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Characterization of Vincristine Transport by the M_r 190,000 Multidrug Resistance Protein (MRP): Evidence for Cotransport with Reduced Glutathione[1]

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ABSTRACT

The M_r 190,000 multidrug resistance protein (MRP) confers resistance to a broad spectrum of natural product drugs. However, it has not been possible to demonstrate that MRP can actively transport unmodified forms of these compounds, although the protein has been shown to transport structurally diverse glutathione (GSH)- and glucuronide-conjugated molecules. Previously, we showed that ATP-dependent uptake of vincristine by MRP-enriched, inside-out membrane vesicles could be stimulated by physiological concentrations of GSH (Loe *et al.*, J. Biol. Chem., *271:* 9675–9682, 1996). We have now established that the ATP/GSH dependent vincristine uptake is both proportional to the level of MRP in the membrane vesicles and can be inhibited by monoclonal antibodies shown previously to inhibit transport of established MRP substrates, such as leukotriene C_4. We also show that short-chain alkyl derivatives of GSH can stimulate drug uptake, which suggests that vincristine transport does not necessarily involve a change in redox state or glutathionylation of the protein. Furthermore, vincristine uptake is accompanied by ATP- and drug-dependent accumulation of GSH, which can also be stimulated to a lesser extent by vinblastine but not daunorubicin or doxorubicin. Although GSH or vincristine alone are very poor inhibitors of MRP-mediated transport of leukotriene C_4, together they act as relatively potent competitive inhibitors. Overall, our data demonstrate that MRP can actively cotransport GSH and unmodified vincristine and that these compounds probably interact, either with the leukotriene C_4 binding site(s) on the protein or with a mutually exclusive site.

INTRODUCTION

Resistance to multiple antineoplastic agents is a major impediment to the successful treatment of many malignant diseases. *In vitro*, the development of multidrug resistance in tumor cells is usually accompanied by increased expression of either the M_r 190,000 MRP[3] or the M_r 170,000 P-glycoprotein (1–4). In some cell lines, increased levels of both proteins have been observed (5–7). MRP and P-glycoprotein belong to the ATP-binding cassette superfamily of transmembrane transport proteins, but their primary structure similarity is limited largely to the generally conserved nucleotide binding domains characteristic of ATP-binding cassette proteins (8).

It has been established by several laboratories that mammalian cells transfected with human MRP, like P-glycoprotein-transfected cells, are resistant to a variety of natural product drugs such as VCR, doxorubicin, daunorubicin, and VP-16, and accumulate lower steady-state levels of these drugs than do control-transfected cells (9–11). Interestingly, lower levels of resistance to other natural product drugs including vinblastine, colchicine, and paclitaxel are observed in MRP transfectants than in P-glycoprotein overexpressing cells but the basis of this differential sensitivity is unknown. Finally, in addition to chemotherapeutic agents, MRP also confers resistance to certain antimonial and arsenical oxyanions, a property not associated with overexpression of P-glycoprotein (9, 12). Thus, although the cross-resistance profiles of these two drug-resistance proteins are similar, they are not identical.

It has long been noted that in cells that overexpress P-glycoprotein, the degree of reduced drug accumulation seldom correlates well with the relative resistance of the cells. This is also true of cells that overexpress MRP, and a generally satisfactory explanation for these observations remains elusive. To avoid some of the problems inherent in studies of intact cells, the transport properties of P-glycoprotein and MRP have been examined more directly in inside-out membrane vesicle systems, which allow for more rigorous analyses of kinetic parameters. In contrast to studies of membrane vesicles enriched for P-glycoprotein (13–15), we and others have been unable to demonstrate MRP-mediated active transport of chemotherapeutic agents such as VCR, daunorubicin, or VP-16 (12, 16–19), and contrary reports claiming to have shown direct transport of these drugs have recently been retracted (20, 21). Consistent with the lack of direct drug transport is the inability to label MRP with photoactive radiolabeled analogues of vinblastine or doxorubicin (9, 22).

In contrast, it has now been well established that *in vitro*, MRP is capable of actively transporting a wide variety of structurally diverse conjugated organic anions (1, 2, 23, 24). Conjugates transported with particularly high affinity include the cysteinyl leukotriene LTC_4 and GSH *S*-conjugates of the potent carcinogen aflatoxin B_1 (16, 17, 25–27). Other compounds shown directly to be substrates of MRP include GSH *S*-conjugates of prostaglandin A_2 and ethacrynic acid as well as the cholestatic 17β-estradiol 17-(β-D-glucuronide) and the bile salts glucuronysylhyodeoxycholate and 3α-sulfatolithocholyltaurine (18, 28–31). Chemotherapeutic agents, such as the *Vinca* alkaloid VCR, are poor inhibitors of LTC_4 transport (16–18). However, we have observed that the ability of several unmodified drugs, including VCR, to inhibit LTC_4 transport can be significantly enhanced by the addition of GSH (17). We have also observed that physiological concentrations of GSH can stimulate MRP-mediated membrane vesicle uptake of some unconjugated substrates, including VCR and aflatoxin B_1 (17, 26).

In the present study, we have further characterized MRP-mediated, GSH-stimulated VCR transport. We have established that it can be inhibited by MRP-specific MAbs and that the transport of VCR is accompanied by drug stimulated cotransport of GSH. We also show that short chain *S*-alkyl GSH conjugates can partially substitute for GSH in stimulating VCR transport, but other structurally related compounds, such as GSH sulfonate or the dipeptides cysteinylglycine and γ-glutamylcysteine, cannot. Finally, we show that VCR and GSH in combination behave as competitive inhibitors of MRP-mediated transport of its high affinity substrate, LTC_4.

MATERIALS AND METHODS

Materials. Generally labeled [^{3}H]VCR (6.9–7.8 Ci/mmol) was obtained from Amersham (Oakville, Ontario, Canada). [Glycine-2-^{3}H]-GSH (40–44 Ci/mmol) and [^{3}H]LTC_4 (165 Ci/mmol) were obtained from Dupont NEN

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[3] The abbreviations used are: MRP, multidrug resistance protein; MAb, monoclonal antibody; GSH, reduced glutathione; GSSG, glutathione disulfide or oxidized GSH; VCR, vincristine; LTC_4, leukotriene C_4; BSO, buthionine sulfoximine; VP-16, etoposide.

