

# Cancer Research



## ***In Vivo* Analysis of Human Multidrug Resistance Protein 1 (MRP1) Activity Using Transient Expression of Fluorescently Tagged MRP1**

Asha Rajagopal, Alok C. Pant, Sanford M. Simon, et al.

*Cancer Res* 2002;62:391-396. Published online January 1, 2002.

**Updated Version** Access the most recent version of this article at:  
<http://cancerres.aacrjournals.org/content/62/2/391>

**Cited Articles** This article cites 22 articles, 15 of which you can access for free at:  
<http://cancerres.aacrjournals.org/content/62/2/391.full.html#ref-list-1>

**Citing Articles** This article has been cited by 5 HighWire-hosted articles. Access the articles at:  
<http://cancerres.aacrjournals.org/content/62/2/391.full.html#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, contact the AACR Publications Department at [permissions@aacr.org](mailto:permissions@aacr.org).

# In Vivo Analysis of Human Multidrug Resistance Protein 1 (MRP1) Activity Using Transient Expression of Fluorescently Tagged MRP1<sup>1</sup>

Asha Rajagopal, Alok C. Pant, Sanford M. Simon,<sup>2</sup> and Yu Chen

Laboratory of Cellular Biophysics, The Rockefeller University, New York, New York 10021

## ABSTRACT

The multidrug resistance protein 1 (MRP1) contributes cellular resistance to a wide array of physiological toxins and chemotherapeutic agents. Its *in vivo* activity has been studied primarily in cells that have been continuously drug selected, culture conditions that might confound the effects of MRP1 expression with the effects of a cell's detoxification machinery. Transient transfection with a MRP1-green fluorescent protein (EGFP) fusion protein allowed us to measure the activity of MRP1 in cells that had insufficient time to induce other chemoprotective proteins. Furthermore, separate transfections with MRP1-yellow fluorescent protein and a fluorescently tagged P-glycoprotein (MDR1-cyan fluorescent protein) permitted the drug-resistant properties of MRP1-expressing cells to be compared with those of MDR1-expressing cells. Our data showed that the expression of MRP1-EGFP results in significantly decreased cellular accumulation of tetramethylrhodamine ethyl ester (TMRE) and daunorubicin, mildly decreased cellular accumulation of mitoxantrone, and decreased nuclear accumulation of doxorubicin. Additionally, MRP1-EGFP expression protected cells from the microtubule depolymerization caused by vincristine and colchicine, but not by vinblastine.

## INTRODUCTION

MRP1<sup>3</sup> is a  $M_r$  190,000 member of the ATP-binding cassette family of transporters (1). The protein has been implicated in the development of multidrug resistance in a variety of cancer cell lines (2). First cloned in 1992 from a daunorubicin-resistant lung cancer cell line (3), MRP1 has been associated with decreased sensitivity to a number of chemotherapeutic agents. Like the well-studied P-glycoprotein (4), MRP1, when overexpressed, can confer varying degrees of resistance to cationic and neutral hydrophobic compounds, including the anthracyclines, the *Vinca* alkaloids, rhodamine 123, and various acetoxymethyl esters of indicator dyes (5, 6). However, unlike P-glycoprotein, MRP1 can also transport anions such as methotrexate, calcein, anti-HIV nucleoside analogues, and heavy metals, as well as anionic conjugates of sulfate, glutathione (*e.g.*, leukotriene C<sub>4</sub>), and glucuronic acid (*e.g.*, 17 $\beta$ -estradiol 17 $\beta$ -D-glucuronide; 7).

Since its discovery nearly a decade ago, MRP1 has been extensively studied in drug-resistant tumor cell lines (8); in transformed cells stably transfected with the cDNA (9); in yeast (10); in microsomes (11); and, more recently, in liposomes containing the purified protein (8). Some *in vivo* studies have found MRP1 to be functional in intracellular compartments (10, 12), while others have seen MRP1 active primarily at the plasma membrane (5). Some studies have

suggested MRP1 to be active against mitoxantrone (13), cadmium (10), vinblastine, and colchicine, whereas others have found MRP1 to confer no increased resistance to these compounds (14, 15). Many of these differing assessments of MRP1 activity could be attributable to the properties of the cell type under investigation. However, other differences may have arisen as a result of the particular assays used to examine MRP1 activity. Whole-cell investigations have been performed either in multidrug-resistant cell lines endogenously expressing MRP1 or in stable, MRP1-transfected lines. Both of these model systems require that cells be continuously cultured in cytotoxins for the multidrug resistance phenotype to be maintained. This form of drug selection has been associated with many changes in cell physiology, including the up-regulation of DNA-repair enzymes, increased drug metabolism, and decreased sensitivity to apoptosis (16, 17). Under these selection conditions, ascertaining the effect of MRP1 expression is made more complex by the presence of other resistance mechanisms.

For these reasons, we have developed a means of studying MRP1 activity in whole cells without protracted drug selection. We have created a protein with EGFP fused to the COOH terminus of MRP1, a construct that permits easy identification of MRP1 expression in cells. After transiently transfecting cells with MRP1-EGFP, we can compare cells expressing MRP1-EGFP with their nonexpressing counterparts and can thereby assess the immediate effect that MRP1 introduction has on a cell's drug resistance properties. This fusion protein allows us to correlate degrees of MRP1 expression, assayed by EGFP fluorescence, with drug accumulation. Furthermore, separate transfections involving MRP1 fused to EYFP (MRP1-EYFP), as well as a MDR1 protein tagged with ECFP (MDR1-ECFP; Ref. 17), allow us to simultaneously compare the activities of the two proteins in the same chemotherapeutic and in the same culture conditions.

## Materials and Methods

**Cell Culture and Transfection.** HeLa cells were propagated in DMEM with 4.5 g/liter glucose and L-glutamine (Cellgro, Herndon, VA) in 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). Transfections of the constructs were done with Fugene 6 as per manufacturer's instructions (Roche Molecular Biochemicals, Brussels, Belgium).

