Evidence for Two Interacting Ligand Binding Sites in Human Multidrug Resistance Protein 2 (ATP Binding Cassette C2)*

Noam Zelcer‡, Maarten T. Huisman§, Glen Reid§, Peter Wielinga‡, Pauline Breedveld‡, Annemieke Kuil‡, Puck Knipscheer‡, Jan H. M. Schellens§, Alfred H. Schinkel§, and Piet Borst‡

From the 3Division of Molecular Biology and Center of Biomedical Genetics, and the Divisions of 8Experimental Therapy and Molecular Carcinogenesis, Netherlands Cancer Institute, Amsterdam 1066 CX, The Netherlands

Multidrug resistance protein 2 (MRP2) belongs to the ATP binding cassette family of transporters. Its substrates include organic anions and anticancer drugs. We have used transport assays with vesicles derived from Sf9 insect cells overproducing MRP2 to study the interactions of drugs, organic anions, and bile acids with three MRP2 substrates: estradiol-17β-d-glucuronide (E17βG), methotrexate, and glutathione-S-dinitrophe

Members of the ABC family of membrane transporters mediate the transport of various substrates across biological membranes at the expense of ATP hydrolysis (1, 2). The ABCC subfamily (3) contains multidrug resistance proteins 1–9 (MRP1–9) along with SUR1, SUR2, and CFTR (1, 2, 4, 5). Interest in the multidrug resistance proteins was sparked by their possible involvement in the clinical resistance of tumors to chemotherapeutic agents. The first member of this family to be cloned, MRP1, confers resistance to a broad spectrum of anticancer drugs when overproduced in cells (6). A common feature of MRPs is that they transport a wide variety of organic anions and compounds that are conjugated with sulfate, glucuronate, or glutathione (GSH) (7, for review, see Refs. 2 and 8–10). How MRPs transport their substrates is not known in detail. MRPs are large membrane-associated proteins, and their structural analysis has proven difficult (11). Although several high resolution structures of bacterial ABC transporters have been determined (12, 13), only low resolution structures are available for the drug transporters MRP1 and MDR1 P-glycoprotein (14–16). In the absence of a detailed structure, the mechanism of transport has been inferred from a combination of transport, binding and mutational studies. Models proposed for MDR1 P-glycoprotein predict three or four drug binding sites or a single complex substrate binding site in which the binding of one compound can affect the binding of another one, the induced-fit model (17–20). Also for MRP1 evidence for more than one ligand binding site was obtained (for review, see Refs. 2 and 21).

The major canalicular organic anion transporter, MRP2 (ABCC2), is closely related to MRP1 (2, 8). The substrate specificities of MRP1 and 2 overlap to a large extent (9, 22–24), but their tissue localizations differ. MRP1 is localized in the basolateral membranes of polarized cells and is present in all tissues, whereas MRP2 is found in the apical membranes of polarized cells and is expressed mainly in the liver, kidney, and intestine. Bakos et al. (25) demonstrated in vesicular transport assays that transport of the GSH conjugate of N-ethylmaleimide by MRP2 is stimulated by several organic anions. Experiments with polarized cells led to a model in which MRP2 cotransports drugs from two distinct drug binding sites (26). Cotransport cannot account for recent observations on MRP1, however (27).

In vectorial transport assays with MDCKII/MRP2 cells we recently observed that the transport of saquinavir is stimulated by probenecid (28). Such drug interactions could potentially affect the oral bioavailability and pharmacokinetics of drugs transported by MRP2. We have therefore studied drug interactions with MRP2 more in detail using transport assays with membrane vesicles from Spodoptera frugiperda (Sf9) insect cells that were infected with a baculovirus construct containing MRP2 (25). Using estradiol-17β-d-glucuronide (E17βG), methotrexate (MTX), and glutathione-S-dinitrophe (GS-DNP) as model substrates, we found stimulation of substrate transport by a range of compounds. We propose that MRP2 contains two distinguishable binding sites: one site from which drug is transported and a second site that allosterically

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†To whom correspondence should be addressed: Dept. of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands. Tel.: 31-20-512-2880; Fax: 31-20-512-2886; E-mail: p.borst@nki.nl.