(Markham, Ontario, Canada). Nucleotides, chemotherapeutic drugs, GSH, GSSG, and GSH derivatives and analogues were purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in DMSO and the final concentration of vehicle in any transport assay did not exceed 1% (v/v). DTT, glucuronic acid, acivicin and (D/L)-BSO were also from Sigma. Paclitaxel was from Omicron BioChemicals (San Antonio, TX) and LTC_4 was purchased from CalBiochem (La Jolla, CA). The murine MRP-specific MAbs QCRL-1, QCRL-2, QCRL-3, and QCRL-4 have been described previously and were purified before use (32).

Cell Culture. The origin and culture conditions of the small cell lung cancer cell line H69, its multidrug resistant MRP-overexpressing variant H69AR, and the revertant H69PR cell line have been described previously (33, 34). The production and maintenance of MRP-transfected (T14) or control vector-transfected (C6) HeLa cell populations were as described (9). The H69AR cells express approximately 4- to 8-fold higher levels of MRP than the transfected T14 HeLa cells (9).

Membrane Vesicle Preparation. Plasma membrane vesicles were prepared as described with modifications (17, 35). Cells were homogenized in buffer containing 50 mM Tris-HCl, 250 mM sucrose, 0.25 mM $CaCl_2$ (pH 7.5), and protease inhibitors (32). Cell pellets were frozen at −70°C for at least 1 h, thawed, and then disrupted by N_2 cavitation. EDTA was added to 1 mM, and after centrifugation at 500 × *g* for 15 min, the supernatant was layered over 35% (w/w) sucrose in 10 mM Tris-HCl and 1 mM EDTA and centrifuged at 100,000 × *g* for 2 h. The interface was collected and washed twice by centrifugation. The membrane pellet was resuspended in transport buffer [50 mM Tris-HCl and 250 mM sucrose (pH 7.5)] and passed 20 times through a 27-gauge needle for vesicle formation.

Membrane Vesicle Transport Studies. ATP-dependent transport of [^{3}H]VCR into the membrane vesicles was measured by a rapid filtration technique (17, 35). Standard transport assays were performed with membrane vesicles (20–25 μg of protein in a 50-μl reaction volume) that were incubated at 37°C in the presence of 200 nM [^{3}H]VCR (0.1 μCi/reaction), 10 mM DTT, 10 mM $MgCl_2$, and 4 mM ATP or AMP in transport buffer containing 1% methanol. Where indicated, GSH was added to 5 mM unless otherwise stated. An ATP-regenerating system was not included, because we had demonstrated previously (17) that its omission does not significantly diminish levels of vesicle-associated VCR. Where indicated, drugs were added to 10 and 100 μM, and MRP-specific MAbs were added to 10 μg/ml. Uptake was stopped by filtration through glass fiber (Type A/E) filters (Gelman Sciences, Dorval, Quebec, Canada) that had been presoaked overnight at 37°C in 10% (w/v) BSA. All of the data were corrected for the amount of [^{3}H]VCR that remained bound to the filter, which was usually <10% of the total radioactivity. In some cases, data were also corrected for the amount of vesicle-associated [^{3}H]VCR in the presence of AMP or GSH alone.

ATP-dependent transport of [^{3}H]GSH into the membrane vesicles was measured as above, with a substrate concentration of 100 μM [^{3}H]GSH (40 nCi/reaction), unless otherwise indicated. To minimize GSH catabolism by γ-glutamyl transpeptidase during transport, membranes were preincubated in 0.5 mM acivicin for at least 1 h before measuring [^{3}H]GSH transport (36). Chemotherapeutic drugs were added to 100 μM where indicated, except for paclitaxel, which was added to 10 μM. Uptake was stopped by filtration through glass fiber (Type A/E) filters. All of the data were corrected for the amount of [^{3}H]GSH that remained bound to the filter, which was <5% of the total radioactivity. In some cases, data were also corrected for the amount of vesicle-associated [^{3}H]GSH in the presence of AMP.

ATP-dependent uptake of [^{3}H]LTC_4 was also measured by rapid filtration as above, except that assays were performed at 23°C in a 50-μl reaction containing 2–4 μg of vesicle protein and 50 nM substrate (50 nCi/reaction; Ref. 17). Uptake was stopped by rapid dilution in ice-cold buffer, and then the reaction mixture was filtered through glass fiber filters that had been presoaked in transport buffer. All of the data were corrected for the amount of [^{3}H]LTC_4 that remained bound to the filter, which was usually 5–10% of the total radioactivity, as well as for the amount of vesicle-associated [^{3}H]LTC_4 in the presence of AMP alone. For kinetic analysis of LTC_4 transport in the presence of VCR and GSH, LTC_4 was added at concentrations ranging from 8 nM to 1 μM, and ATP-dependent LTC_4 uptake was determined as above.

RESULTS

[^{3}H]VCR Uptake in Membrane Vesicles. The time course, ATP- and GSH-dependence of [^{3}H]VCR accumulation by membrane vesicles prepared from MRP-transfected HeLa T14 cells are shown in Fig. 1*A*. Accumulation was measured at 37°C at an initial concentration of 200 nM [^{3}H]VCR in the presence of 4 mM ATP or AMP and/or 5 mM GSH. The increase in vesicle-associated [^{3}H]VCR uptake in the absence of GSH (or in the presence of GSH but not ATP) was rapid and reached steady state (~3.5–4 pmol/mg) within 60 s. In the presence of both GSH and ATP, vesicle-associated [^{3}H]VCR increased markedly and approached steady-state levels of ~8–9 pmol/mg) within ~120 s. Thus, the net GSH-dependent [^{3}H]VCR accumulation in the presence of ATP was approximately 4.7 pmol/mg vesicle protein. Steady-state levels of vesicle-associated [^{3}H]VCR were maintained for up to 40 min (data not shown). Analyses of data from early time points indicated ATP/GSH-dependent uptake was no longer linear after the first time point of 8 s. This precluded obtaining reliable estimates of initial rates. The low levels of [^{3}H]VCR uptake in T14 vesicles in the absence of both ATP and GSH were similar to those observed for control C6 membrane vesicles with or without ATP and GSH over the time course shown (data not shown). Thus, no ATP/GSH-dependent uptake of [^{3}H]VCR was detected with control vesicles.