**Construction and Expression of Vectors.** Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Human MRP1 cDNA was obtained in the cloning vector pGEM-11zf (gift of Gary Kruh, Fox Chase Cancer Center, Philadelphia, PA). To generate an expression plasmid for wild-type MRP1, designated pMRP1, MRP1 cDNA was subcloned between the *SacI* and *XbaI* sites of pEGFP-N1 (Clontech, Palo Alto, CA), a step that excised the EGFP coding sequence and created a pEGFP-N1-backbone plasmid with MRP1 placed under the control of the cytomegalovirus promoter. To generate the MRP1-EGFP fusion protein, standard mutagenesis techniques were used on pMRP1 to replace the MRP1 stop codon with an *AgeI* site, the site at which EGFP was introduced. pMRP1-ECFP and pMRP1-EYFP were created by replacing EGFP with ECFP or EYFP (Clontech).

The MDR1-GFP fusion vector was made using site-directed mutagenesis to replace the 3' stop codon with a *SalI* site. The MDR1 open reading frame was then inserted into pEGFP-N1 (Clontech), as described previously (17). MDR1-ECFP was made by replacing the EGFP with ECFP from pECFP (Clontech).

**Western Blot Analysis.** MRP1-transfected and MRP1-EGFP-transfected cells were dissociated with Cell Stripper (Cellgro) and solubilized with 1% Triton X-100. The nuclear debris was removed by a low-speed centrifugation

Received 7/10/01; accepted 11/13/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by American Cancer Society Grant RPG-98-177-01-CDD (to S. M. S.), NIH Grant R01CA81257 (to S. M. S.), NIH Medical Student Training Program Grant GM07739 (to Y. C.), and Institutional National Research Science Award T32 GM07982-20 (to A. R.)

<sup>2</sup> To whom requests for reprints should be addressed, at The Rockefeller University, Box 304, 1230 York Avenue New York, NY 10021. Phone: (212) 327-8130; Fax: (212) 327-7542; E-mail: simon@mail.rockefeller.edu.

<sup>3</sup> The abbreviations used are: ABC, ATP-binding cassette; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; MRP, multidrug resistance protein; TMRE, tetramethylrhodamine ethyl ester perchlorate; BSO, L-buthionine-(S)-sulfoximine; MTOC, microtubule-organizing center.

and the supernatant was resolved on a 4–20% gradient gel, using SDS PAGE. After electrotransfer onto a membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using a semidry electroblotter, proteins were immunoblotted with either the MRPr1 anti-MRP1 rat monoclonal antibody (Alexis Biochemicals, San Diego, CA) and an alkaline phosphatase-conjugated antirat IgG antibody (Sigma Chemical Co.) or directly with the alkaline phosphatase conjugated Living Colors Peptide Antibody (Clontech). Protein molecular weight markers from Amersham were used to distinguish relative electrophoretic mobilities.

**Fluorescence Microscopy.** Wide-field fluorescence microscopy was performed with an IX-70 Olympus microscope using a 1.4 N.A. oil-immersion objective, and an ORCA-cooled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) as described previously (17, 18). Wide-field fluorescence microscopy with deconvolution was performed using a DeltaVision deconvolution microscope with a 1.4 N.A. oil-immersion  $\times 60$  objective. The following excitation and emission filters were used for wide-field fluorescent microscopy: ECFP:  $\lambda_{\text{ex}} = 400\text{--}430$  nm,  $\lambda_{\text{em}} = 460\text{--}500$  nm; EGFP:  $\lambda_{\text{ex}} = 480\text{--}490$  nm,  $\lambda_{\text{em}} = 500\text{--}550$  nm; TMRE:  $\lambda_{\text{ex}} = 530\text{--}560$  nm,  $\lambda_{\text{em}} = 570\text{--}650$  nm. Confocal microscopy was performed on either an upright Axioplan 2 microscope or an inverted Axiovert 100 microscope, each with a LSM 510 confocal attachment (Carl Zeiss, Thornwood, NY), using a 1.2 N.A. water-immersion  $\times 63$  objective. The following excitation laser lines and emission filters were used: EGFP:  $\lambda_{\text{ex}} = 488$  nm,  $\lambda_{\text{em}} = 500\text{--}530$  nm; daunorubicin and doxorubicin:  $\lambda_{\text{ex}} = 488$  nm,  $\lambda_{\text{em}} = 580$  nm LP; and mitoxantrone:  $\lambda_{\text{ex}} = 633$  nm,  $\lambda_{\text{em}} = 650$  nm LP.

Live cell imaging of fluorescent drugs was performed on cells 48–72 h after transfection. Cells were incubated with fluorescent drugs [TMRE (Molecular Probes, Eugene, OR), daunorubicin (Calbiochem, La Jolla, CA), doxorubicin (Calbiochem), or mitoxantrone (Sigma Chemical Co.)] in Opti-MEM with HEPES and L-glutamine (Life Technologies, Inc., Gaithersburg, MD) in a 5% CO<sub>2</sub> incubator at 37°C for 15 min. To inhibit MRP1 activity, cells were incubated with 25  $\mu\text{M}$  BSO for 24 h prior to imaging.

For immunofluorescence of microtubules, transfected cells were incubated in 600 nM vincristine, 600 nM vinblastine, or 2  $\mu\text{M}$  colchicine for 60 min, and fixed with ice-cold methanol. Microtubules were stained using a Cy3-labeled anti- $\beta$  tubulin antibody (Sigma Chemical Co.) according to the manufacturer's instructions. For the immunolabeling of MRP1, cells were fixed with ice-cold methanol, incubated with the anti-MRP1-antibody MRPr1 at a 1:1000 dilution, washed, and subsequently incubated with an Alexa 594 conjugated antirat antibody (Molecular Probes).

**Flow Cytometry.** Flow cytometry was performed on a FACSort (Becton Dickinson, San Jose, CA). Adherent cells were nonenzymatically dissociated using Cell Stripper, resuspended in Opti-MEM with fluorescent drugs and incubated at 37°C for 30 min, and stored on ice until sample acquisition (not more than 30 min). The cells were harvested and resuspended in ice-cold PBS with a DNA stain to label dead cells [either 10 nM TOTO-3 iodide (Molecular Probes)<sup>4</sup> or 1  $\mu\text{M}$  propidium iodide (Sigma Chemical Co.)] immediately before data acquisition. The following wavelengths were used for excitation and emission: EGFP:  $\lambda_{\text{ex}} = 488$  nm,  $\lambda_{\text{em}} = 500\text{--}520$  nm; daunorubicin, doxorubicin, TMRE, propidium iodide:  $\lambda_{\text{ex}} = 488$  nm,  $\lambda_{\text{em}} = 564\text{--}606$  nm; and mitoxantrone, TOTO-3  $\lambda_{\text{ex}} = 633$  nm,  $\lambda_{\text{em}} = 650$  nm LP.

