FUNCTIONAL ANALYSIS OF AN $\alpha$-HELICAL REGION IN THE HUMAN MULTIDRUG AND ORGANIC ANION TRANSPORTER MRP1

by

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ABSTRACT

Multidrug resistance protein 1 (MRP1/ABCC1) is a 190 kDa phosphoglycoprotein that mediates the efflux of structurally diverse endo- and xenobiotics across biological membranes, and is known to play roles in drug disposition and resistance. The goal of the present study was to examine the functional importance of the region proximal to transmembrane helix 17 (TM17) of MRP1 by mutational analysis of seven conserved amino acids in this region. Thus, Glu\textsuperscript{1253}, Glu\textsuperscript{1255}, Val\textsuperscript{1261}, Glu\textsuperscript{1262}, Arg\textsuperscript{1263}, Glu\textsuperscript{1266}, and Tyr\textsuperscript{1267} were initially replaced by Ala, and after expression in HEK293T cells, the properties of the mutant proteins were investigated. All of the mutant proteins were expressed at levels comparable to wild-type MRP1, indicating that these residues are not critical for MRP1 biosynthesis. Vesicular transport assays showed that Ala-substitution of Glu\textsuperscript{1253} and Glu\textsuperscript{1262} significantly reduced 17\(\beta\)-estradiol 17-(\(\beta\)-D-glucuronide) (E\textsubscript{217}\(\beta\)G) and leukotriene C\textsubscript{4} (LTC\textsubscript{4}) transport by 30-75\% (p < 0.05), while Ala-substitution of Glu\textsuperscript{1255} and Glu\textsuperscript{1266} had no effect. Transport activity of the same-charge mutant E\textsubscript{1253}D was comparable to wild-type MRP1, while transport by E\textsubscript{1262}D remained reduced (by 50-75\%) (p < 0.05). Kinetic analysis suggests that E\textsubscript{1253}A and E\textsubscript{1262}A exhibit reduced E\textsubscript{217}\(\beta\)G uptake as a result of a decreased uptake affinity (\(K_m\)), while the reduced transport of E\textsubscript{1262}D was associated with a reduction in \(V_{max}\). Reciprocal mutations of potential interhelical bonding partners of Glu\textsuperscript{1253} and Glu\textsuperscript{1262} (Lys\textsuperscript{1141} and Arg\textsuperscript{1142}, respectively), identified by examination of an atomic homology model of MRP1, did not significantly enhance MRP1 function. This suggests that even if bonding interactions exist between the side-chains of these two pairs of amino acids, the interactions are not exclusive. These findings also suggest that Glu\textsuperscript{1253} and Glu\textsuperscript{1262} have unique and complex roles in substrate
binding and/or translocation. Ala-substitution of Val$_{1261}$, Arg$_{1263}$ and Tyr$_{1267}$ caused a small reduction in E$_2$17βG transport (by 25-35%) (p < 0.05), while reductions in LTC$_4$ transport were somewhat more substantial (by 30-55%) (p < 0.05). In conclusion, these studies have provided the first evidence of the functional importance of anionic residues in the COOH-proximal region of TM17 of MRP1.
STATEMENT OF CO-AUTHORSHIP

This thesis is based on research conducted by Steven V. Molinski under the supervision of Dr. Susan P.C. Cole. All data were obtained and analyzed by Steven V. Molinski. Kathy Sparks provided assistance with tissue culture work. Dr. Gwenaëlle Conseil provided assistance when optimizing the transfection efficiency of MRP1 expression vectors, and generated K1141E and R1142E MRP1 mutant cDNA expression vectors as described in Conseil et al. (2006).
ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

ABSTRACT..................................................................................................................................................... ii
STATEMENT OF CO-AUTHORSHIP ................................................................................................................ iv
ACKNOWLEDGEMENTS..................................................................................................................................... v
TABLE OF CONTENTS................................................................................................................................... vi
LIST OF FIGURES...................................................................................................................................... x
LIST OF TABLES....................................................................................................................................... xii
LIST OF ABBREVIATIONS....................................................................................................................... xiii

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.1 ATP-Binding Cassette Trasporter Superfamily............................................................. 1
1.2 Multidrug Resistance ........................................................................................................ 6
1.3 MRP1-Mediated Multidrug Resistance .................................................................. 7
1.4 Modulation of MRP1-Mediated Multidrug Resistance ...................................... 10
1.5 Pharmacological, Toxicological, and Physiological Roles of MRP1 .............. 13
   1.5.1 Pharmacological Roles of MRP1 ................................................................. 13
   1.5.2 Toxicological Roles of MRP1 .................................................................... 14
   1.5.3 Physiological Roles of MRP1 .................................................................... 16
1.6 Structure of MRP1 ......................................................................................................... 19
1.7 MRP1 Transport Mechanism .................................................................................. 22
1.8 Atomic Homology Models of MRP1 ...................................................................... 24
1.9 MRP1 Substrate Specificity and Structure-Function Studies of MRP1 .......... 28
1.10 Rationale, Hypotheses, and Objectives............................................................... 30
CHAPTER II: MATERIALS AND METHODS