To examine the correlation between VCR transport and levels of MRP expression, [^{3}H]VCR uptake was also measured in vesicles isolated from multidrug resistant H69AR cells which express 4- to 8-fold more MRP than T14 cells (9). In the presence of ATP (or GSH and AMP), vesicle-associated [^{3}H]VCR determined at 8 s was 1.6–2.3 pmol/mg and approached a steady-state level of 6–8 pmol/mg by 3–5 min. In the presence of both ATP and GSH, vesicle-associated

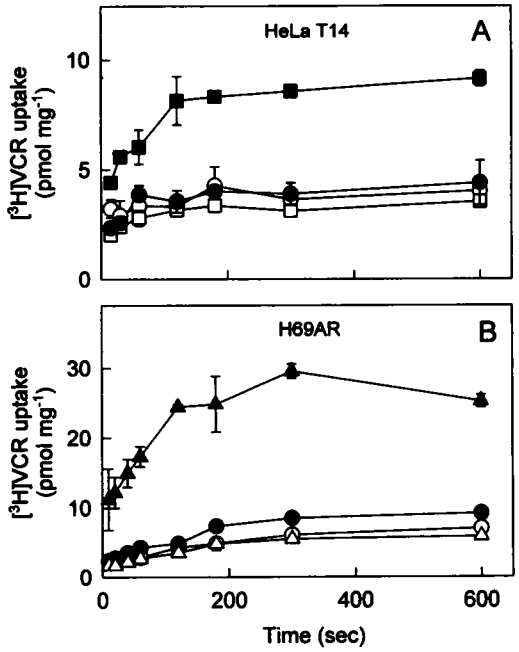

Fig. 1. The time course of [^{3}H]VCR uptake by membrane vesicles from MRP-expressing HeLa T14 transfectants and drug-selected H69AR cells. Membrane vesicles derived from MRP-transfected HeLa cells (*A*) or MRP-overexpressing drug-selected H69AR cells (*B*) were incubated with 200 nM [^{3}H]VCR in transport buffer for the times indicated. The first time point was taken at 8 s. Closed symbols (●, ▲, and ■) represent uptake in the presence of 4 mM ATP; open symbols (○, △, and □) represent uptake in the presence of 4 mM AMP. Squares (□ and ■; *A*) or triangles (△ and ▲; *B*) represent VCR uptake in the presence of 5 mM GSH; circles (*A* and *B*) indicate uptake in the presence of ATP (●) or AMP (○), respectively. Data were corrected for background caused by [^{3}H]VCR binding to filters. Data points are means of triplicate determinations (±SE) in a typical experiment.

[^{3}H]VCR at 8 s increased to ~11 pmol/mg and reached a steady-state level of 23–24 pmol/mg by 120 s (Fig. 1*B*). Thus, the net ATP- and GSH-dependent [^{3}H]VCR transport in H69AR vesicles was approximately 17 pmol/mg, compared with 4.7 pmol/mg in T14 vesicles, which is consistent with the relative levels of MRP in the two membrane vesicle preparations. The results are also consistent with those that we have obtained previously when comparing the relative rates of uptake of the MRP substrates LTC_4, 17β-estradiol 17-(β-D-glucuronide), and aflatoxin B_1-GSH by H69AR and T14 membrane vesicles (17, 26, 28). No ATP/GSH-dependent [^{3}H]VCR uptake was observed for membrane vesicles derived from drug sensitive H69 or revertant H69PR cells (data not shown).

Effect of VCR and GSH Concentration on [^{3}H]VCR Transport by Membrane Vesicles. To determine the dependence of VCR uptake on the concentration of drug and GSH, ATP-dependent [^{3}H]VCR uptake was measured at a constant concentration of GSH (5 mM) and at concentrations of [^{3}H]VCR ranging from 40 to 1.2 nM. Drug uptake was also measured at a constant concentration of VCR (200 nM) and GSH concentrations ranging from 0 to 20 mM. Saturation was not achieved under the conditions of the assay, probably because the uptake was measured at 10 min when equilibrium was already established. Net ATP/GSH-dependent [^{3}H]VCR uptake in H69AR membrane vesicles increased with increasing concentrations of VCR up to 1.2 μM (the highest concentration tested), to reach approximately 70 pmol/mg (Fig. 2*A*). Similarly, as the GSH concentration was increased from 1 to 5 mM, ATP-dependent [^{3}H]VCR (200 nM) uptake by H69AR and T14 membrane vesicles increased from 3 to 8 pmol/mg and from 1 to 4 pmol/mg, respectively (Fig. 2*B*). No change in VCR uptake was observed at concentrations of GSH between 5 and 20 mM. The effect of GSH on [^{3}H]VCR uptake at concentrations less than 1 mM was not detectable under the conditions of the assay.

Inhibition of [^{3}H]VCR Transport by MRP-specific MAbs. Several MRP-specific MAbs have been shown to inhibit MRP-mediated transport of LTC_4, 17β-estradiol 17-(β-D-glucuronide), and aflatoxin B_1-GSH (17, 26, 28). In the present study, we tested whether or not they would also inhibit GSH-stimulated VCR transport, and the results are shown in Fig. 3. As observed previously for other MRP substrates, three MAbs that recognize distinct conformation-dependent epitopes in the first (MAbs QCRL-2 and QCRL-3) and second (MAb QCRL-4) nucleotide-binding domains of MRP[4] completely inhibited ATP/GSH-dependent VCR transport by T14 vesicles at a concentration of 10 μg/ml and a vesicle protein concentration of 500 μg/ml. In contrast, MAb QCRL-1, which recognizes a linear epitope in a part of the linker region of MRP that we have shown is not required for LTC_4 transport function (37, 38), had no effect.