## RESULTS

**MRP1-EGFP Fusion Protein Is Correctly Folded and Localized.** The short-term effects of expressing MRP1 on the concentration and distribution of chemotherapeutics in cells were studied with transient transfection. To identify the expression and to study the cellular distribution of the protein, we used a MRP1-EGFP fusion protein. Two controls were used to determine whether EGFP fluorescence could be used as an indicator of the localization of MRP1. The first control was to determine whether all of the cellular EGFP was present as part of the MRP1 fusion protein, a task accomplished by immunoblotting lysates of cells transfected with either wild-type MRP1 or MRP1-EGFP using antibodies to either MRP1 or EGFP (Fig. 1A–B). In cells transfected with MRP1, the anti-MRP1 antibody

recognized a doublet that migrated between  $M_r$  160,000 and  $M_r$  250,000, (Fig. 1A, *first lane*), a weight range encompassing the reported size of MRP1 in both its unglycosylated and fully glycosylated states (19). In cells transfected with MRP1-EGFP, the anti-MRP1 antibody recognized a slightly heavier doublet, that would be consistent with the addition of a  $M_r$  27,000 EGFP COOH-terminal fusion (Fig. 1A, *second lane*). The anti-EGFP antibody recognized a similar doublet in the MRP1-EGFP transfected cells (Fig. 1B, *second lane*), along with several proteins of lower molecular weight. The anti-EGFP antibody also recognized these lower molecular weight bands in cells transfected with wild-type MRP1 (Fig. 1B, *first lane*), suggesting that these lower molecular weight bands are the result of a nonspecific cross-reaction and do not represent degraded products containing EGFP. Because both antibodies recognized identical molecular weight bands in MRP1-EGFP transfected lanes, and because no fragment of the fusion protein was recognized by either antibody, it is likely that MRP1-EGFP is being expressed in its entirety. EGFP expression thus correlates directly with the presence of MRP1.

The second control was to determine whether the subcellular localization of MRP1 was affected by the placement of EGFP at its COOH terminus. HeLa cells were transfected with MRP1-EGFP and observed by confocal microscopy. Within 24–48 h after transfection, cells revealed plasma membrane-localized EGFP fluorescence, with minor staining in the perinuclear region (Fig. 1C). Punctate regions of brighter fluorescence follow the cell periphery along invaginations of the membrane, demonstrating the degree to which MRP1-EGFP localizes to the plasma membrane. Immunolabeling with an anti-MRP1 antibody resulted in a distribution of immunofluorescence that was similar to the EGFP fluorescence (Fig. 1D). When the EGFP fluorescence was merged with the anti-MRP1 derived fluorescence, there was nearly complete colocalization of the anti-MRP1 and EGFP signals (Fig. 1E). To follow this pattern in greater detail, the relative fluorescence intensities of the two signals were plotted, using a representative line across the cell as a reference (Fig. 1, E and F). Both the anti-MRP1 and the anti-EGFP signals share the same general distribution along the length of the cell, with a marked drop in intensity in the center, corresponding to the nucleus. An absence of signal in the nucleus once again indicates that EGFP has not been cleaved from MRP1, because free EGFP is small enough to diffuse through the nuclear pore. Immunolabeling of anti-MRP1 in wild-type MRP1 transfected cells revealed a similar pattern of fluorescence distribution (data not shown). Thus, it seems likely that the COOH-terminal addition of EGFP did not affect the localization of MRP1 and that EGFP fluorescence is a good indicator of the presence of MRP1.

**Activity of the MRP1-EGFP Fusion Protein.** MRP1 expression is associated with the decreased intracellular accumulation of many compounds, some of which are naturally fluorescent. Therefore, it is possible to assess the effect of MRP1-EGFP expression on the cellular accumulation and distribution of fluorescent substrates using EGFP fluorescence as a marker for MRP1-EGFP expressing cells. The effect of MRP1-EGFP on the accumulation of the nonfluorescent microtubule-depolymerizing agents vincristine, vinblastine, and colchicine can be measured by the extent to which these drugs are able to disrupt microtubule structure in MRP1-EGFP expressing cells. Because transient transfection generates large numbers of both expressing and nonexpressing cells, cells that express EGFP-fluorescence can be directly compared with nonexpressing cells, both of which are side by side, in the same culture dish, serving as an internal control.

The specificity of MRP1-EGFP activity was determined by comparing the activity of the fluorescent MRP1 to that of another protein involved in multidrug resistance, P-glycoprotein (MDR1). To make direct comparisons of the two proteins, MDR1 was tagged with ECFP (17) and MRP1 was tagged with EYFP. Cells were separately trans-

<sup>4</sup> A. P. Pant, Y. Chen, and S. M. Simon. Using long-wavelength, cell-impermeant, cyanine DNA dyes to label dead cells, manuscript in preparation.

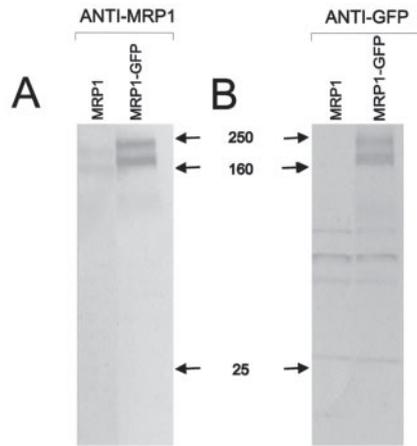
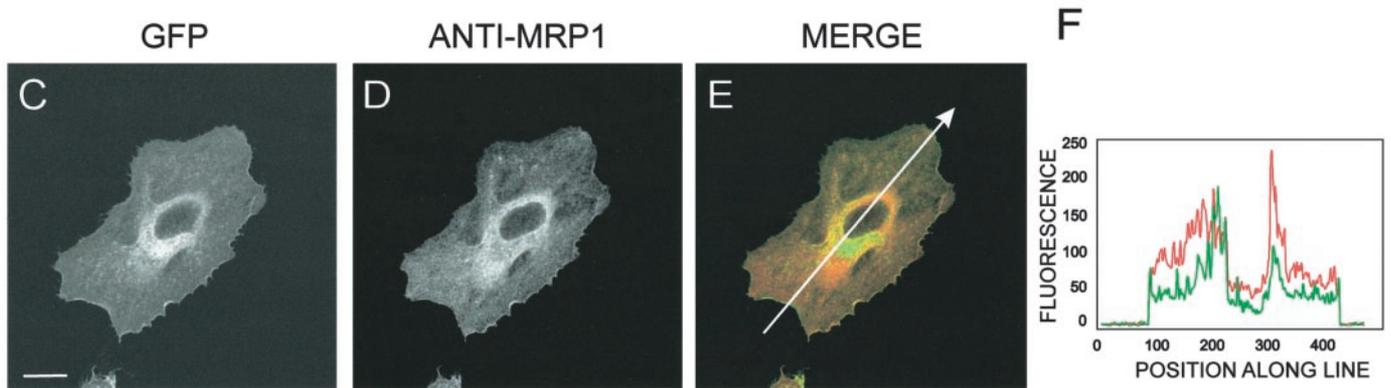