‡ The abbreviations used are: ABC, ATP binding cassette; E17βG, estradiol-17β-d-glucuronide; GS-DNP, glutathione-S-dinitrophe; MDCK, Madin-Darby canine kidney; MRP, multidrug resistance protein; MTX, methotrexate; NNL-glucuronide, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronide; SU2, apparent half-maximal rate; Sf9, Spodoptera frugiperda.

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regulates the former. Analogous results have been independently obtained by Bodo and colleagues and are presented in the accompanying manuscript.

EXPERIMENTAL PROCEDURES

Materials—40.5 Ci/mmol [3H]HIB, 21 Ci/mmol [3H]pencillin G, and 20 Ci/mmol [3H]Clidomethacin were obtained from PerkinElmer Life Sciences. 9 Ci/mmol [3H]MTX was obtained from Amersham Biosciences. 13.6 μCi/mg [3H]saquinavir was from Roche Applied Science. Vials of omeproza (Loses®, AstraZeneca) and pantoprazole (Pantozol, Altana Pharma BV) were obtained from the pharmacy of The Netherlands Cancer Institute and dissolved in saline according to the manufacturer’s instructions. Creatine phosphate and creatine kinase were obtained from Roche, and RC-L55 and OE-67 filters were from Schleicher & Schuell. All other chemicals and reagents were purchased from Sigma.

Cell Lines and Culture Conditions—SF9 insect cells in suspension were grown in SF-900 II SFM medium in the absence of serum (Invitrogen). The MDCKII control and MR2-overproducing lines were described previously (24) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units of penicillin/streptomycin/ml. Cells were grown at 37 °C with 5% CO2 under humidifying conditions.

Protein Analysis and Immunoblotting—Membrane vesicle preparations were diluted in buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris–HCl, pH 7.4), supplemented by a mixture of protease inhibitors used at the concentration recommended by the manufacturer (Roche). The indicated amount of protein was size fractionized on a 7.5% SDS-polyacrylamide gel and subsequently blotted overnight in a tank blotting system. Blots were probed with anti-MRP1, anti-MRP2, anti-MRP3, anti-MRP4 (ECL, Amersham Biosciences). 1:250), M2II5 (1:250) and M3II9 (1:250), respectively, as described previously (24, 29). Signals were visualized with chemiluminescence (ECL, Amersham Biosciences).

Transepithelial Transport Assays—Transepithelial transport assays were done as described previously (28). Briefly, Cells were seeded on microporous polycarbonate membrane filters (Transwell 3414, Costar, Corning, NY) at a density of 1.0 × 106 cells/well in 2 ml of complete medium. Medium from both compartments containing 5 μCi [3H]MTX was added to check for leakage through the cell layers. Cells were incubated at 37 °C until use.

Preparation of Membrane Vesicles—Membrane vesicles from SF9 cells were obtained after infection with an MR1- (25), MR2- (25), MR3- (29), or MR4- (30, 31) cDNA-containing baculovirus at a multiplicity of infection of 1. After incubation at 27 °C for 3 days, cells were harvested by centrifugation at 3,000 rpm for 5 min. The pellet was resuspended in ice-cold hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μM pepstatin) and incubated at 4 °C for 90 min. The suspension was centrifuged at 4 °C at 100,000 × g for 40 min, and the pellet was homogenized in ice-cold TS buffer (50 mM Tris–HCl, 250 mM sucrose, pH 7.4) using a tight-fitting Dounce homogenizer. After centrifugation at 500 × g at 4 °C for 10 min, the supernatant was centrifuged at 4 °C at 100,000 × g for 40 min. The pellet was resuspended in TS buffer and passed through a 27-gauge needle 25 times. The vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at −80 °C until use.

Vesicular Transport Assays—Vesicular transport assays were performed as described previously (28). Vesicles were obtained by centrifugation of 100 mM KCl, 50 mM HEPES/KOH, pH 7.4, in the presence or absence of 4 mM ATP (32). Similar results were obtained with a Tris/sucrose buffer (not shown). The time- and concentration-dependent uptake of substrates into membrane vesicles was studied following the rapid filtration method as described previously (29).