Effect of GSH Derivatives on [^{3}H]VCR Transport in MRP-enriched T14 Membrane Vesicles. Previously, we showed that [^{3}H]VCR transport was not increased by other sulfhydryl reducing agents such as DTT, L-cysteine or 2-mercaptoethanol (17). We have now examined the ability of several GSH derivatives and other small anionic molecules to enhance [^{3}H]VCR transport. Seven compounds structurally related to GSH (*S*-methyl-GSH, *S*-ethyl-GSH, *S*-butyl-GSH, GSH ethyl ester, GSH sulfonate, and the dipeptides cysteinylglycine and γ-glutamylcysteine) were tested, as were glucuronic acid and BSO, an irreversible inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis. With the exception of the low molecular weight *S*-alkyl GSH derivatives, none of these compounds stimulated [^{3}H]VCR uptake (Table 1). *S*-Methyl-GSH, *S*-ethyl-GSH and *S*-butyl-GSH supported [^{3}H]VCR uptake to steady-state levels that were 30, 25, and 19%, respectively, of those observed

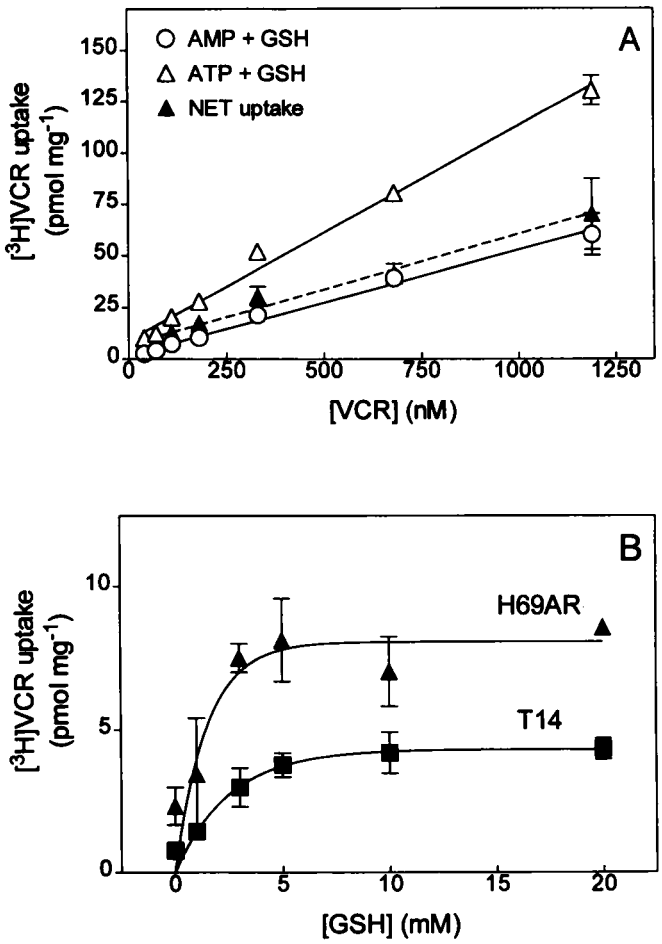

Fig. 2. The effect of VCR and GSH concentration on [^{3}H]VCR transport by membrane vesicles. In *A*, [^{3}H]VCR transport by H69AR membrane vesicles was measured at VCR concentrations ranging from 40 nM to 1.2 μM for 10 min at 37°C. GSH was included in all of the incubations at a concentration of 5 mM. ○, uptake in the presence of 4 mM AMP; △, uptake in the presence of 4 mM ATP. Net uptake (▲) was determined by subtracting the level of uptake in the presence of AMP from that measured in the presence of ATP at each concentration of VCR. *B*, [^{3}H]VCR (initial concentration 200 nM) transport by T14 (■) or H69AR (▲) membrane vesicles was measured at various GSH concentrations (0–20 mM) for 10 min at 37°C. Data represent net [^{3}H]VCR transport that was determined by subtracting the level of uptake in the presence of AMP from that measured in the presence of ATP at each GSH concentration. Data points are means (±SE) of triplicate determinations in a typical experiment.

in the presence of GSH. The inability of the dipeptides γ-glutamylcysteine and cysteinylglycine to stimulate [^{3}H]VCR transport indicates that all three amino acids of GSH are required. The lack of stimulation by glucuronic acid is consistent with our earlier observations that this compound (at concentrations up to 10 mM) failed to enhance the inhibition of MRP-mediated LTC_4[5] or 17β-estradiol 17-(β-D-glucuronide) transport by chemotherapeutic agents (28). BSO also had no effect on uptake of [^{3}H]VCR.

Inhibition of [^{3}H]VCR Transport by Chemotherapeutic Agents. We also investigated whether other chemotherapeutic agents to which MRP confers resistance could inhibit or compete for GSH-dependent [^{3}H]VCR uptake. [^{3}H]VCR uptake in T14 membrane vesicles was measured in the presence of drugs to which intact T14 cells display moderate (daunorubicin, doxorubicin, VP-16) and low levels of resistance (vinblastine, paclitaxel, colchicine (9). Unlabeled VCR at concentrations of 10 and 100 μM (50- and 500-fold molar excess) decreased [^{3}H]VCR uptake by 77 and 95%, respectively, whereas

[4] D. R. Hipfner, M. Gao, R. G. Deeley, and S. P. C. Cole, unpublished observations.

[5] D. W. Loe, R. G. Deeley, and S. P. C. Cole, unpublished observations.