Fig. 1. The expression and localization of MRP1-EGFP in HeLa cells. In A–B, to determine whether all cellular EGFP was part of the MRP1-EGFP fusion protein, cell lysates of MRP1-EGFP or MRP1 transfected cells were immunoblotted with either an anti-EGFP or an anti-MRP1 antibody. Molecular masses of the bands were determined by using standard protein markers. The anti-MRP1 antibody recognized a doublet that migrated between 160 and 250 kDa in MRP1 transfected lysates (A, left lanes). In MRP1-EGFP cell lysates, both of the antibodies recognized a doublet of reduced electrophoretic mobility that was consistent with a 30-kDa addition to the wild-type protein; no proteolysis products of MRP1-EGFP were recognized by either antibody. Lower molecular-weight bands were recognized nonspecifically in both MRP1- and MRP1-EGFP-transfected cells. In C–F, to confirm the colocalization of MRP1 and EGFP in MRP1-EGFP transfected cells, cells were fixed, probed with an anti-MRP1 antibody, and examined under confocal microscopy for (C) endogenous EGFP fluorescence and (D) immunofluorescence against MRP1. In E, EGFP fluorescence was merged with the fluorescence derived from labeled MRP1 to determine the extent of the colocalization. In F, the relative fluorescence intensities of both labels were plotted for a representative line drawn along the merged image. Scale bar, 20  $\mu$ m.



fectured with either MRP1-EYFP or MDR1-ECFP, and then replated together, generating a mixed population of cells: some expressing neither protein, others expressing various levels of MDR1-ECFP, and still others expressing various levels of MRP1-EYFP. Because EYFP and ECFP can be spectrally distinguished, the relative activities of the two drug-resistant proteins, MRP1 and MDR1, could be directly compared.

**MRP1-EGFP Shows Activity Profiles Analogous to MRP1 with TMRE, a Positively Charged Dye.** Rhodamine 123 is a positively charged membrane-potential dye that stains the negatively charged

mitochondria of living cells. Previous studies have suggested that it is substrate for MRP1 (20). However, because of the spectral overlap between rhodamine 123 and EYFP, we assayed the activity of MRP1 against the homologous dye TMRE. HeLa cells that were transfected with either MRP1-EYFP or MDR1-ECFP were subsequently examined for TMRE accumulation (Fig. 2A–D). In the field of cells seen in Fig. 2A–D, one expressed MRP1-EYFP (Fig. 2A, bottom center) and three expressed MDR1-ECFP (Fig. 2B, one on the left and two on the top center). TMRE fluorescence was observed in only three cells (Fig. 2C, right side), none of which expressed MRP1-EYFP or

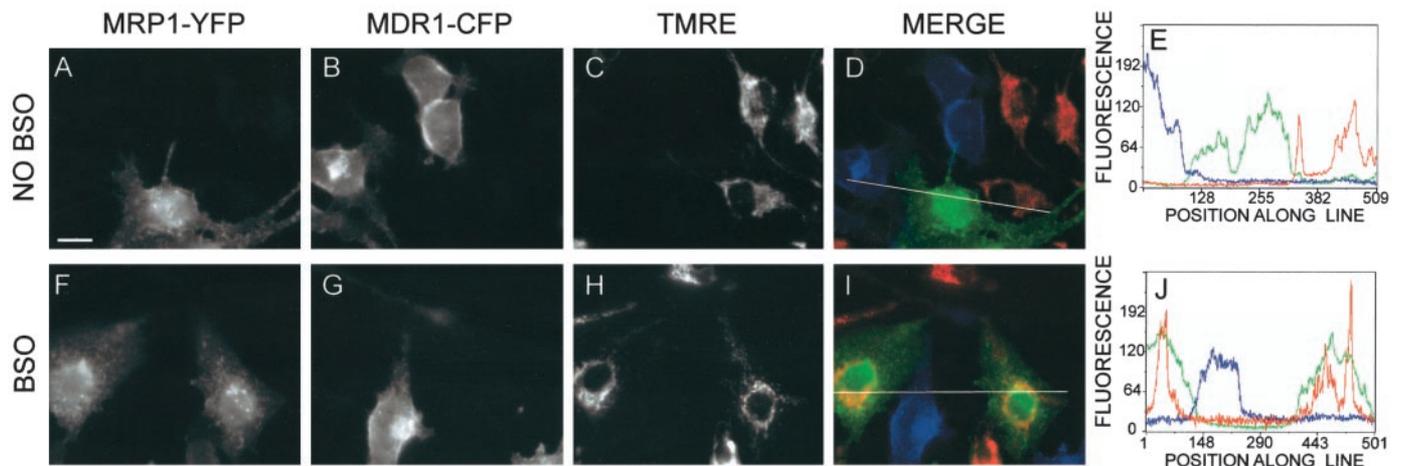


Fig. 2. The activity of MRP1-EYFP is analogous to wild-type MRP1. HeLa cells were separately transfected with MRP1-EYFP and MDR1-ECFP, plated together, and then visualized 48 h after transfection under wide-field fluorescence microscopy. All of the cells were incubated in 50 nM TMRE 15 min prior to visualization. In A and F, cells expressing MRP1 were visualized using EYFP fluorescence; in B and G, cells expressing MDR1 were visualized using ECFP fluorescence; in C and H, cells accumulating TMRE were visualized using rhodamine fluorescence. In D, cells expressing either MRP1 (green) or MDR1 (blue) excluded TMRE (red) as is evident in the merged image. In E, a line plot of the fluorescence intensities of a representative region in the merged image demonstrates the exclusion of TMRE (red line) from the cells expressing MRP1 (green line) or MDR1 (blue line). In F–J, cells were incubated with the MRP1-inhibitor BSO (25  $\mu$ M) for 24 h. In I, MRP1-EYFP cells accumulated TMRE, whereas MDR1-ECFP cells did not. In J, a line scan quantifies the degree to which TMRE was excluded from MDR1-ECFP cells but not from MRP1-EYFP cells. Scale bar, 20  $\mu$ m.