2.1 Materials ........................................................................................................ 35
2.2 Secondary Structure Predictions ........................................................................ 36
2.3 Sequence Alignments and In Silico Illustrations ............................................... 36
2.4 Vector Construction and Site-Directed Mutagenesis ........................................... 36
2.5 Cell Culture ..................................................................................................... 38
2.6 Transfections of MRP1 Expression Vectors in HEK293T Cells ......................... 39
2.7 Preparation of Membrane Vesicles from MRP1 Transfectants .......................... 39
2.8 Determination of MRP1 Protein Levels in Transfected Cells ............................. 40
2.9 MRP1-Mediated Transport of $^3$H-Labeled Substrates by Membrane Vesicles ................................................................. 41
2.10 Kinetic Analysis of $[^3H]E_{217}βG$ Transport ............................................... 42
2.11 Photolabeling of MRP1 by $[^3H]LTC_4$ ......................................................... 43
2.12 Statistical Analyses ....................................................................................... 44

CHAPTER III: FUNCTIONAL ANALYSIS OF GLU$^{1253}$, GLU$^{1255}$, GLU$^{1262}$ AND GLU$^{1266}$ MRP1 MUTANTS

3.1 Introduction ....................................................................................................... 45
3.2 Results ............................................................................................................... 47
   3.2.1 Secondary Structure Predictions, Sequence Alignments, and In Silico Illustrations of the Region COOH-Proximal to TM17 ............... 47
   3.2.2 Expression, and E$_{217}βG$ and LTC$_4$ Transport Activities of Ala-Substituted MRP1 Mutant Proteins ................................................. 53
3.2.3 Expression and Transport Activities of MRP1 mutants Same- and Opposite-Charge Substitutions of Glu\textsuperscript{1253} and Glu\textsuperscript{1262} .............................. 56

3.2.4 Kinetic Analysis of [\textsuperscript{3}H]E\textsubscript{2}17\textgreek{G} Uptake by E1253A, E1262A, and E1262DMRP1 Mutants ................................................................. 59

3.2.5 Photolabeling of E1253A, E1262A and E1262D MRP1 Mutants by [\textsuperscript{3}H]LTC\textsubscript{4} ............................................................................................................. 62

3.2.6 Expression, and E\textsubscript{2}17\textgreek{G} and LTC\textsubscript{4} Transport Activities of K1141E/E1253K and R1142E/E1262R Double Reciprocal MRP1 Mutants ........................................................................................................ 63

3.3 Discussion ........................................................................................................ 70

CHAPTER IV: FUNCTIONAL ANALYSIS OF ALA-SUBSTITUTED VAL\textsuperscript{1261}, ARG\textsuperscript{1263} AND TYR\textsuperscript{1267} MRP1 MUTANTS

4.1 Introduction .................................................................................................. 76

4.2 Results ....................................................................................................... 77

4.2.1 Secondary Structure Predictions, Sequence Alignments, and \textit{In Silico} Illustrations of the Region COOH-Proximal to TM17 .............................. 77

4.2.2 Expression, and E\textsubscript{2}17\textgreek{G} and LTC\textsubscript{4} Transport Activities of Ala-Substituted MRP1 Mutant Proteins ......................................................... 80

4.3 Discussion .................................................................................................. 85

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Implications for MRP1 Structure .............................................................. 87
5.2 Limitations of MRP1 Homology Models and Reciprocal Mutagenesis........ 90
5.3 Concluding Remarks....................................................................................... 91

REFERENCES ................................................................................................................. 94

APPENDIX

A1.1 Uncharacterized MRP1 Mutants E1079A, R1263K, and E1263E
Generated and Related to this Study................................................................. 112

A1.2 MRP1 Mutants R1202E, R1202E/E1204R, and R1202D/E1204K
Generated During the Course of this Master's Thesis in Collaboration with
Marina Chan.......................................................................................................... 112
LIST OF FIGURES

Figure 1.1 A predicted membrane topology model of MRP1 ........................................... 4
Figure 1.2 Phylogenetic tree of the human ABCC transporter subfamily ....................... 5
Figure 1.3 Chemical structures of some MRP1 substrates ........................................... 9
Figure 1.4 An atomic homology model of MRP1 based on the crystal structure of
Sav1866 from S. aureus ........................................................................................... 21
Figure 1.5 A hypothetical transport cycle of MRP1-mediated substrate efflux .......... 25
Figure 1.6 Helical projection of amino acids in the TM17-proximal region ............... 32