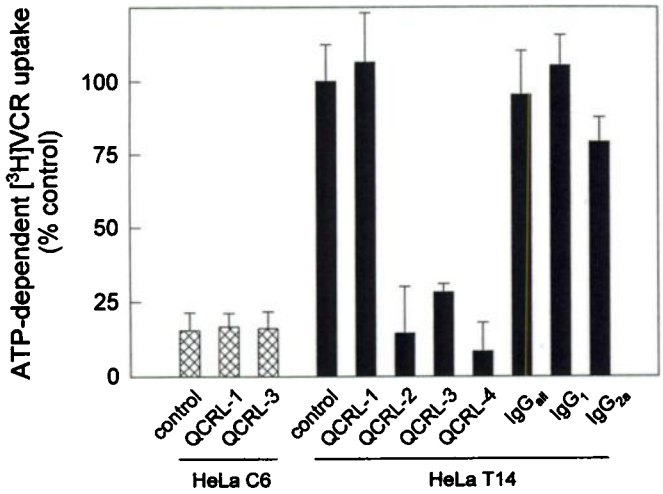

Fig. 3. Inhibition of [^{3}H]VCR transport in membrane vesicles by MRP-specific MAbs. Membrane vesicles derived from control HeLa C6 (*hatched bars*) or MRP-transfected T14 cells (*solid bars*) were pre-incubated for 1 h on ice in the presence of the indicated MRP-specific MAbs or control immunoglobulin (10 μg/ml). [^{3}H]VCR (200 nM) transport was subsequently determined in transport buffer (containing 5 mM GSH, 10 mM DTT, 10 μg/ml MAb or control immunoglobulin, and either AMP or ATP). Samples were incubated for 10 min at 37°C as before. Data were corrected for background by subtracting uptake in the presence of AMP from that observed in the presence of ATP and then calculated and plotted as % control net uptake (±SE) of triplicate determinations in a single experiment. The control [^{3}H]VCR uptake level for T14 membrane vesicles in the experiment shown was 6.4 ± 0.8 pmol/mg at 10 min.

Table 1 *The effect of various GSH derivatives on [^{3}H]VCR uptake in MRP-enriched membrane vesicles*

Membrane vesicles prepared from MRP-transfected T14 HeLa cells were incubated with 200 nM [^{3}H]VCR in transport buffer for 10 min at 37°C in the presence of GSH, BSO, glucuronic acid, or one of various GSH derivatives, all at 5 mM. Incubations were carried out in the presence of AMP or ATP, and ATP-dependent [^{3}H]VCR uptake was determined and calculated as % control VCR uptake in the presence of 5 mM GSH. The values shown are means of triplicate determinations (±SE) in a single experiment. The control level of [^{3}H]VCR uptake in this experiment was 6.4 ± 1.4 pmol/mg.

Compound	[^{3}H]VCR uptake (% control)
None	11 ± 7
GSH (control)	100 ± 23
S-methyl-GSH	30 ± 10
S-ethyl-GSH	25 ± 11
S-butyl-GSH	19 ± 3
Cysteinylglycine	10 ± 5
γ-Glutamylcysteine	3 ± 3
GSH ethyl ester	6 ± 3
GSH sulfonate	4 ± 4
BSO	6 ± 3
Na glucuronate	6 ± 3

similar concentrations of another *Vinca* alkaloid, vinblastine, diminished uptake by 55 and 98% (Fig. 4). Of the two anthracyclines tested, daunorubicin decreased uptake to the greatest extent (55 and 92% at 10 and 100 μM, respectively) compared with 40 and 66% reductions by the same concentrations of doxorubicin. VP-16 and paclitaxel were less effective, reducing uptake by 17–20% at 10 μM, and colchicine had little or no effect on GSH-dependent [^{3}H]VCR transport at either 10 or 100 μM.

[^{3}H]GSH Transport in MRP-enriched T14 Membrane Vesicles. To determine whether the uptake of VCR was associated with the transport of GSH, we examined the time course of [^{3}H]GSH uptake (at an initial concentration 100 μM) by vesicles from T14 MRP-transfectants in the presence and absence of drug. In the absence of VCR, GSH uptake was similar in the presence of either AMP or ATP and was approximately 0.2 nmol/mg at 20 min (Fig. 5*A*). Thus, no net ATP-dependent [^{3}H]GSH uptake was detectable, consistent with previous reports that GSH by itself is not a substrate of MRP (39). However, in the presence of 100 μM VCR and ATP, [^{3}H]GSH uptake increased to 0.38 and 0.58 nmol/mg at 20 and 60 min, respectively (Fig. 5*A*). Therefore, net MRP-mediated VCR-stimulated GSH uptake (after the subtraction of uptake in the presence of ATP alone) was 0.19 and 0.32 nmol/mg GSH at 20 and 60 min, respectively. Accumulation of GSH did not reach steady state even after 60 min and VCR-stimulated [^{3}H]GSH uptake was osmotically sensitive (data not shown). The dependence of [^{3}H]GSH transport on VCR concentration was also determined (Fig. 5*B*). Below 5 μM, VCR had no detectable effect on [^{3}H]GSH transport. However, [^{3}H]GSH transport increased with increasing drug concentration between 5 and 100 μM VCR to reach a maximum of approximately 0.4 nmol/mg over a 20-min period.

We also compared the same panel of drugs used to compete for or inhibit VCR uptake for their ability to stimulate the transport of GSH. All of the drugs were tested at 100 μM, except paclitaxel, which was tested at 10 μM, and the results are shown in Fig. 5*C*. VCR showed the greatest stimulation of GSH transport (approximately 0.27 nmol/mg above control). In contrast, vinblastine, VP-16, and colchicine showed very low levels of stimulation (0.06–0.08 nmol/mg GSH above control); and daunorubicin, doxorubicin, and paclitaxel did not stimulate [^{3}H]GSH uptake.

Characterization of VCR and GSH Inhibition of [^{3}H]LTC$_4$ Transport in T14 MRP-enriched Membrane Vesicles. To further investigate the observation that GSH enhanced the ability of VCR and vinblastine to inhibit MRP-mediated LTC_4 transport (17), we examined the concentration-dependence of the inhibition with respect to both VCR and GSH at a constant LTC_4 concentration of 50 nM (Fig. 6). GSH alone was a poor inhibitor of LTC_4 transport with an IC_{50} of approximately 12 mM (Fig. 6*A*). However, the IC_{50} for GSH was reduced 60-fold to approximately 0.2 mM in the presence of 100 μM VCR, a concentration of drug that by itself inhibits LTC_4 transport by approximately 50% (Fig. 6*B*). Similarly, in the presence of 5 mM GSH, the IC_{50} for VCR decreased from 100 to 5–6 μM.