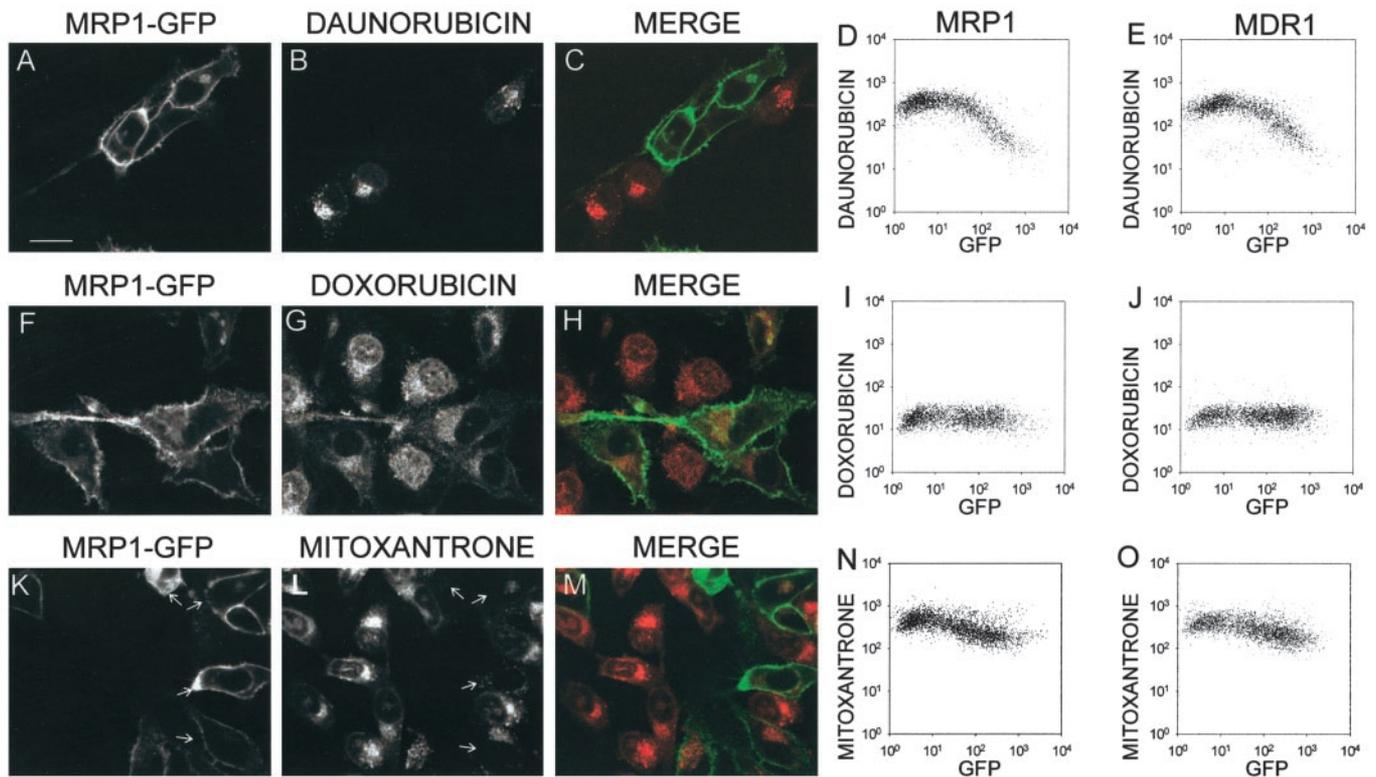


Fig. 3. MRP1 activity against weakly-basic chemotherapeutic agents. HeLa cells were transiently transfected with either MRP1-EGFP or MDR1-EGFP and assayed 48 h after transfection for the accumulation of fluorescent chemotherapeutic drugs. *A–E*, 10  $\mu$ M daunorubicin. *B*, daunorubicin accumulated in the nuclear and perinuclear regions of cells that did not express MRP1-EGFP whereas (*A*) cells expressing MRP1-EGFP excluded the chemotherapeutic. This pattern is evident in the merged image (*C*) as well as in (*D*) flow cytometry of MRP1 activity, in which MRP1-EGFP expression is inversely related to the level of daunorubicin fluorescence present in the cell. *E*, flow cytometry of cells transfected with MDR1-EGFP revealed similar activity. *F–J*, 10  $\mu$ M doxorubicin. Whereas cellular doxorubicin levels were not affected by the expression of MRP1-EGFP, as assayed by flow cytometry (*I*), doxorubicin was excluded from the nucleus of cells expressing MRP1, seen in the merged image (*H*), in which *red* denotes doxorubicin and *green* denotes MRP1-EGFP. In *J*, flow cytometry was also performed on MDR1-EGFP expressing cells for comparison. *K–O*, 2  $\mu$ M mitoxantrone. MRP1 expression resulted in a slight decrease in mitoxantrone accumulation, an observation made clear in the merged image (*M*) as well as by flow cytometry analysis (*N*). MDR1 activity was used as a point of comparison (*O*). *Arrows*, MRP1-EGFP expressing cells; *scale bar*, 20  $\mu$ m.

MDR1-ECFP, as evident in the merged image (Fig. 2*D*). Because cells expressed either drug-resistance protein or accumulated TMRE, the expression of MDR1 or MRP1 was responsible for lowering the accumulation of TMRE. The line profile of ECFP, EYFP, and TMRE fluorescence intensities also indicates that TMRE fluorescence was diminished to background levels in cells expressing either MDR1-ECFP or MRP1-EYFP (Fig. 2*E*).