RESULTS

Effects of Drugs and Organic Anions on E6,17βG Transport by MR2—Membrane vesicles were prepared from SF9 insect cells transfected with a recombinant baculovirus coding for MR1, MR2, or MR3, and 0.5 μg of protein was loaded per lane and size fractionated on a 7.5% SDS-polyacrylamide gel. MR1, 2, or 3 was detected as described under “Experimental Procedures.”

Sulfanitran, the strongest stimulator of MR2-mediated E6,17βG transport, also stimulated the vectorial transport of saquinavir, a recently described MR2 substrate (28), across polarized MDCKII monolayers demonstrating that it also stimulates MR2 in intact cells (Fig. 3). Comparison of B and D of Fig. 3 shows that sulfanitran increases transport of saquinavir in the apical direction, decreases transport in the basolateral direction, and substantially decreases the intracellular concentration of saquinavir. Saquinavir is too hydrophobic to study in the vesicular transport assay, but in transepithelial transport assays, we have shown previously that in addition to sulfanitran, other stimulators of vesicular transport also stimulate MR2 in intact cells: transport of saquinavir is stimulated by both sulfipyrazone and probenecid (28), and sulfanitran had no effect on transport of E6,17βG by MR2 (Fig. 2).

The stimulation by the compounds studied was specific for MR2. Neither lansoprazole nor saquinavir at their maximal MR2-stimulatory concentration stimulated transport of E6,17βG in wild type, MR1, MR3, or MR4 vesicles, and sulfanitran had no effect on transport of E6,17βG in wild type vesicles either (data not shown). Furosemide and acetaminophen-glucuronide even inhibited MR3-mediated transport of E6,17βG (Fig. 4), whereas these compounds stimulated transport of E6,17βG by MR2 (Fig. 2). Sulfanitran, the compound that...
stimulated MRP2 transport most, had only a minimal effect on MRP3 (Fig. 4).

**Effects of Drugs on Transport of GS-DNP by MRP2**—GSH conjugates are another class of molecules transported by MRP2. We therefore tested whether transport of GS-DNP, a model GSH conjugate and a known substrate of MRP2, could be stimulated like transport of E217/H9252/G. The results are summarized in Table I. Like E217/H9252/G, GS-DNP transport is stimulated by sulfanitran and indomethacin albeit to a lower extent. Sulfapyrazone stimulates GS-DNP transport in vesicular transport assays, similar to what we found previously in MDCKII/MRP2 cells (24). Furosemide at its maximal stimulatory concentration (500 μM) has only a marginal effect on GS-DNP transport, in contrast to its effect on E217/H9252/G transport. Moreover, whereas probenecid strongly stimulated the transport of E217/H9252/G, it inhibited GS-DNP transport as is the case for MTX as well.

### Table I. Effects of Drugs on GS-DNP Transport by MRP2

| Drug                | Control | MRP2    |
|---------------------|---------|---------|
| Sulfanitran         | 100     | 150     |
| Indomethacin        | 150     | 200     |
| Sulfapyrazone       | 200     | 250     |
| Furosemide          | 100     | 110     |
| Probenecid          | 50      | 100     |
| MTX                 | 100     | 100     |

**Fig. 2. Effects of organic anions and drugs on the transport of 1 μM E217G by MRP2.** Membrane vesicles containing MRP2 were incubated with 1 μM [3H]E217G for 2 min at 37 °C in the presence or absence of the indicated compounds. The ATP-dependent transport is plotted as a percentage of the control value. Each point and error are the mean ± S.E. of experiments in triplicate.