The mode of inhibition of [^{3}H]LTC_4 transport by VCR and GSH was further investigated by measuring LTC_4 transport (at substrate concentrations from 8 nM to 1 μM) in the presence of a constant concentration of GSH (5 mM) and three concentrations of VCR (0, 3, and 6 μM). A similar experiment was also carried out at a constant concentration of VCR (100 μM) and three concentrations of GSH (0, 200, and 400 μM). Lineweaver-Burk analyses of the data indicate that

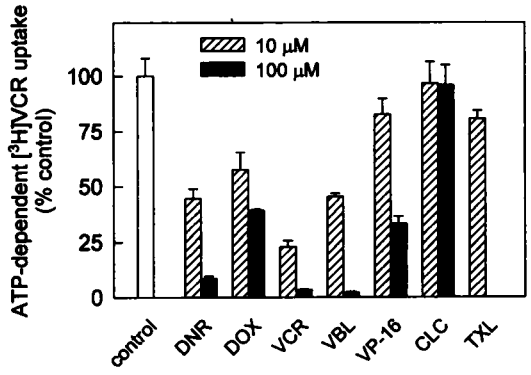

Fig. 4. The effect of chemotherapeutic agents on [^{3}H]VCR transport by MRP-enriched T14 membrane vesicles. The ability of drugs to inhibit [^{3}H]VCR (200 nM) uptake was measured in the presence of GSH (5 mM) for 10 min at 37°C. Uptake in the presence of AMP, GSH, and drug (10 μM, *hatched bars*; 100 μM, *solid bars*) was subtracted in all of the cases. Results were then calculated as a % of control values obtained in the absence of drug (*open bar*). Bars represent means (±SE) of triplicate determinations in a typical experiment. The control level of [^{3}H]VCR uptake into T14 membrane vesicles in the experiment shown was 8.7 ± 1.4 pmol/mg. *DNR*, daunorubicin; *DOX*, doxorubicin; *VBL*, vinblastine; *CLC*, colchicine; *TXL*, paclitaxel.

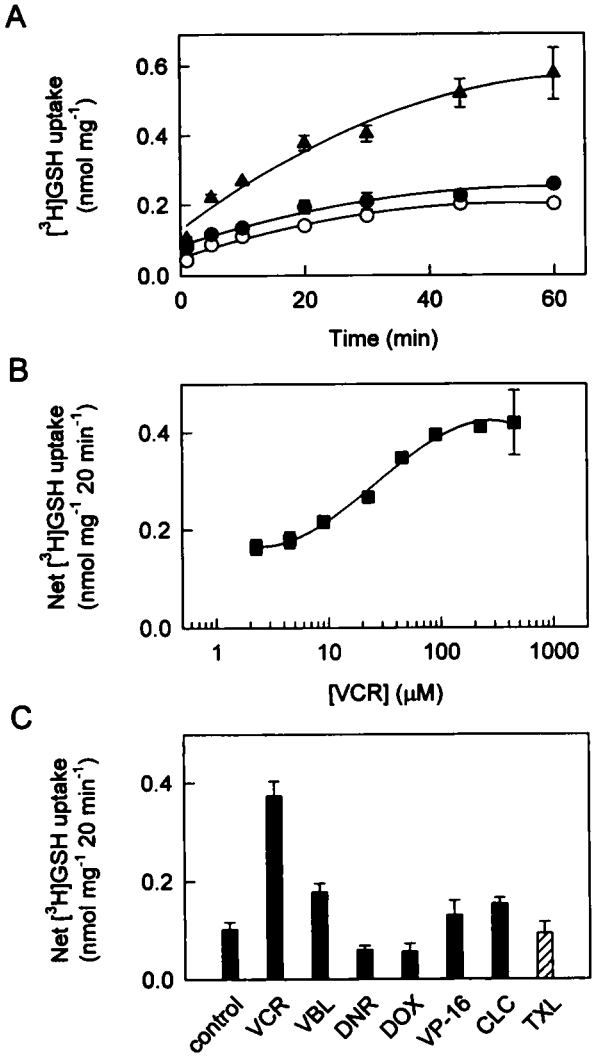

Fig. 5. The time course and effect of VCR and other chemotherapeutic agents on [^{3}H]GSH uptake by MRP-enriched T14 membrane vesicles. In *A*, membrane vesicles were incubated with 100 μM [^{3}H]GSH in transport buffer at 37°C for the times indicated. DTT (10 mM) was included in all of the assays to minimize GSSG formation. ○, uptake in the presence of 4 mM AMP; ●, uptake in the presence of 4 mM ATP alone; ▲, uptake in the presence of ATP and 100 μM VCR. In *B*, membrane vesicles were incubated with 100 μM [^{3}H]GSH in transport buffer at 37°C for 20 min in the presence of the indicated concentrations of VCR (■). GSH uptake in the presence of AMP alone was subtracted in each case. In this experiment, [^{3}H]GSH uptake in the presence of ATP but in the absence of VCR was 0.12 ± 0.01 nmol/mg vesicle protein at 20 min (not shown). In *C*, membrane vesicles were incubated with 100 μM [^{3}H]GSH in transport buffer at 37°C for 20 min in the presence of solvent vehicle or the indicated chemotherapeutic drugs at 100 μM (*solid bars*), except TXL, which was at 10 μM (*hatched bar*). DTT (10 mM) was included in all of the assays to minimize GSSG formation. The bars represent values obtained after subtraction of uptake in the presence of AMP (which was similar for all of the drugs) and are means (±SE) of triplicate determinations in a typical experiment. *DNR*, daunorubicin; *DOX*, doxorubicin; *VBL*, vinblastine; *CLC*, colchicine; *TXL*, paclitaxel.

VCR and GSH together behave as competitive inhibitors of LTC_4 transport, with apparent K_i values of 2.5 ± 0.3 μM (VCR; Fig. 6*C*) and 111 ± 8 μM (GSH; Fig. 6*D*), respectively.