BSO is reported to be an inhibitor of MRP1 activity because it depletes the cell of glutathione, a cofactor thought to be necessary for MRP1 function (7). On the administration of BSO, cells expressing MRP1-EYFP accumulated TMRE (Fig. 2, *F* and *H*), but cells expressing MDR1-ECFP did not (Fig. 2, *G* and *H*). Both the merged image (Fig. 2*I*) and the line scan (Fig. 2*J*) make this pattern evident: BSO specifically reversed the activity of the fluorescently tagged MRP1 protein. Because MRP1-EYFP is active against a previously characterized substrate, and because its activity can be distinguished from that of MDR1 by an MRP1-specific inhibitor, it is likely that the fluorescent tag has not significantly affected the activity of MRP1.

**Anthracyclines.** We next assayed the activity of MRP1-EGFP against the anthracyclines daunorubicin and doxorubicin, both thought to be MRP1 substrates (15). When transiently transfected cells were incubated in 10  $\mu$ M daunorubicin, cells expressing the protein (Fig. 3*A*) showed no detectable drug accumulation (Fig. 3*B*), whereas neighboring, nonexpressing cells accumulated daunorubicin in nuclear and perinuclear regions (Fig. 3, *B* and *C*). To obtain a quantitative relationship between MRP1-EGFP expression and daunorubicin accumulation, total cellular EGFP and

daunorubicin fluorescence were measured for a large number of cells using flow cytometry. Cells exhibiting higher MRP1-EGFP fluorescence showed lower daunorubicin fluorescence, with the highest expressing cells showing 10-fold decreased daunorubicin fluorescence compared with nonexpressing cells (Fig. 3*D*). Cells transfected with MDR1-EGFP showed a similar relationship between expression and daunorubicin accumulation (Fig. 3*E*).

Although MRP1 was able to reduce the daunorubicin levels inside a cell, it exhibited only a marginal effect on the total cellular accumulation of doxorubicin, an anthracycline closely related in structure to daunorubicin. Cells with the highest levels of MRP1-EGFP expression showed only a 2-fold reduction in total doxorubicin fluorescence, as assayed by flow cytometry (Fig. 3*I*). Despite this attenuated activity on the total cellular accumulation of doxorubicin, cells expressing MRP1-EGFP revealed an altered intracellular distribution of the chemotherapeutic. As seen under confocal microscopy, MRP1-EGFP expressing cells had significantly reduced doxorubicin fluorescence inside the nucleus but had levels of doxorubicin fluorescence in the cytoplasm similar to that of nonexpressing cells (Fig. 3, *F* and *G*). In contrast, the nucleus of nonexpressing cells was brightly fluorescent with the drug, as was the perinuclear region (Fig. 3*H*). Because the cellular targets of doxorubicin are located primarily inside the nucleus, this altered distribution away from the nucleus may account for MRP1-mediated doxorubicin resistance. Expression of MDR1-EGFP had similar effects on doxorubicin distribution; cells expressing MDR1-EGFP had diminished drug accumulation in the nucleus (data not shown), whereas the total intracellular drug fluorescence remained unchanged (Fig. 3*J*). Altered patterns of

doxorubicin accumulation have been previously associated with multidrug-resistant cells (21).

**Mitoxantrone.** Previously published reports have differed in their assessment of MRP1 activity against the anthracenedione mitoxantrone. Some have suggested that MRP1 expression alone confers resistance to the chemotherapeutic (13, 22), whereas others have seen no effect of MRP1 expression on mitoxantrone resistance (15). When transiently transfected HeLa cells were incubated in 2  $\mu\text{M}$  mitoxantrone, cells with elevated MRP1-EGFP levels (Fig. 3K, *cell in the upper center with an arrow*), showed diminished levels of mitoxantrone accumulation (Fig. 3L). However, cells with lower levels of MRP1 (Fig. 3K, *bottom center*), had levels of mitoxantrone that were similar to nonexpressing cells (Fig. 3L). In the merged image (Fig. 3M), we can clearly distinguish a spectrum of drug accumulation that corresponds to the degree of MRP1-EGFP expressed in the cell. Flow cytometry reveals that MRP1 activity against mitoxantrone (Fig. 3N) is considerably diminished when compared with daunorubicin (Fig. 3D). Cells expressing the highest level of MRP1-EGFP showed less than a 10-fold reduction in drug accumulation (Fig. 3N), a reduction comparable with MDR1-EGFP expressing cells (Fig. 3O). This weak activity may account for the differing assessments of mitoxantrone as a substrate of MRP1.

**Microtubule-depolymerizing Agents.** Because the microtubule-depolymerizing chemotherapeutics vincristine, vinblastine, and colchicine are not fluorescent, the transport functions of MRP1 were assayed by the relative degrees of microtubule depolymerization in cells expressing and not expressing the protein. After an incubation in

600 nM vincristine, HeLa cells, transiently transfected with either MDR1-ECFP or MRP1-EYFP, were fixed and then stained with a fluorescent antitubulin antibody. The field of cells in Fig. 4A–E includes one cell strongly expressing MRP1-EYFP (Fig. 4A), two cells strongly expressing MDR1-ECFP (Fig. 4B), and a number of surrounding cells expressing neither protein (Fig. 4D). The microtubules of nonexpressing cells were severely disrupted by vincristine, so much so that they had altered cell morphology (Fig. 4D). These cells were round and considerably smaller than their MRP1-EYFP- or MDR1-ECFP-tagged counterparts. However, the cell expressing MRP1-EYFP and the two cells expressing MDR1-ECFP showed relatively little microtubule depolymerization; each cell retained an intricate microtubule network. Moreover, whereas the MTOC was intact in the MDR1-ECFP expressing cells, it was no longer apparent in the MRP1-EYFP expressing cells. Therefore, MRP1 provides some protection against the depolymerizing effects of vincristine, if not as much as does MDR1.

Next, we assayed the effect of MRP1-EYFP on colchicine activity. Cells incubated in 2  $\mu\text{M}$  colchicine were unable to maintain microtubule integrity in the absence of a MRP1 or MDR1 (Fig. 4I). These cells were diffusely stained with the antitubulin antibody, but they showed no discernable microtubule structure. Expression of MDR1-ECFP, however, rescued this loss; the ECFP-tagged cell (Fig. 4G) had relatively undamaged microtubules, with the MTOC still intact (Fig. 4H). Similarly, the two MRP1-EYFP-expressing cells (Fig. 4F) had a complex network of microtubules with the MTOC quite evident (Fig. 4J).