**Fig. 3. Effect of sulfanitran on the transepithelial transport of saquinavir by MRP2.** An MDCKII neomycin-resistant control clone and an MRP2 transfectant were incubated with 5 μM [14C]saquinavir in the absence (A and B) or presence (C and D) of 500 μM sulfanitran as described under “Experimental Procedures.” Transport of saquinavir from the apical to the basolateral (C) and from the basolateral to apical (D) directions was determined. Each point is the mean ± S.E. of experiments in triplicate. Values within figures represent the percent of radioactivity found in the monolayer after a 4-h incubation with saquinavir in the apical (Ap) or basolateral (Bl) compartments.

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![Graph A: E217G transport vs. concentration](image1.png)

![Graph B: E217G transport vs. concentration](image2.png)

![Graph C: E217G transport vs. concentration](image3.png)

![Graph D: E217G transport vs. concentration](image4.png)

**Fig. 4. Allosteric Regulation of MRP2-mediated Transport**

![Graph E: Saquinavir Transport](image5.png)

![Graph F: Transport of saquinavir](image6.png)
penicillin G or pantoprazole, another stimulator of E217G transport of indomethacin and probenecid by MRP2 (data not shown). Indeed, in these assays we detected marginal vesicles at high rates, preventing transport measurements. This by MRP2. Negative results in vesicular transport assays are stimulate transport of [14C]indomethacin (at concentrations up to 50 μM) by MRP2, making cotransport unlikely.

To investigate the mechanism of stimulation further, we determined the rate of transport of E217G by MRP2 as a function of substrate concentration in the absence or presence of 100 μM indomethacin or 1 mM sulfanitran (Fig. 5). The transport of E217G by MRP2 was not consistent with simple Michaelis-Menten kinetics, but the plot of reaction velocity versus substrate concentration was clearly sigmoidal with an estimated apparent half-maximal rate (S_{1/2}) at 120 μM E217G (Fig. 5A). In the presence of either of the two stimulators, the curve was shifted to a more hyperbolic shape with apparent S_{1/2} values of 65 and 16 μM in the presence of 100 μM indomethacin and 1 mM sulfanitran, respectively (Fig. 5, B and C). The maximal rate of transport remained relatively unchanged. The degree of stimulation of E217G transport at low substrate concentration (1 μM) by these compounds correlates well with the increased affinity for this substrate (Figs. 2 and 5). At 200 μM E217G transport was not stimulated by 100 μM indomethacin and was only stimulated by 10% by 1 mM sulfanitran (data not shown) suggesting that at this concentration of substrate MRP2 is close to saturation. We note, however, that Bodo et al. found higher rates of E217G transport at 1 mM than at 200 μM, the maximal concentration that we were able to test because of solubility problems.

As a control, we also determined the concentration-dependent transport of E217G by MRP1, for which we found saturation kinetics with a K_m of 3.1 ± 0.3 μM and a V_max of 38 ± 1 pmol/mg/min. This further strengthens the notion that the requirements for optimal transport of the same substrate by MRP1 and MRP2 are different (27), even though the substrate specificity of these transporters largely overlaps.

**Characterization of MTX Transport by MRP2—MTX** is transported by MRP2, but MRP2 has such a low affinity for this substrate that we were unable to determine reliable kinetic parameters for this transport process (not shown and Refs. 25 and 33). Sulfinpyrazone and indomethacin stimulate MTX transport, but to a much lower extent than the transport of E217G (Fig. 6A). In contrast, E217G, GS-GS, and probenecid only inhibited MTX transport by MRP2 (Fig. 6B). E217G at a concentration of 200 μM inhibits the transport of MTX by 80%, suggesting that these two substrates share a common step in transport. In this light, the absence of a substantial inhibitory or stimulatory effect of MTX (at a concentration up to 4.4 mM) on transport of 1 μM E217G by MRP2 (Fig. 2F) is unexpected. A possible explanation is that MTX is a weak stimulator of E217G transport and that at low E217G concentrations (allosteric) stimulation and inhibition (by competition) of E217G transport by MTX balance out. Indeed, MTX did inhibit the transport of high concentrations of E217G (200 μM) and of 1 μM E217G stimulated by 1 mM sulfanitran (Fig. 7). Moreover, trimetrexate, a structural analog of MTX, stimulates transport of 1 μM E217G by 320 ± 10% at a concentration of 300 μM (data not shown). This is compatible with the hypothesis that MTX itself might have a weak stimulatory effect as well. Following the reasoning applied to MTX, GSH, another low affinity substrate of MRP2 (34), should be able to inhibit E217G and MTX transport under appropriate conditions. However, GSH at concentrations up to 10 mM had no effect on the transport of 200 μM E217G or 100 μM MTX by MRP2 (not shown).