Figure 3.1 Secondary structure and in silico illustration of the TM17-proximal region of
MRP1 ................................................................................................................... 46
Figure 3.2 Structural predictions and sequence alignments of the TM17-proximal region
of MRP1 ............................................................................................................. 48
Figure 3.3 Putative relative locations and side-chain positions of Glu<sup>1253</sup>, Glu<sup>1255</sup>, Glu<sup>1262</sup>
and Glu<sup>1266</sup> in a homology model of MRP1 .................................................. 50
Figure 3.4 In silico illustration of potential paired interhelical interactions involving side-
chains of Glu<sup>1253</sup> and Lys<sup>1141</sup>, Glu<sup>1255</sup> and Arg<sup>1138</sup>, Glu<sup>1262</sup> and Arg<sup>1142</sup>, and
Glu<sup>1266</sup> and Arg<sup>1075</sup> .................................................................................... 52
Figure 3.5 Expression levels and vesicular uptake of <sup>3</sup>H-labeled organic anions by
Ala-substituted Glu<sup>1253</sup>, Glu<sup>1255</sup>, Glu<sup>1262</sup> and Glu<sup>1266</sup> MRP1 mutants ........ 54
Figure 3.6 Expression levels and vesicular uptake of <sup>3</sup>H-labeled organic anions by same-
and opposite-charge substitutions of Glu<sup>1253</sup> and Glu<sup>1262</sup> MRP1 mutants .... 57
Figure 3.7 Kinetic analysis of $[^3H]E_{217}\beta G$ uptake by Ala-substituted Glu$^{1253}$ and Glu$^{1262}$, and Asp-substituted Glu$^{1262}$ MRP1 mutants ................................................................. 60

Figure 3.8 Photolabeling of Ala-substituted Glu$^{1253}$ and Glu$^{1262}$, and Asp-substituted Glu$^{1262}$ MRP1 mutant proteins by $[^3H]LTC_4$ ...................................................... 64

Figure 3.9 Expression levels and vesicular uptake of $E_{217}\beta G$ and LTC$_4$ by MRP1 mutants containing single and double exchange mutations of Lys$^{1141}$ and Glu$^{1253}$ ................................................................. 65

Figure 3.10 Expression levels and vesicular uptake of $E_{217}\beta G$ and LTC$_4$ by MRP1 mutants containing single and double exchange mutations of Arg$^{1142}$ and Glu$^{1262}$ ................................................................. 67

Figure 4.1 Structural predictions and sequence alignments of Val$^{1261}$, Arg$^{1263}$, and Tyr$^{1267}$ of MRP1 ........................................................................................................ 78

Figure 4.2 Putative relative locations and side-chain positions of Val$^{1261}$, Arg$^{1263}$, and Tyr$^{1267}$ in a homology model of MRP1 ................................................................. 79

Figure 4.3 *In silico* illustration of potential interhelical interactions involving side-chains of Val$^{1261}$ and Val$^{1083}$, Arg$^{1263}$ and Glu$^{1079}$, and Tyr$^{1267}$ and Phe$^{1063}$ ... 81

Figure 4.4 Expression levels and vesicular uptake of $E_{217}\beta G$ and LTC$_4$ by Ala-substituted Val$^{1261}$, Arg$^{1263}$, and Tyr$^{1267}$ MRP1 mutants ................................................................. 83
LIST OF TABLES

Table 1.1 Genetic disorders associated with mutant ABC transporters ......................... 2
Table 1.2 Reported tissue distribution of MRP1 mRNA and protein ............................ 15
Table 1.3 Selected substrates of MRP1 and reported $K_m$ values ................................. 17

Table 3.1 Estimated distances between side-chains of Glu$^{1253}$, Glu$^{1255}$, Glu$^{1262}$ and Glu$^{1266}$, and other amino acids which potentially form interhelical bonds in MRP1 .................................................................................................................. 51
Table 3.2 Summary of kinetic parameters of $[^3]$H$\beta$G uptake by Ala-substituted Glu$^{1253}$ and Glu$^{1262}$, and Asp-substituted Glu$^{1262}$ MRP1 mutants .......................... 61

Table 4.1 Estimated distances between the side-chains of Val$^{1261}$, Arg$^{1263}$ and Tyr$^{1267}$, and other amino acids in other helices which might form bonding interactions ........................................................................................................................................... 82
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ALD</td>
<td>Adrenoleukodystrophy</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile salt export pump</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>E$_1$3SO$_4$</td>
<td>Estrone 3-sulfate</td>
</tr>
<tr>
<td>E$_2$17$\beta$G</td>
<td>17$\beta$-estradiol 17-($\beta$-D-glucuronide)</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>K$_{ir}$</td>
<td>Inward rectifying potassium channel</td>
</tr>
<tr>
<td>LTC$_4$</td>
<td>Leukotriene C$_4$</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MSD</td>
<td>Membrane spanning domain</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>NBS</td>
<td>Nucleotide binding site</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TSB</td>
<td>Tris-sucrose buffer</td>
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</table>
CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.1 ATP-Binding Cassette Transporter Superfamily

ATP-binding cassette (ABC) transporters are polytopic membrane proteins that are found in all species, and require ATP to translocate structurally diverse endo- and xenobiotic compounds across the lipid bilayer of cell membranes (Higgins, 1992). Mammalian ABC transporters are involved in many different physiological processes, including: 1) cellular efflux of signaling and bioactive molecules (e.g. eicosanoids, bile acids, and steroid conjugates), 2) modulating absorption, distribution, and elimination of nutrients and their metabolites, and 3) preventing accumulation, and aiding elimination of drugs and chemical toxins and their metabolites from a variety of tissues in the human body (Szakács et al., 2008). When certain ABC transporters are expressed in human tumour cells, the latter function can also lead to a multidrug resistance (MDR) phenotype (Gerlach et al., 1987; Gottesman et al., 2002; Leonard et al., 2003). Therefore, these proteins are of great physiological, pharmacological, and pathological importance.