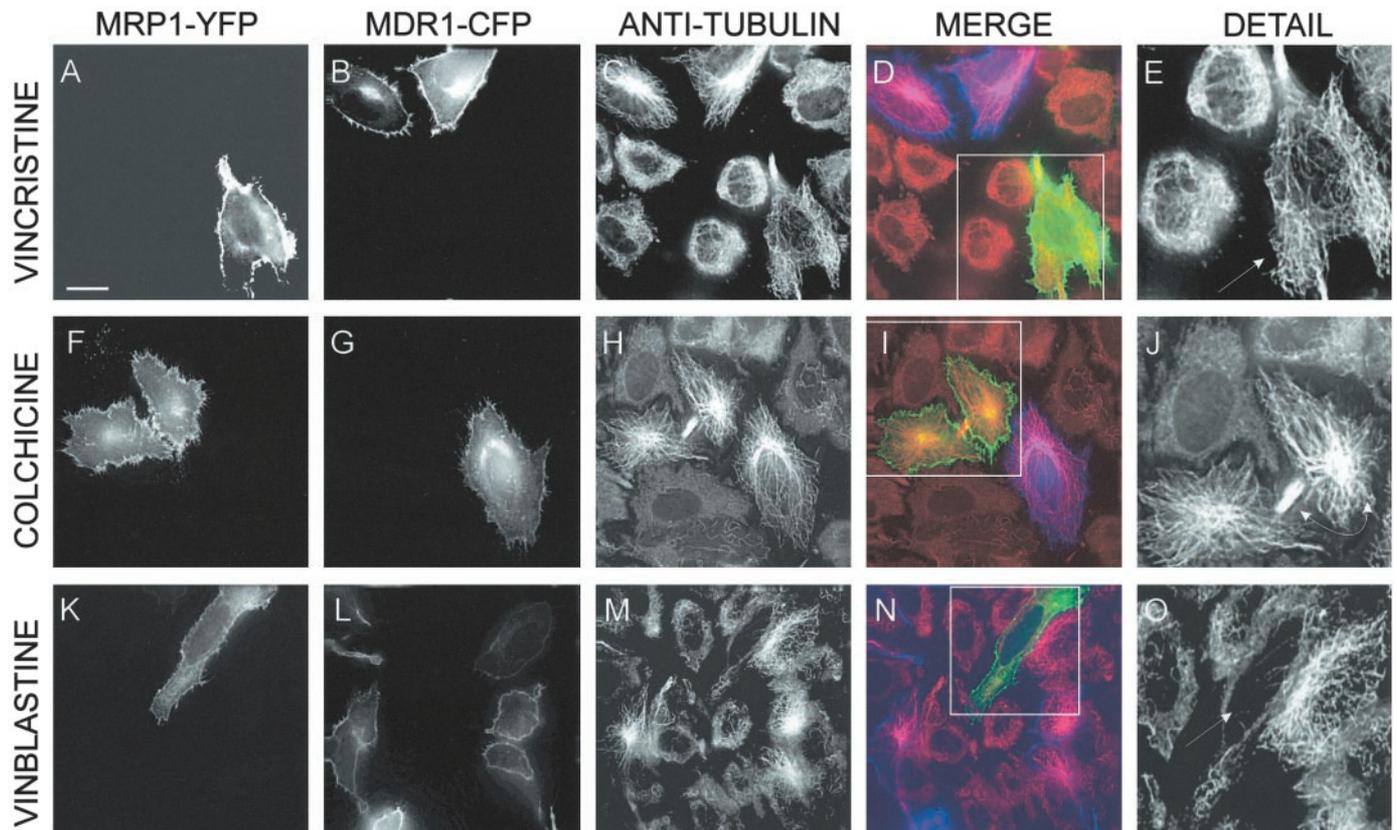


Fig. 4. MRP1 activity against microtubule-depolymerizing agents. HeLa cells, separately transfected with MRP1-EYFP and MDR1-ECFP, were incubated with microtubule-disrupting chemotherapeutics and examined for the extent of microtubule damage with a fluorescent antitubulin antibody 48 h posttransfection. A–E, 600 nM vincristine. Cells expressing MRP1-EYFP were afforded some protection against vincristine's effects, and cells with MDR1-ECFP were relatively unaffected by vincristine. In contrast, there was substantial disruption of the microtubules in cells that did not express either of the multidrug resistance proteins. F–J, 2  $\mu\text{M}$  colchicine. MRP1-EYFP expressing cells, as well as MDR1-ECFP expressing cells, maintained a complex microtubule structure in the presence of colchicine, whereas nonexpressing cells had almost completely depolymerized microtubules. K–O, 600 nM vinblastine. Cells expressing MRP1-EYFP and nonexpressing cells showed almost complete depolymerization of their microtubules, whereas cells expressing MDR1-ECFP were much less affected by the drug. White arrows, MRP1-EGFP-expressing cells. Scale bar, 20  $\mu\text{m}$ .

Finally, we examined the effect of expressing MRP1-EYFP on vinblastine activity. Cells lacking both multidrug resistance proteins were clearly affected by vinblastine; they had no MTOC and considerably disrupted microtubules (Fig. 4N). All four of the cells that express MDR1-ECFP showed relatively undisrupted microtubule structures, with a MTOC somewhat visible in each cell (Fig. 4M). In contrast, the cell expressing MRP1-EYFP could not be distinguished from the nonexpressing cells (Fig. 4O). As we see in the detail of the antitubulin-stained field (Fig. 4O), the center cell, with clearly disrupted microtubule structure, was the one that expressed MRP1-EYFP. The adjacent cell with a largely intact MTOC was expressing MDR1-ECFP. Surrounding cells, like the cell expressing MRP1, had microtubules that had been severely damaged by vinblastine. It seems, therefore, that MRP1, unlike MDR1, provides little to no protection against the effects of vinblastine. A differential effect of MRP1 expression on vincristine and vinblastine has been suggested previously (15).

## DISCUSSION

Transient transfection with a fluorescently tagged MRP1 provides a means of assessing MRP1 activity that obviates the need for continuous culture in chemotherapeutic or antibiotic agents. The fluorescent tag can be used as a reliable indicator of the presence of MRP1 because all detectable EGFP is present as part of the fusion protein. Moreover, the fusion protein has intracellular localization patterns that are indistinguishable from wild-type MRP1. This fusion protein thereby allows us to determine whether the transient introduction of MRP1 can alter a cell's response to a chemotherapeutic challenge, and it does so in a way that allows levels of MRP1-EGFP expression to be correlated with the intracellular concentration of fluorescent substrates. Furthermore, separate transfections with a ECFP-tagged MDR1 and a EYFP-tagged MRP1 permit easy comparisons of MRP1 and MDR1 activities. Indeed, fluorescent tags of this sort would allow the activities of any two functionally related proteins to be directly compared and easily distinguished.