**DISCUSSION**

Our work shows complex effects of various drugs and organic anions on MRP2. For transport of E217G the plot of reaction

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**Fig. 4. Effects of drugs on 1 μM E217G transport by MRP3.** Membrane vesicles containing MRP3 were incubated with 1 μM [3H]E217G for 2 min at 37 °C in the presence or absence of the indicated compounds. The ATP-dependent transport is plotted as a percentage of the control value. Each point and error are the mean ± S.E. of experiments in triplicate.

**TABLE I**

| Compound | Concentration (μM) | ATP-dependent transport % |
|----------|-------------------|--------------------------|
| Sulfanitran | 1,000 | 253 ± 10 |
| Indomethacin | 100 | 274 ± 11 |
| Furosemide | 500 | 111 ± 3 |
| MTX | 2,200 | 82 ± 1 |
| Probenecid | 1,000 | 62 ± 3 |
| E217G | 5 | 100 ± 3 |
| Sulfinpyrazone | 1,000 | 152 ± 2 |

Effects of Stimulators of MRP2-mediated E217G Transport on the Affinity of MRP2 for Substrate—Evers et al. (26) demonstrated that sulfinpyrazone stimulates transport of GSH by MRP2 and that vinblastine transport is accompanied by GSH transport at an approximate ratio of 1:1. Sulfinpyrazone also stimulates transport of E217G by MRP2 (Fig. 2C). Compounds that stimulate E217G transport by MRP2 might therefore be cotransported with this substrate, as proposed previously (26). We have tested this in vesicular and trans epithelial transport assays. We did not detect vesicular transport of [3H]penicillin G at concentrations of up to 1 mM either in the absence or presence of varying concentrations of E217G (data not shown). Similarly, in vesicular transport assays we did not detect transport of [14C]indomethacin (at concentrations up to 50 μM) by MRP2. Negative results in vesicular transport assays are not conclusive, however, as the substrate may leak out of the vesicles at high rate, preventing transport measurements. This is not a problem in transepithelial transport assays with MDCKII/MRP2 cells. Indeed, in these assays we detected marginal transport of indomethacin and probenecid by MRP2 (data not shown). In the same assays, we did not detect transport of penicillin G or pantoprazole, another stimulator of E217G transport by MRP2 (Fig. 2, and data not shown). These results indicate that these compounds are either not transported by MRP2 or are poor substrates, even though they strongly stimulate transport of E217G by MRP2, making cotransport unlikely.

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**Effects of drugs and organic anions on GS-DNP transport by MRP2**

Membrane vesicles containing MRP2 were incubated with 15 nM [3H]GS-DNP for 5 min at 37 °C in the presence or absence of the indicated compounds. Each value is the mean ± S.E. of experiments in triplicate expressed as the percentage of transport in the absence of stimulator/inhibitor.

| Compound | Concentration (μM) | ATP-dependent transport % |
|----------|-------------------|--------------------------|
| Sulfanitran | 1,000 | 253 ± 10 |
| Indomethacin | 100 | 274 ± 11 |
| Furosemide | 500 | 111 ± 3 |
| MTX | 2,200 | 82 ± 1 |
| Probenecid | 1,000 | 62 ± 3 |
| E217G | 5 | 100 ± 3 |
| Sulfinpyrazone | 1,000 | 152 ± 2 |
Allosteric Regulation of MRP2-mediated Transport

The effect of a transport modulator depends on the substrate transported. Probenecid strongly stimulates transport of E_217βG (Fig. 2) but inhibits transport of MTX (Fig. 6) and GS-DNP (Table I). Indomethacin and sulfanitran stimulate E_217βG transport more than transport of GS-DNP or MTX. Furosemide strongly stimulates E_217βG transport but has no effect on GS-DNP transport. It even inhibits GS-N-ethylmaleimide transport (25). These observations are compatible with the idea that each substrate-modulator pair forms unique interactions within the complex drug binding sites of MRP2, a possibility raised previously for MRP1 (27, 32).