In addition to regulating the disposition of endogenous and exogenous organic molecules and conferring resistance to multiple chemotherapeutics, mutations in certain human ABC transporters are responsible for a number of genetic disorders (Table 1.1). Among these are Harlequin-type ichthyosis (ABCA12), Dubin-Johnson Syndrome (MRP2/ABCC2), Pseudoxanthoma elasticum (MRP6/ABCC6), and cystic fibrosis (CFTR/ABCC7) (Akiyama, 2006; Kartenbeck et al., 1996; Ringpfeil et al., 2000; Riordan et al., 1989).
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Protein (common name)</th>
<th>Genetic Disorder</th>
<th>References</th>
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<tr>
<td>ABCA1</td>
<td></td>
<td>Tangier disease</td>
<td>Dean, 2005</td>
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<tr>
<td>ABCA3</td>
<td></td>
<td>Newborn respiratory distress syndrome</td>
<td>Klugbauer and Hofmann, 1996</td>
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<td>Stargardt disease</td>
<td>Dean, 2005</td>
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<td>ABCA12</td>
<td>TAP1</td>
<td>Harlequin-type ichthyosis</td>
<td>Akiyama et al., 2006</td>
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<td>ABCB2</td>
<td>TAP2</td>
<td>Ankylosing spondylitis</td>
<td>Feng et al., 2009</td>
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<td>Feng et al., 2009</td>
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<td>ABCB4</td>
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<td>Progressive familial intrahepatic cholestasis type 3</td>
<td>Dean, 2005</td>
</tr>
<tr>
<td>ABCB7</td>
<td>BSEP</td>
<td>X-linked sideroblastosis and anemia</td>
<td>Dean, 2005</td>
</tr>
<tr>
<td>ABCB11</td>
<td></td>
<td>Progressive familial intrahepatic cholestasis type 2</td>
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<tr>
<td>ABCC2</td>
<td>MRP2</td>
<td>Dubin-Johnson Syndrome</td>
<td>Kartenbeck, et al., 1996</td>
</tr>
<tr>
<td>ABCC6</td>
<td>MRP6</td>
<td>Pseudoxanthoma elasticum</td>
<td>Ringpfeil et al., 2000</td>
</tr>
<tr>
<td>ABCC7</td>
<td>CFTR</td>
<td>Cystic fibrosis</td>
<td>Riordan et al., 1989</td>
</tr>
<tr>
<td>ABCC8</td>
<td>SUR1</td>
<td>Familial persistent hyperinsulinemic hypoglycemia of infancy</td>
<td>Dean, 2005</td>
</tr>
<tr>
<td>ABCC9</td>
<td>SUR2</td>
<td>Dilated cardiomyopathy with ventricular tachycardia</td>
<td>Dean, 2005</td>
</tr>
<tr>
<td>ABCC11</td>
<td>MRP8</td>
<td>Dry ear wax, body odour</td>
<td>Yoshiura et al., 2006</td>
</tr>
<tr>
<td>ABCD1</td>
<td>ALD</td>
<td>Adrenoleukodystrophy</td>
<td>Dean, 2005</td>
</tr>
<tr>
<td>ABCG5</td>
<td></td>
<td>Sitosterolemia</td>
<td>Rudkowska and Jones, 2008</td>
</tr>
<tr>
<td>ABCG8</td>
<td></td>
<td>Sitosterolemia</td>
<td>Rudkowska and Jones, 2008</td>
</tr>
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</table>

Table 1.1: Genetic disorders associated with mutant ABC transporters.
The human ABC transporter superfamily is comprised of 49 genes that are
distributed among 7 phylogenetic branches designated A through G (Dean and Allikmets,
2001). The basic functional structure of ABC proteins consists of two membrane
spanning domains (MSDs) and two nucleotide binding domains (NBDs) (Higgins, 1992),
and some members contain an additional MSD, the function of which is not yet clear
(Bakos et al., 1998; Westlake et al., 2005). The domains of functional ABC transporters
are usually arranged as MSD-NBD-MSD-NBD, while the third MSD can be found at the
N-terminus of 7 members of the ABC‘C’ subfamily (i.e. ABCC1, ABCC2, ABCC3,
ABCC6, ABCC8, ABCC9, and ABCC10) (Figure 1.1) (Higgins, 1992; Deeley et al.,
2006). Many mammalian ABC proteins are translated as one long polypeptide, while
some are translated into two polypeptides each consisting of either an NBD-MSD (e.g.
ABCG2 (breast cancer resistance protein, BCRP)) or an MSD-NBD (e.g. TAP1/TAP2
(transporter associated with antigen processing 1 and 2)), and thus these 2-domain
‘subunits’ dimerize to form a functional 4-domain transporter.