After brief incubations in compounds reported previously to be MRP1 substrates, HeLa cells expressing various levels of MRP1-EGFP were examined for altered patterns of intracellular drug distribution, as well as for cytotoxic damage. In accordance with previous findings, MRP1-EGFP expression resulted in substantially diminished accumulation of TMRE and daunorubicin and somewhat reduced levels of mitoxantrone. However, MRP1-EGFP had little effect on the total doxorubicin concentrations inside the cell. Surprisingly, cells expressing MRP1-EGFP did have significantly lowered doxorubicin fluorescence inside the nucleus, a reduction which may be responsible for MRP1-mediated resistance against this chemotherapeutic. Whether this altered nuclear distribution points to MRP1 activity away from the plasma membrane is yet to be resolved. When MRP1 expression was assayed for its ability to protect against the microtubule-depolymerizing effects of three chemotherapeutic agents, the protein was found to substantially attenuate the damage caused by colchicine, somewhat guard against vincristine-induced microtubule disruption, and have no detectable effect against vinblastine.

## ACKNOWLEDGMENTS

We thank Dr. Gary Kruh for generously providing the MRP1 cDNA plasmid, Alison North for assistance with fluorescence microscopy, and Collin Thomas for suggestions on the manuscript.

## REFERENCES

- Cole, S. P., and Deeley, R. G. Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. *Bioessays*, 20: 931–940, 1998.
- Borst, P., Evers, R., Kool, M., and Wijnholds, J. The multidrug resistance protein family. *Biochim. Biophys. Acta*, 1461: 347–357, 1999.
- Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deeley, R. G. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science (Wash. DC)*, 258: 1650–1654, 1992.
- Gottesman, M. M., and Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, 62: 385–427, 1993.
- Zaman, G. J., Flens, M. J., van Leusden, M. R., de Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., and Broxterman, H. J. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc. Natl. Acad. Sci. USA*, 91: 8822–8826, 1994.
- Feller, N., Kuiper, C. M., Lankelma, J., Ruhdal, J. K., Scheper, R. J., Pinedo, H. M., and Broxterman, H. J. Functional detection of MDR1/P170 and MRP/P190-mediated multidrug resistance in tumour cells by flow cytometry. *Br. J. Cancer*, 72: 543–549, 1995.
- Hipfner, D. R., Deeley, R. G., and Cole, S. P. Structural, mechanistic and clinical aspects of MRP1. *Biochim. Biophys. Acta*, 1461: 359–376, 1999.
- Mao, Q., Deeley, R. G., and Cole, S. P. Functional reconstitution of substrate transport by purified multidrug resistance protein MRP1 (ABCC1) in phospholipid vesicles MAO2000. *J. Biol. Chem.*, 275: 34166–34172, 2000.
- Zaman, G. J., Lankelma, J., van Tellingen, O., Beijnen, J., Dekker, H., Paulusma, C., Oude Elferink, R. P., Baas, F., and Borst, P. Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc. Natl. Acad. Sci. USA*, 92: 7690–7694, 1995.
- Tommasini, R., Evers, R., Vogt, E., Mornet, C., Zaman, G. J., Schinkel, A. H., Borst, P., and Martinou, E. The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor I. *Proc. Natl. Acad. Sci. USA*, 93: 6743–6748, 1996.
- Loe, D. W., Almquist, K. C., Deeley, R. G., and Cole, S. P. C. Multidrug resistance protein (MRP)-mediated transport of leukotriene C(4) and chemotherapeutic agents in membrane vesicles. *J. Biol. Chem.*, 271: 9675–9682, 1996.
- Van Luyn, M. J., Muller, M., Renes, J., Meijer, C., Scheper, R. J., Nienhuis, E. F., Mulder, N. H., Jansen, P. L., and de Vries, E. G. Transport of glutathione conjugates into secretory vesicles is mediated by the multidrug-resistance protein 1. *Int. J. Cancer*, 76: 55–62, 1998.
- Morrow, C. S., Smitherman, P. K., Diah, S. K., Schneider, E., and Townsend, A. J. Coordinated action of glutathione S-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1 and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells. *J. Biol. Chem.*, 273: 20114–20120, 1998.
- Litman, T., Brangi, M., Hudson, E., Fetsch, P., Abati, A., Ross, D. D., Miyake, K., Resau, J. H., and Bates, S. E. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J. Cell Sci.*, 113(Pt 11): 2011–2021, 2000.
- Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M., Deeley, R. G. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res.*, 54: 5902–5910, 1994.
- Chen, Y., Schindler, M., and Simon, S. M. A mechanism for tamoxifen-mediated inhibition of acidification. *J. Biol. Chem.*, 274: 18364–18373, 1999.
- Chen, Y., and Simon, S. M. *In situ* biochemical demonstration that P-glycoprotein is a drug efflux pump with broad specificity. *J. Cell Biol.*, 148: 863–870, 2000.
- Chen, Y., Pant, A. C., and Simon, S. M. P-glycoprotein does not reduce substrate concentration from the extracellular leaflet of the plasma membrane in living cells. *Cancer Res.*, 61: 7763–7769, 2001.
- Bakos, E., Hegedus, T., Hollo, Z., Welker, E., Tusnady, G. E., Zaman, G. J., Flens, M. J., Varadi, A., and Sarkadi, B. Membrane topology and glycosylation of the human multidrug resistance-associated protein. *J. Biol. Chem.*, 271: 12322–12326, 1996.
- Twentyman, P. R., Rhodes, T., and Rayner, S. A comparison of rhodamine 123 accumulation and efflux in cells with P-glycoprotein-mediated and MRP-associated multidrug resistance phenotypes. *Eur. J. Cancer*, 30A: 1360–1369, 1994.
- Altan, N., Chen, Y., Schindler, M., and Simon, S. M. Defective acidification in human breast tumor cells and implications for chemotherapy. *J. Exp. Med.*, 187: 1583–1598, 1998.
- Schneider, E., Horton, J. K., Yang, C. H., Nakagawa, M., and Cowan, K. H. Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. *Cancer Res.*, 54: 152–158, 1994.