Although the substrate specificities of MRP1, 2, and 3 largely overlap, these transporters handle some substrates/inhibitors in different ways. The affinity of MRP1 (this study and Ref. 37) and MRP3 (29) for E_217βG is relatively high, and transport follows Michaelis-Menten kinetics, in contrast to our result for MRP2. Stimulators of MRP2-mediated E_217βG transport may even inhibit transport by MRP3. Furosemide and acetaminophen glucuronide are examples. Although glycocholic and taurocholic acid can stimulate transport of E_217βG by MRP2, they only inhibit transport of etoposide glucuronide (38) and MTX (39) by MRP3. These results suggest that MRPs bind a similar range of compounds, but not in the same manner.

On the basis of our findings we propose that MRPs bind a similar but nonidentical ligand binding sites: one site from which substrate is transported (S site) and a second site that is able to modulate transport (M site). Binding of a modulator to the M site induces a structural change that results in a better fit of the substrate at the S site. Compounds that only stimulate transport of E_217βG (e.g., sulfanitran) bind only to the M site. Compounds that display a "bell-shaped" stimulation of MRP2-mediated E_217βG and MTX transport (e.g., indomethacin) bind at a low concentration predominantly to the M site. At a higher concentration they compete for binding with E_217βG at the S site as well. Whether such a stimulating compound is also transported by MRP2 must depend on its interactions with the substrate site. The stimulators penicillin G and pantoprazole are not detectably transported by MRP2, but sulfanitran and saquinavir are (26, 28). The GSH conjugates we tested (GS-DNP and GSSG) only inhibited the transport of both E_217βG and MTX. This suggests that these GSH conjugates, both known to be transported by MRP2 (25, 34), bind MRP2 predominantly at the S site in a way that competes with binding of other transported substrates. Some of the compounds that stimulate E_217βG transport also stimulate the transport of GS-DNP, indicating that binding of GS-DNP to the S site leaves the M site accessible to modulators that are able to fit velocity versus substrate concentration is sigmoidal (Fig. 5), indicative of at least two drug binding sites that interact in a positively cooperative manner. Many compounds stimulate E_217βG transport at low substrate concentrations, and for two stimulators, sulfanitran and indomethacin, we have shown that they increase the affinity of MRP2 for substrate with no significant effect on the V_{max}.

Compounds that stimulate transport of substrates by MRP2 are not necessarily transported by MRP2. In vesicular transport assays we do not detect transport of [3H]penicillin G and [14C]indomethacin. Using vectorial transport assays with MDCKII/MRP2 cells we found only marginal transport of indomethacin and no transport of pantoprazole, another stimulator. Taurocholate is a good stimulator of E_217βG transport by human (Fig. 2D) and rat Mrp2, but it is not transported by rat Mrp2 (35), as is the case with furosemide (36). Taken together, these observations indicate that transport of a compound by MRP2 is not a prerequisite for its ability to stimulate the transport of another compound.

Fig. 5. Concentration-dependent transport of E_217βG by MRP2 and effect of 100 μM indomethacin or 1 mM sulfanitran. Membrane vesicles containing MRP2 were incubated at 37 °C for 30 s with 40 nM [3H]labeled E_217βG and unlabeled E_217βG to the final concentration indicated in the figure. The ATP-dependent transport by MRP2 was determined in the absence of stimulator (A) or in the presence of 100 μM indomethacin (B) or 1 mM sulfanitran (C). The dashed line in A, B, and C represents a computer-fitted curve to the experimental points. Each point and error are the mean ± S.E. of an experiment in triplicate.
Allosteric Regulation of MRP2-mediated Transport

FIG. 6. Effects of organic anions and drugs on 100 μM MTX transport by MRP2. Membrane vesicles containing MRP2 were incubated with 100 μM [3H]MTX for 5 min at 37 °C in the presence or absence of various concentrations of the indicated compounds. The ATP-dependent transport is plotted as a percentage of the control value. Each point and error are the mean ± S.E. of experiments in triplicate.

