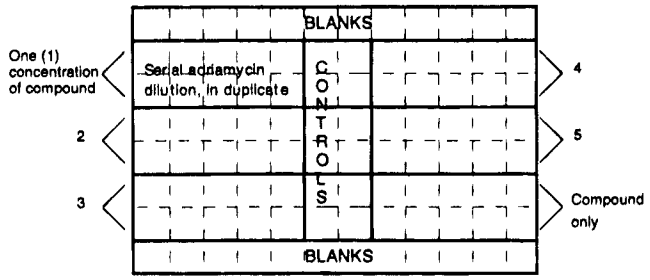


Figure 5. Setup of a 96-well plate to test a compound at up to five different concentrations and to determine the cytotoxicity of the compound. One concentration of the compound being tested is added to 10 wells. After a 30-min incubation, a serial dilution of Adriamycin is added in duplicate to these wells. This process can be repeated up to five times on one 96-well plate. To determine the cytotoxicity of the compound, a serial dilution in duplicate of the compound alone is added to 10 wells. The top and bottom rows are blanks, media only; and the middle columns are controls, cells only.

Bicinchoninic Acid (BCA) Assay. Cells were seeded into 96-well microtiter dishes. MCF-7/ADR cells were seeded at a concentration of 3000 cells/well (15 000 cells/mL), and MCF-7/WT cells were seeded at 1000 cells/well (5000 cells/mL). These plating densities were used to compensate for a difference in growth rates. Cells were seeded in 200 μL of medium per well and were then incubated at 37 °C for 24 h prior to the addition of compound.

The compounds (or extracts) were solubilized first with a volume of DMSO that gave a concentration of less than 0.1% DMSO in the well and then were further diluted with media. On one 96-well plate, up to five concentrations of a compound were added to resistant cells. Cells were incubated at 37 °C with the compound for 30 min, and then serial dilutions of Adriamycin were added to each concentration of compound. This allowed the dose at which Adriamycin is 50% effective (ED_{50}) to be determined for each concentration of the compound. The compound alone was also added to the resistant cells to determine its cytotoxicity. As long as these requirements were met, the exact setup was not crucial. Figure 5 demonstrates our setup.

Each test also required the determination of the cytotoxicity of Adriamycin on both the resistant and wild-type cells, necessary for the calculations and as a reference. Verapamil was tested at a single concentration of 10 μg/mL (2.2 μM) with Adriamycin to ensure that the standard degree of MDR reversal that can be achieved by verapamil for each run is comparable.

After a 6-day incubation period, media were removed from the wells, the wells were washed with phosphate-buffered saline (PBS), and the amount of protein present was assayed as follows. Nonidet P-40 1% (10 μL) was added to each well of cells to solubilize the cells and provide the required volume of protein sample for this assay. To each well, 200 μL of the mixed BCA reagent was added, and the 96-well plate was allowed to incubate at 37 °C for 30 min. Purple-tinted wells indicated the presence of cellular protein and viable

cells. The plate was then read at 570 nm in a microtiter plate reader (Dynatech Microplate Reader MR 600, Chantilly, VA). ED_{50} values were calculated using linear regression, and additional calculations were performed as described under Calculations. The data were then graphed to depict the varying degrees of reversal of resistance by the compounds or crude extracts tested.

When conducting this assay we sometimes attempted to increase solubility of the extract or compound being tested by trying solvents other than DMSO, such as EtOH or MeOH/CH_2Cl_2 in the initial solution. We also usually conducted this assay at least twice to determine the effectiveness of a pure compound at reversing MDR. For the second test a more defined concentration range was chosen to provide better reproducibility. Furthermore, concentrations of the compound/extract were chosen that better represented concentrations below the ED_{20} values for that compound to assess the effectiveness of the compound/extract as an MDR-reversing agent independent of its cytotoxicity.

Calculations. To ensure that each run provided valid data, Adriamycin (ADR) was used as a reference compound, the ED_{50} value of Adriamycin for verapamil at 10 μg/mL (2.2 μM) was determined, and the following calculations were performed:

A. Quality Control. The resistance factor, to ensure that the resistant cells have maintained a resistant phenotype is defined as follows:

$$\text{resistance factor} = \frac{ED_{50} \text{ of ADR only on MCF-7/ADR cells}}{ED_{50} \text{ of ADR only on MCF-7/WT cells}} = 10^2 - 10^3$$