DISCUSSION

The inability to detect ATP-dependent transport of chemotherapeutic agents by MRP-enriched membrane vesicles, coupled with demonstrable transport of glutathione- and glucuronide-conjugated anions, such as LTC_4 and 17β-estradiol 17-(β-D-glucuronide), led to the suggestion that MRP was a drug conjugate transporter (16, 17, 25). However, there are several reasons why the ability to transport drug conjugates is unlikely to provide a general explanation of the ability of MRP to confer resistance to such a structurally diverse spectrum of antineoplastic agents in such a wide variety of cell types. Most compelling is the well-established observation that Phase II conjugation plays a relatively minor role in the *in vivo* and *in vitro* metabolism of these compounds (31, 40–42). In addition, Phase I and Phase II biotransformation reactions are known to occur primarily in the liver (and to a lesser extent in other tissues), and it is highly improbable that all of the cell types in which overexpression of MRP causes resistance are competent to carry out these reactions with the required efficiency and completeness. On the other hand, depletion of cellular GSH levels by treatment with BSO has been observed to improve the efficacy of some natural product drugs both in cultured cells and in mice bearing tumors that express elevated levels of MRP (40, 43–46). These findings, together with the observation that MRP increases drug efflux from intact cells but is apparently unable to transport the same compounds in isolated plasma membrane vesicles, suggested that either the efflux of certain drugs might require activation of MRP by GSH (or some other endogenous anionic molecule) or that some type of cotransport mechanism might be involved. Support for these ideas was provided by the observation that physiological concentrations of GSH significantly enhanced the ability of vinblastine and VCR to inhibit MRP-mediated ATP-dependent transport of LTC_4 (17). We subsequently demonstrated the direct uptake of unmodified VCR (as well as unmodified aflatoxin B_1; REF. 26) by MRP-enriched vesicles in an ATP and GSH-dependent manner (17). We have now shown that GSH-stimulated ATP-dependent VCR uptake by membrane vesicles from MRP-transfected HeLa cells and drug-selected H69AR cells is proportional to the level of MRP in the two vesicle preparations and that the level of stimulation is dependent on GSH concentration in the physiologically relevant range of 1–5 mM. In addition, GSH-stimulated uptake can be inhibited by MRP-specific MAbs, shown previously to inhibit transport of LTC_4 and aflatoxin B_1-GSH. Together, these observations provide strong additional evidence that VCR uptake in the presence of GSH is mediated by MRP.

Because of the presence of potential vicinal thiols in MRP (47), we considered the possibility that GSH may influence VCR transport by altering the redox state of the protein. We have shown that other sulfhydryl reducing agents cannot substitute for GSH suggesting that this is unlikely (17, 26). The demonstration that short chain *S*-alkyl derivatives of GSH can also enhance VCR uptake confirms that reduction or glutathionylation of the protein is not involved and that a thiolate anion is not essential for the stimulation of VCR transport (Table 1). The lack of stimulation by dipeptides such as cysteinylglycine and γ-glutamycysteine also indicates that the tripeptide structure of GSH is a requirement for stimulation of VCR transport to occur.

GSH has been reported previously not to be transported by MRP (39). Consequently, it was unclear whether stimulation of VCR transport was a result of GSH binding (48), or whether GSH was also transported along with the drug. Our data show that VCR does stimulate ATP-dependent transport of GSH in a concentration dependent fashion (Fig. 5). Hydrophobic compounds such as VCR are capable of relatively high rates of passive diffusion across vesicular membranes. Consequently, the rate of ATP-dependent drug transport into vesicles is rapidly counterbalanced by the rate of passive efflux. However, to maintain the vesicular concentration of VCR, active transport of drug must continue and under the assay conditions used, we have established that steady state can be maintained for at least 40 min. The rate of passive efflux of GSH from the vesicles is expected to be negligible compared with that of VCR and, consistent with a cotransport mechanism, we detected continued drug-dependent net uptake of [^{3}H]GSH throughout this period (Fig. 5). In the absence of VCR, we detected no ATP-dependent GSH transport, as reported