The human ABC‘C’ subfamily consists of 13 members, including the multidrug
resistance proteins MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4
(ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), MRP7 (ABCC10), MRP8 (ABCC11),
MRP9 (ABCC12) and the pseudogene MRP10 (ABCC13), as well as CFTR (ABCC7),
and the sulfonylurea receptors SUR1 (ABCC8) and SUR2 (ABCC9) (Figure 1.2) (Dean
and Allikmets, 2001). CFTR is a cAMP-gated chloride channel, and SUR1 and SUR2 are
regulators of inwardly rectifying potassium channels (K_{ir}) (Riordan et al., 1989; Bryan et
al., 2007), while the nine MRPs appear to be mainly involved in transporting organic
anions out of cells, as is discussed below (Deeley et al., 2006). MRP1 (ABCC1) is
Figure 1.1: A predicted membrane topology model of MRP1.

A topology model of MRP1 showing MSD0, MSD1, MSD2, NBD1, and NBD2 coloured in light gray, green, blue, yellow, and magenta, respectively. TM17 is coloured in red, and the orange star denotes the TM17-proximal region investigated in this thesis. The ‘Y-shaped’ sticks represent N-glycosylation sites. MSD, membrane spanning domain; TM, transmembrane α-helix; NBD, nucleotide binding domain.
Figure 1.2: Phylogenetic tree of the human ABCC transporter subfamily.

Alignments were performed using ClustalW v2.0 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Shown in parentheses are the common protein names for each ABCC transporter. The pseudogene ABCC13 has been omitted from this analysis.
the MRP family member most strongly implicated in mediating MDR in malignant
disease (Abe et al., 1994; Borst, 1999; Berger et al., 2005; Haber et al., 2006; Ozben et
al., 2006).

1.2 Multidrug Resistance
MDR is a major obstacle to overcome during cancer chemotherapy since the appearance
of this phenotype is typically associated with treatment failure and a poor prognosis
(Gerlach et al., 1986; Gottesman et al., 2002). Cells which exhibit MDR are able to
survive exposure to normally lethal doses of multiple cytotoxic agents. Thus, MDR to a
broad range of structurally and functionally diverse agents might explain why
chemotherapeutic regimens that use 'cocktails' of multiple anticancer drugs with different
targets are not always more effective than single agent treatments (Gottesman et al.,
2002).

There are two major forms of MDR, inherent and acquired. Inherent MDR refers
to the ability of a cell to exhibit relative resistance without having to modify its own
intracellular processes. These processes prevent adequate doses of active anticancer drugs
from reaching their target site (Luqmani, 2005; McCarthy, 2009). Acquired MDR is a
multi-factorial process, allowing for tumours, which are initially sensitive to cytotoxic
agents, to develop resistance to a spectrum of unrelated drugs through multiple
mechanisms and pathways. Thus, acquired MDR refers to the ability of a cell to adapt to
its local environment by modifying its genetic expression profile (i.e. upregulating genes
that promote survival and/or downregulating genes that do not), such that the amount of
drug that reaches its target is reduced, or the apoptotic and other pathways that are set into motion by the drug/target interaction are blunted (Bradley et al., 1988).

MRP1 together with P-glycoprotein (P-gp) (ABCB1) and ABCG2 are major players in intrinsic and acquired MDR in many tumours (Borst, 1999; Pankunlu et al., 2003; Leonard et al., 2003; Burger et al., 2005; Pajic et al., 2009). There has been much research aimed at modifying drug efflux mediated by these transporters in human cancers as one approach to combat this deadly phenotype.

1.3 MRP1-Mediated Multidrug Resistance

MRP1 was discovered in 1992 during investigations into the cause of MDR in a human small cell lung carcinoma cell line, designated H69AR (Mirski et al., 1987; Cole et al., 1992). An unusual characteristic of this cell line was that it did not overexpress P-gp, the only protein demonstrated to cause MDR at that time (Mirski et al., 1987; Cole et al., 1991; Cole et al., 1992). An mRNA transcript, encoded by a gene later designated \textit{ABCC1}, was found to be markedly overexpressed, and was predicted to encode a protein containing 1531 amino acids with a minimum molecular mass of 170 kDa (Cole et al., 1992). \textit{ABCC1} was localized to chromosome 16p13.11-p13.12, and determined to contain 31 exons that span 192.84 kb (Cole et al., 1992; Grant et al., 1997; Dean and Allikmets, 2001). The 6.5 kb mRNA transcript encoded by \textit{ABCC1} was translated into a ~190 kDa phosphoglycoprotein when expressed in mammalian cells (Cole et al., 1994; Almquist et al., 1995). In addition, MRP1 was shown to confer resistance to, and mediate the ATP-dependent efflux of a broad range of structurally diverse anticancer compounds,
including anthracyclines, *Vinca* alkaloids, antifolates, and an epipodophyllotoxin in cultured cells (Figure 1.3) (Cole et al., 1994; Deeley et al., 2006).

Subsequent studies aimed at determining the prevalence of MRP1 in human tumours were able to show a correlation of MRP1 protein expression with the stage of disease, providing some evidence for a role for MRP1 in clinical MDR (Filipits et al., 1999; Haber et al., 2006; Ohsawa et al., 2005; Larbcharoensub et al., 2008; Hendig et al., 2009). Thus, MRP1 is expressed at relatively high levels in certain tumours, including those derived from lung, colorectal, testis, and breast tissues (Berger et al., 2005; Meijer et al., 1999; Bart et al., 2004; Filipits et al., 1999).