The reversal factor, to ensure that the standard degree of MDR reversal that can be achieved by verapamil for each run is comparable, is defined as follows:

$$\text{reversal factor} = \frac{ED_{50} \text{ of ADR only on MCF-7/ADR cells}}{ED_{50} \text{ of ADR with 10 } \mu\text{g of verapamil on MCF-7/ADR cells}} \simeq 10^2$$

B. Relative Efficacy of MDR Efflux Blocker. After data were acquired, the following calculations were performed so that the data could be represented as a graph. The y-axis was labeled Cytotoxicity Enhancement Factor (CEF),

$$\text{CEF} = \frac{ED_{50} \text{ of ADR only on MCF-7/ADR cells}}{ED_{50} \text{ of ADR with efflux blocker on MCF-7/ADR cells}}$$

with values indicating the increase in Adriamycin toxicity to the resistance cells with efflux blocker relative to the toxicity of Adriamycin without blocker. The x-axis was labeled Cytotoxicity Factor (CF)

$$\text{CF} = \frac{\text{concn of efflux blocker } (\mu\text{M})}{2 \times ED_{50} \text{ of efflux blocker alone } (\mu\text{M})} = 0-0.2 \quad (4)$$

with values representing the concentrations chosen for an efflux blocker relative to the ED_{100} of that blocker. Concentrations of the MDR efflux blocker were chosen that were below or equivalent to the ED_{20} value of the blocker alone on resistant cells to minimize the potential synergistic cytotoxicity interactions unrelated to the MDR efflux mechanism. In addition, because the chosen concentrations were divided by the ED_{100} values of the efflux blocker alone on resistant cells, the slope of the line decreases with increasing cytotoxicity of the blocker, showing decreasing effectiveness. From the graph it can be seen easily which compounds are better at reversing MDR; steep lines indicate better reversal. This approach will not lead us to isolate cytotoxic compounds from the crude extract with mechanisms that have nothing to do with the MDR reversal of adriamycin.

Coulter Counted Assay. A 24-well plate was seeded with 12 wells of resistant cells and 12 wells of wild-type cells. After a 24-h incubation period, Adriamycin was added in duplicate to each cell line at five, ten-fold dilutions. After 6 days of incubation, medium was aspirated from each well and washed with 1.0 mL of PBS without Ca^{2+} or Mg^{2+}. Then 200 μL of 0.05% trypsin/0.02% EDTA was added to each well and incubated at 37 °C for 10 min. The cells were removed and placed into glass tubes, and each well was washed with 200 μL of PBS without Ca^{2+} or Mg^{2+}, which was also removed and added to its respective tube. Enough PBS was then added to the tubes to make a total volume of 1.0 mL. Tubes were vortexed thoroughly to eliminate clumping of cells, and cell suspensions were diluted 100-fold with Isoton II. The cells from each well were counted twice using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) with an amplification of 2, aperture current of 1, and upper and lower thresholds of 100.0 and 5.0, respectively. ED_{50} values were calculated using linear regression.

Fluorometry Measurements. Six flaskes each of resistant and wild-type cells were prepared by adding cells to 15 mL of medium at a 1:3 split. Six flasks allowed for five concentrations and one control. After 24 h of incubation at 37 °C, five Adriamycin concentrations (0.1, 0.4, 0.8, 1.0, 2.0 μg/mL) were added to the flasks, and the cells were incubated for another 24 h, again at 37 °C. Cells were harvested by pouring off medium and washing each flask twice with 5 mL of PBS without Ca^{2+} or Mg^{2+} and then adding 5 mL of trypsin/EDTA. The trypsin/EDTA was removed after washing over the cells so that about 1 mL remained in the flask. The flasks were incubated at 37 °C for about 10 min, until the cells fell off, and the number of cells/mL was then determined by hemocytometer. The appropriate volume of cells in trypsin/EDTA from the flasks was transferred to 13 × 75 mm tubes so that there were approximately 2.0×10^6 cells in each tube. Enough PBS without Ca^{2+} or Mg^{2+} was added to each test tube to make a total volume of 3.0 mL, and the tubes were vortexed before transferring cell solutions to 3-mL cuvettes. Adriamycin fluorescence was determined using an excitation wavelength of 470 nm, an emission wavelength of 590 nm, an excitation band pass of 2 nm, and an emission band pass of 1 nm on an SLM Aminco 8000C (Urbana, IL) fluorometer. An offset feature was also used to decrease background signal from the turbid cell suspensions.