FIG. 7. Transport of E217βG by MRP2 at saturating substrate concentrations is inhibited by MTX. Membrane vesicles containing MRP2 were incubated at 37 °C for 30 s with 200 μM [3H]E217βG (black bars) or with 1 μM [3H]E217βG together with 1 mM sulfanitrant (hatched bars) in the presence of increasing concentrations of MTX. The ATP-dependent transport of E217βG by MRP2 was determined, and each bar represents the mean ± S.E. of experiments in triplicate.

together with GS-DNP and stimulate its transport (e.g. sulfanitrant). We surmise that probenecid, in contrast, cannot fit together with GS-DNP to form a stimulator-substrate pair and therefore only inhibits transport of GS-DNP.

MTX represents another class of MRP2 substrates (25, 33). Ito et al. (40) reported that MTX inhibits transport of E217βG by MRP2 with an approximate IC50 of ± 1 mM, whereas we found that transport of 1 μM E217βG by MRP2 is slightly stimulated by MTX up to 4.4 mM. We have no explanation for this discrepancy, but our results are readily explained by our model. The affinity of MTX for the substrate site is low, making it a poor competitive inhibitor of transport. Moreover, MTX weakly stimulates transport of E217βG (Fig. 2F), indicating that it binds to the M site as well. Hence, MTX will inhibit E217βG transport only under conditions where it can no longer stimulate it, i.e. close to E217βG substrate saturation (Fig. 7). Using a similar reasoning, we also expected to find conditions where GSH, a low affinity substrate of MRP2 (34, 41), would inhibit E217βG or MTX transport, but no effect was found up to 10 mM GSH. In contrast, 3 mM GSH has been shown to inhibit transport of NNAL-glucuronide by MRP2 nearly completely (27). A speculative explanation for these large differences is that GSH binding within the drug-transporting site of MRP2 disrupts the binding of some substrates (e.g. NNAL-glucuronide) but not of other ones (e.g. E217βG), as proposed previously by Leslie et al. (27).

Complex interactions between multiple drug binding sites in ABC transporters have been described already for P-glycoprotein (17–20, 42, 43) and MRP1 (27, 44–49). The two-site model proposed here is patterned on results obtained for the cytochrome P-450 monooxynogenases, which contain a single complex binding site able to bind two ligands (50, 51). There are other explanations for apparent cooperativity, however (52). Structural studies on ligand-bound MRP2 will be required to determine how the protein works.

Given the complex heterotropic positive drug interactions found for MRP1 and MRP2, it is necessary to reconsider the results interpreted as cotransport of drug and GSH reported previously (26, 45, 46). If GSH can bind both to the M site and the S site of MRP2, as shown unambiguously for MRP1 (47, 48), apparent cotransport could be the result of cross-stimulation, in which GSH in the M site stimulates transport of drug in the S site and vice versa. At present, we see no compelling evidence for the alternative that the M site can function as a transport site, but given the postulated presence of at least two transport sites in P-glycoprotein, this alternative remains open for the MRPs. It follows that we cannot exclude cotransport either, as proposed previously by Evers et al. (26). It should be noted that the transport of E217βG by human (23) and rat MRP2 (23, 53) has been analyzed before. In both cases saturation kinetics and Km values of 4–7 μM were reported (23, 53). More experiments are required to resolve the discrepancy with our results.

Some of the drugs studied here are used in patients (e.g. glibenclamide for the treatment of diabetes), and the allosteric properties of MRP2 could therefore result in adverse or beneficial drug-drug interactions, as pointed out before (25, 28). On the one hand, stimulation of intestinal and hepatic MRP2 could lead to a decrease in bioavailability of drugs and thus to a lower treatment efficacy. On the other hand, stimulation of the secretion of toxic metabolites could be benefi-
Allosteric Regulation of MRP2-mediated Transport

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