Several studies have investigated whether MRP1 expression can be used as a prognostic indicator for determining the effectiveness of cancer chemotherapies. For example, Filipits et al. (2007) collected tumour samples from ~800 patients with completely resected non-small cell lung cancer, and by immunohistochemical analysis with the MRP1-specific antibody MRPr1, showed that although MRP1 was frequently expressed, it was not a good predictive tool for determining clinical outcomes in cisplatin-based adjuvant chemotherapy. This is not necessarily unexpected, since there is no evidence that MRP1 confers resistance to cisplatin (Sharp et al., 1998). Another immunohistochemical study of 115 ovarian carcinoma patients also concluded that response to chemotherapy, as well as prognoses did not correlate well with MRP1 expression levels. Again, however, this study involved samples from patients receiving platinum-based chemotherapy (Arts et al., 1999).

In contrast to the above two studies, MRP1 protein expression was found to be a good predictive indicator of poor clinical outcome in several smaller studies of patients
Figure 1.3: Chemical structures of some MRP1 substrates.

A, anticancer agents; B, established, and potential physiological substrates.
with nasopharyngeal carcinoma (Larbcharoensub et al., 2008), primary neuroblastoma (Haber et al., 2006), nodal diffuse large B-cell lymphoma (Ohsawa et al., 2005), breast carcinoma (Filipits et al., 1999), and retinoblastoma (Hendig et al., 2009). Interestingly, in the study by Larbcharoensub et al. (2008), cisplatin-based chemotherapy was employed, and thus MDR could not be attributed to MRP1 alone. Nevertheless, these authors concluded that MRP1 protein expression could be used as a prognostic marker in certain tumour tissue samples, while noting that there are currently no guidelines or standardized testing for MRP1 expression in clinical studies. Additionally, other proteins involved in MDR, such as P-gp and ABCG2, could also be used as clinical prognostic markers, but again there are currently no standardized guidelines for this approach, although recommendations have been made for P-gp (Orina et al., 2009).

Positive correlations in certain tumour tissues provide a potential target for improving response to cancer chemotherapy, in that the suppression of MRP1 expression or inactivation of its drug efflux activity might be expected to enhance the chemosensitivity of cells in the patient’s tumour(s). However, it should be kept in mind that other ABC efflux transporters, including P-gp and ABCG2, can also be overexpressed in conjunction with MRP1, making it even more difficult to correlate disease state solely with MRP1-based MDR, and thus further complicating the course of treatment (Huang and Sadée, 2005).

1.4 Modulation of MRP1-Mediated Multidrug Resistance
Antisense oligonucleotides can be used to target MRP1 mRNA in order to reduce MRP1 protein levels so that, in theory, the response to chemotherapy can be improved. The use
of antisense oligonucleotides has been shown to reduce the effects of MRP1 \textit{in vitro}. For example, Stewart \textit{et al.} (1996) used an MRP1 antisense phosphorothioate oligonucleotide targeted to the coding region to reduce MRP1 mRNA levels in lung cancer cells by 90%, as well as MRP1 protein by 50%. Similarly, Canitrot \textit{et al.} (1996) found that a modified 2’-deoxy analog of the same antisense oligonucleotide greatly reduced (70%) MRP1 protein levels in transfected HeLa cells. In addition, Niewiarowski \textit{et al.} (2000) demonstrated that MRP1 mRNA and protein levels can be significantly reduced in leukemia cells (75% and 50%, respectively), using antisense oligonucleotides targeted to different regions of the MRP1 mRNA, and Kuss \textit{et al.} (2002) showed that MRP1 protein levels can be significantly reduced (by 60%) in an \textit{in vivo} mouse-human xenograft model of neuroblastoma. More recently, Matsumoto \textit{et al.} (2004) showed that antisense oligonucleotides, again targeting different sequences, significantly reduced MRP1 mRNA levels (by 85%) in an etoposide resistant glioma cell line, and Pakunlu \textit{et al.} (2004) demonstrated that antisense oligonucleotides significantly reduced (by 35%) MRP1 protein levels in the H69AR small cell lung cancer cell line. Although proof of concept has been demonstrated by these \textit{in vitro} and \textit{in vivo} studies, suppression of MRP1 will not be as effective for multifactorial (i.e. transporter and non-transporter-mediated) clinical MDR. Furthermore, this approach may not be feasible due to poor delivery at target sites.

A variety of small molecules of both natural and synthetic origin have also been investigated as MRP1 inhibitors primarily \textit{in vitro}. Compounds such as LTD$_4$ receptor (CysLT1 and CysLT2) antagonists (e.g. MK571 and BAY u9773), polyhydroxylated sterols (e.g. acetate agosterol A), dietary bioflavonoids, tricyclic isoxazole derivatives (e.g. LY475776), benzothiophene derivatives (e.g. LY329146), as well as
pyrazolopyrimidines (e.g. CBLC4H10 (Reversan)) have been shown to inhibit MRP1-mediated multidrug resistance by interacting with the transporter. The IC_{50} values for many of these compounds are in the low μM range, while others are considerably more potent (e.g. LY475776 is in the low nM range, although it is dependent on GSH for its activity) (Mao et al., 2002; Boumendjel et al., 2005; Burkhart et al., 2009; Maeno et al., 2009).