Acknowledgments. We would like to thank Dr. Craig Fairchild (NCI, National Institutes of Health, Bethesda, MD 20892) for the MCF-7 cell lines. We would also like to thank Dr. Kinam Park for use of the fluorometer and Jim Wesley for his aid and patience while operating the fluorometer. The cytotoxicity test-

ing was conducted at the Cell Culture Laboratory, Purdue University, which is partially supported by the National Cancer Institute (P30 CA23168). We are grateful to the National Cancer Institute for financial support of this research (U01 CA 50743).

References and Notes

(1) Cano-Gauci, D. F.; Riordan, J. R. *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*; Plenum Press: New York, 1991; Chapter 18, p 340.
(2) Shen, D.-W.; Cardarelli, C.; Hwang, J.; Cornwell, M.; Richert, N.; Ishii, S.; Pastan, I.; Gottesman, M. M. *J. Biol. Chem.* **1986**, *261*, 7762–7770.
(3) Roy, S. N.; Horwitz, S. B. *Cancer Res.* **1985**, *45*, 3856–3863.
(4) Fojo, A. T.; Ueda, K.; Slamon, D. J.; Poplack, D. G.; Gottesman, M. M.; Pastan, I. *Proc. Natl. Acad. Sci.* USA **1987**, *84*, 265–269.
(5) Akiyama, S.-I.; Fojo, A.; Hanover, J. A.; Pastan, I.; Gottesman, M. M. *Somat. Cell Mol. Genet.* **1985**, *11*, 117–126.
(6) Dano, K. *Biochim. Biophys. Acta* **1973**, *323*, 466–483.
(7) Kellen, J. A. *Reversal of Multidrug Resistance in Cancer*; CRC Press: Boca Raton, 1994; Chapter 5.
(8) Roninson, I. B.; Noonan, K. E.; Choi, K.; Morse, B. S.; Chen, C.-J.; Chin, J.; Stern, R. K.; Safa, A. R.; Tsuruo, T.; Ogawa, M. *Drug Resistance as a Biochemical Target in Cancer Chemotherapy*, Bristol-Myers Squibb Cancer Symposia; Academic Press: San Diego, 1992; Vol. 13, Chapter 2, p 29–32.
(9) Gottesman, M. M.; Pastan, I. *Annu. Rev. Biochem.* **1993**, *62*, 387.
(10) Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res.* **1981**, *41*, 1967–1972.
(11) Miller, T. P.; Grogan, T. M.; Dalton, W. S.; Spier, C. M.; Scheper, R. J.; Salmon, S. E. *J. Clin. Oncol.* **1991**, *9*, 17–24.
(12) Salmon, S. E.; Dalton, W. S.; Grogan, T. M.; Plezia, P.; Lehnert, M.; Roe, D. J.; Miller, T. P. *Blood* **1991**, *78*, 44–50.
(13) Tsuruo, T.; Kawabata, H.; Nagumo, N.; Iida, H.; Kitatani, Y.; Tsukagoshi, S.; Sakurai, Y. *Cancer Chemother. Pharmacol.* **1985**, *15*, 16–19.
(14) Gnanpathi, R.; Grabowski, D. *Cancer Res.* **1983**, *43*, 3696–3699.
(15) Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res.* **1982**, *42*, 4730–4733.
(16) You, M.; Ma, X.; Mukherjee, R.; Farnsworth, N. R.; Cordell, G. A.; Kinghorn, A. D.; Pezzuto, J. H. *J. Nat. Prod.* **1994**, *57*, 1517–1522.
(17) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76–85.
(18) Cowan, K. H.; Baptist, G.; Tulpule, A.; Biranda, K. S.; Myers, C. E. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9328–9332.
(19) McKeehan, W. L.; Ham, R. G. *J. Cell. Biol.* **1976**, *71*, 727–734.
(20) Inaba, M.; Johnson, R. K. *Biochem. Pharmacol.* **1987**, *27*, 2123–2130.
(21) Beck, W. T.; Cirtain, M. C.; Glover, C. J.; Felsted, R. L.; Safa, A. R. *Biochem. Biophys. Res. Commun.* **1988**, *153*, 959–966.
(22) Plumb, J. A.; Wishart, G. C.; Setanioans, A.; Morison, J. G.; Hamilton, T.; Bicknell, S. R.; Kaye, S. B. *Biochem. Pharm.* **1994**, *47*, 257–266.
(23) Yoshimura, A.; Shudo, M.; Ikeda, S.-I.; Ichikawa, M.; Sumizawa, T.; Akiyama, S.-I. *Cancer Lett.* **1990**, *50*, 45–51.

NP960024C