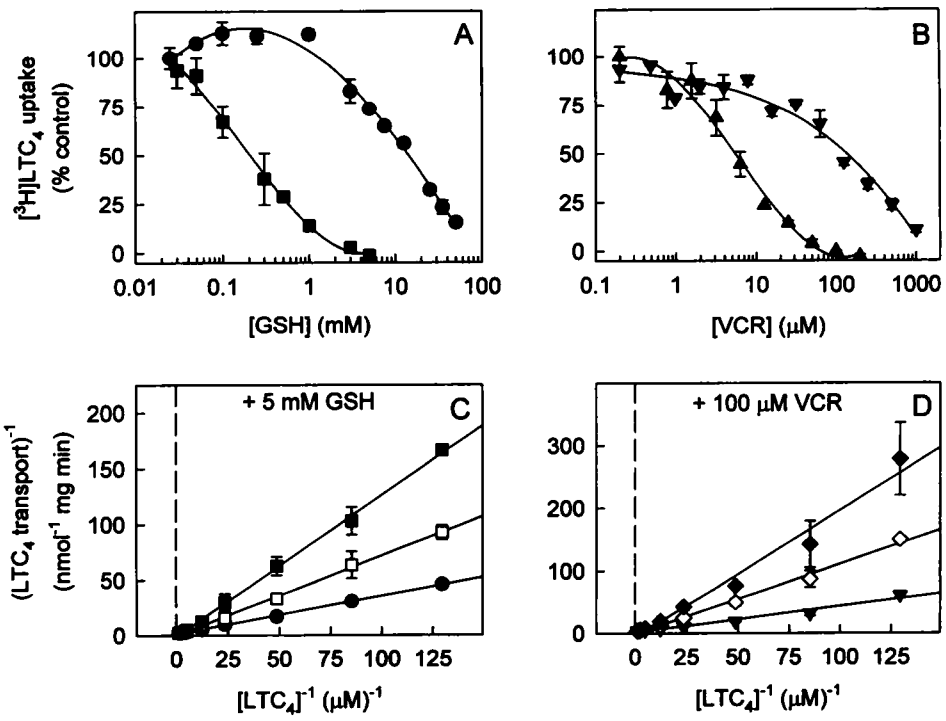

Fig. 6. The effect of VCR and GSH on [^{3}H]LTC$_4$ transport by T14 MRP-enriched membrane vesicles. In *A*, ATP-dependent [^{3}H]LTC$_4$ transport (at an initial substrate concentration of 50 nM) at 23°C was determined at various concentrations of GSH (20 μM–50 mM) in the presence (■) or absence (●) of 100 μM VCR. Control uptake rates were 115 ± 13 and 71 ± 1 pmol/mg/min, respectively. In *B*, ATP-dependent [^{3}H]LTC$_4$ (50 nM) transport was determined at various concentrations of VCR (0.2 μM–1 mM) in the presence (▲) or absence (▼) of 5 mM GSH. Control uptake rates were 98 ± 1 and 63 ± 3 pmol/mg/min, respectively. In *C*, LTC$_4$ (8 nM–1 μM) transport was determined in the presence of 5 mM GSH and various concentrations of VCR (0 μM, ●; 3 μM, □; 6 μM, ■). Double-reciprocal plots were generated and an apparent K_i of 2.5 ± 0.3 μM was calculated from the apparent K_m (151 nM) and V_{max} (452 pmol/mg/min) in the presence of 5 mM GSH and the indicated concentrations of VCR. In *D*, LTC$_4$ (8 nM–1 μM) transport was determined in the presence of 100 μM VCR and various concentrations of GSH (0 μM, ▼; 200 μM, ◇; 400 μM, ◆). Double-reciprocal plots were generated and an apparent K_i of 111 ± 8 μM was calculated from the apparent K_m (186 nM) and V_{max} (452 pmol/mg/min) in the presence of 100 μM VCR and the indicated concentrations of GSH. In all cases, data points represent means (±SE) of triplicate determinations in a typical experiment.

previously (39). However, GSSG is a known substrate for MRP, raising the possibility that [^{3}H]GSSG was being transported rather than [^{3}H]GSH (19, 39). This is extremely unlikely for several reasons. All of the transport reactions were carried out in the presence of 10 mM DTT to minimize possible contamination with GSSG. In addition, we have established that GSSG does not enhance the transport of VCR and is in fact inhibitory at concentrations greater than 100 μM (data not shown). Finally, if the [^{3}H]GSH was contaminated with [^{3}H]GSSG, we would expect to see time-dependent uptake of radioisotope in the absence of VCR, but this was not observed.

Reliable estimation of the stoichiometry of VCR and GSH transport is difficult because of the extremely brief period for which VCR uptake is linear. However, if the GSH-stimulated uptake of VCR at the earliest time point of 8 s is taken as a minimal estimate of initial rate, a value of ~20 pmol/min/mg is obtained for T14 vesicles (Fig. 1*A*). This is approximately the same as the rate of uptake of GSH uptake calculated over a 5-min period (Fig. 5*A*). In addition to the caveat mentioned above, these data are based on determinations at only one concentration of drug and GSH. Consequently, they are certainly not sufficiently reliable to conclude that there is a 1:1 stoichiometry with respect to GSH and VCR transport. However, they do indicate that the transport rates of both compounds are not vastly different, and that a true cotransport mechanism may be involved.

Despite the lack of structural similarity between the MRP substrates LTC$_4$ and 17β-estradiol 17-(β-D-glucuronide), the two compounds compete reciprocally for transport (28). The competition observed between many conjugated compounds with diverse structures suggests the possibility that there could be a bipartite binding site on MRP, one region of which can accommodate anionic moieties such as GSH and glucuronide, and another with which a wide range of hydrophobic structures may interact (17). Consequently, it was of interest to examine the inhibitory characteristics of VCR and GSH alone, and in combination, with respect to their ability to compete for LTC$_4$ transport. As reported previously (17, 18), VCR alone or GSH alone was a very poor inhibitor of MRP-mediated LTC$_4$ transport with IC$_{50}$s of 100 μM and 12 mM, respectively (Fig. 6, *A* and *B*). However, in the presence of a physiological concentration of GSH, the inhibitory potency of VCR increased markedly, and the drug acted as a competitive inhibitor with an apparent K_i of 2.5 ± 0.3 μM (Fig. 6*C*). This value is almost an order of magnitude lower than the K_i for 17β-estradiol 17-(β-D-glucuronide) obtained under comparable conditions (28). Similarly, in the presence of VCR, GSH also acted as a competitive inhibitor with a K_i of approximately 100 μM (Fig. 6*D*). Thus, the data indicate that VCR and GSH probably interact with the same site(s) on MRP as LTC$_4$, or at least with mutually exclusive sites. The observation that the presence of both compounds dramatically decreases each of their apparent K_i values, strongly suggests that they are not binding completely independently. Whether this is because of an interaction between VCR and GSH or indicative of a cooperative interaction with the protein is currently under investigation.

Studies with intact cells have provided additional support for MRP-mediated drug/GSH cotransport. For example, VP-16 has been reported to increase the efflux of GSH from normal mouse embryonic stem cells but not from stem cells in which both *mrp* alleles have been knocked out (49). In addition, we and others have observed (43, 44, 46, 50) that overexpression of MRP in some cell lines, but not all, is associated with a significant (2- to 6-fold) decrease in intracellular GSH levels. Thus, the available evidence from studies with both membrane vesicles and intact cells strongly supports a MRP-mediated GSH cotransport mechanism for some drugs. However, it appears premature to propose this as a general mechanism by which MRP transports all of the drugs to which it confers resistance. This is particularly true with respect to the anthracycline antibiotics, inasmuch as GSH displays little or no ability to either enhance the transport of daunorubicin or doxorubicin directly or to enhance the ability of these drugs to inhibit MRP-mediated transport of LTC$_4$ (17). In the current study, VCR and, to a lesser degree, vinblastine stimulated ATP-dependent [^{3}H]GSH uptake, whereas doxorubicin and daunorubicin did not (Fig. 5), despite the fact that they inhibited [^{3}H]VCR transport almost as well as vinblastine. We have also found that the treatment of either drug-selected or MRP-transfected cells

with BSO restores sensitivity to VCR far more effectively than to doxorubicin (43, 50). Thus, overall, our data are consistent with the apparently greater importance of GSH in MRP-mediated transport of VCR compared with the anthracyclines.

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