Agosterol A and its analogs have been shown to competitively inhibit MRP1-mediated LTC4 transport in a GSH-dependent manner in vitro, while in vivo results have not yet been reported (Ren et al., 2003). Similarly, both LY475776 and LY329146 were also able to competitively inhibit MRP1-mediated LTC4 transport in a GSH-dependent manner in vitro, while LY171883 and BAY u9773 were able to competitively inhibit MRP1-mediated E_{217β}G transport, further suggesting an inhibitory role for these small molecules (Mao et al., 2002; Norman et al., 1999; Maeno et al., 2009).

Dietary bioflavonoids are never specific inhibitors of MRP1 (or other ABC proteins, and other enzymes), and often inhibit the activity of MRP2 and P-gp, as well as other intracellular proteins (e.g. topoisomerase II, an enzyme crucial for chromosome segregation) (Di Pietro et al., 2002; Morris and Zhang, 2006). In addition, the inhibitory action of some bioflavonoids is enhanced in the presence of GSH (Leslie et al., 2003a). It has been suggested that bioflavonoids might be used in conjunction with other modulatory agents in order to achieve synergistic inhibition of MRP1 (Boumendjel et al., 2005). The inhibitory effects of MK571 are also not specific for MRP1, as it is reported to modulate the activity of many MRPs, including MRP1, MRP2, MRP3, and MRP4 with varying potencies (Gekeler et al., 1995; Chen et al., 1999). Such relatively non-specific
inhibitors are likely to be of little clinical value since they are likely to alter other physiologically relevant processes in addition to MRP1-mediated MDR (Bakos et al., 2000).

The newly reported pyrazolopyrimidine Reversan was able to inhibit MRP1-mediated export of vincristine and etoposide, in murine models of neuroblastoma (syngeneic and human xenografts) with no apparent off-target toxicities (Burkhart et al., 2009). These data support the idea that small molecules targeting MRP1 could potentially be used to combat tumours exhibiting MRP1-mediated MDR by increasing their sensitivity to conventional cytotoxic chemotherapeutic agents.

1.5 Pharmacological, Toxicological, and Physiological Roles of MRP1

As mentioned previously, in addition to conferring resistance in malignant cells, MRP1 also plays important roles in drug and toxin disposition in normal cells (Leslie et al., 2005; Szakács et al., 2008). Thus, some of the main roles of MRP1 include tissue defense from toxic compounds, as well as mediating the passage of endo- and xenobiotics through cellular and tissue barriers, and these are discussed briefly below.

1.5.1 Pharmacological Roles of MRP1

MRP1 protein is found on the basolateral membrane of polarized epithelial and endothelial cells in a wide range of tissues, as well as the apical membrane of specialized endothelial cells in the blood-brain barrier (reviewed by Deeley et al., 2006). MRP1 mRNA and protein is present at relatively low levels in all tissues throughout the body, and relatively higher levels are found in the lung, testis, skeletal and cardiac muscles,
kidney, prostate, and the placenta (Table 1.2) (Deeley et al., 2006; Wijnholds et al., 1997; Cole et al., 1992; Kruh et al., 1995; Szakács et al., 2008). This tissue distribution pattern of MRP1 is consistent with its role in regulating the accumulation of pharmacological agents at a number of blood-organ interfaces, like the blood-cerebral spinal fluid (CSF)-barrier and the blood-testis barrier, that create so-called pharmacological sanctuary sites in the body (Leslie et al., 2005). To date, in vivo studies have been very useful in determining the pharmacological consequences of deficient MRP1 protein expression. Studies involving Mrp1 knockout mice have shown that certain tissues (e.g. lung, testis) exhibit an increased sensitivity to acute doses of some chemotherapeutic agents, including etoposide and vincristine, although sensitivity to other agents, including cisplatin and sodium arsenite, was not changed (Wijnholds et al., 1997; Lorico et al., 1996; Rappa et al., 1999; Johnson et al., 2001; van Tellingen et al., 2003).

1.5.2 Toxicological Roles of MRP1

MRP1 is also important in the cellular efflux of a number of toxins including the glucuronide conjugate of the hydroxylated metabolite (NNAL) of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a carcinogen found in cigarette smoke (Leslie et al., 2001a). NNK is first metabolized to NNAL and after glucuronidation, the NNAL-O-Gluc conjugate can be exported by MRP1 across the plasma membrane. However, despite being an organic anion, NNAL-O-Gluc requires the presence of GSH (or a non-reducing analog, e.g. S-Me-GSH) to be transported, for reasons that are still not well understood (Leslie et al., 2001a). Interestingly, MRP2 also transports NNAL-O-Gluc but without a
### Table 1.2: Reported tissue distribution of MRP1 mRNA and protein.

‘+', ‘++', and ‘+++’ denote low, moderate, and high levels of expression, respectively. Data were obtained from reviews by Leslie et al., 2001b; Leslie et al., 2005; Deeley et al., 2006; Ballatori et al., 2009. These values represent whole tissue analysis, and thus do not reflect cell-type specific expression within these tissues. It should be noted that variability among studies as well as inconsistencies in the literature has been reported.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>MRP1 mRNA</th>
<th>MRP1 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood-brain barrier</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Brain</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Breast</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Colon</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>(very low)</td>
</tr>
<tr>
<td>Lung</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Marrow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ovary</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pancreas</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Placenta</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Prostate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Skin</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Small intestine</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Testis</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>
requirement for GSH. Furthermore, MRP1 is able to transport the GSH conjugate of the
liver and lung carcinogen (and mycotoxin) aflatoxin B₁ (Zaman et al., 1995).

In addition, MRP1 also mediates the transport of many unconjugated toxins. For
example, MRP1 has been shown to protect cells from sodium arsenite, sodium arsenate,
and potassium antimony tartrate (Cole et al., 1994; Loe et al., 1997; Rappa et al., 1997).

It is also interesting to note that certain exogenous chemicals are reported to
induce MRP1 protein expression. For example, the hepatotoxin carbon tetrachloride has
been shown to dose-dependently increase Mrp1 protein levels in mice liver, while tert-
butyl hydroquinone, and quercetin have been shown to induce MRP1 protein expression
in a human breast cancer cell line (Aleksunes et al., 2006; Yamane et al., 1998; Schrenk
et al., 2001). Although several chemicals appear to alter the expression patterns of MRP1,
the mechanism(s) by which they do so is not fully understood.

1.5.3 Physiological Roles of MRP1

It has been well established that MRP1 transports GSH, glucuronide, and sulfate
conjugates of certain endobiotics, even though there are no apparent common structural
features of these moieties, other than their organic anion nature (Figure 1.3) (Loe et al.,
1996a). Well characterized endogenously formed substrates of human MRP1 include
17β-estradiol 17-(β-D-glucuronide) (E₂17βG), estrone 3-sulfate (E₁3SO₄), and LTC₄
(Table 1.3) (Deeley et al., 2006; Cole and Deeley, 2006; Leier et al., 1994). Despite their
different chemical structures, E₂17βG and LTC₄ are competitive inhibitors of each other’s
transport by MRP1 (Jedlitschky et al., 1996; Loe et al., 1996a). However, many site-
directed mutagenesis studies have identified regions of MRP1 which exhibit substrate
### Table 1.3: Selected substrates of MRP1 and reported $K_m$ values.

‘*’ denotes $K_m$ determined in the presence of GSH; ‘**’ denotes $K_m$ determined in the presence of apigenin. ‘***’ denotes $K_m$ determined in the presence of verapamil. $N/D$, not determined. $K_m$ values were determined by *in vitro* vesicular transport assays in all cases.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Apparent $K_m$ (μM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous metabolites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin*</td>
<td>0.01</td>
<td>Rigato <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>Monoglucuronosyl bilirubin</td>
<td>N/D</td>
<td>Jedlitschky <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Bisglucuronosyl bilirubin</td>
<td>N/D</td>
<td>Jedlitschky <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>DHEAS*</td>
<td>5.0</td>
<td>Zelcer <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>$E_1^{3}SO_4$</td>
<td>4.2</td>
<td>Qian <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>$E_1^{3}SO_4^*$</td>
<td>0.7</td>
<td>Qian <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>$E_2^{17}\beta$G</td>
<td>1.5; 2.5</td>
<td>Jedlitschky <em>et al.</em>, 1996; Loe <em>et al.</em>, 1996a</td>
</tr>
<tr>
<td>GSH</td>
<td>3400</td>
<td>Salerno <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>GSH**</td>
<td>116</td>
<td>Leslie <em>et al.</em>, 2003a</td>
</tr>
<tr>
<td>GSH***</td>
<td>83</td>
<td>Loe <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>GSSG</td>
<td>93</td>
<td>Leier <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>LTC$_4$</td>
<td>0.1</td>
<td>Jedlitschky <em>et al.</em>, 1994; Loe <em>et al.</em>, 1996b</td>
</tr>
<tr>
<td>PGA$_2$-SG</td>
<td>1.0</td>
<td>Evers <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>4-Hydroxynonenal-SG</td>
<td>1.6</td>
<td>Renes <em>et al.</em>, 2000</td>
</tr>
<tr>
<td><strong>Exogenous compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-Dinitrophenyl-SG</td>
<td>3.6</td>
<td>Jedlitschky <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Aflatoxin B$_1$-SG</td>
<td>0.2</td>
<td>Loe <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Depsipeptide FK228</td>
<td>N/D</td>
<td>Xiao <em>et al.</em>, 2005</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>2150</td>
<td>Zeng <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Metolachlor-SG</td>
<td>290</td>
<td>Leslie <em>et al.</em>, 2001b</td>
</tr>
<tr>
<td>NNAL-0-glucuronide*</td>
<td>39</td>
<td>Leslie <em>et al.</em>, 2001a</td>
</tr>
<tr>
<td>Ethacrynic acid-SG</td>
<td>28</td>
<td>Zaman <em>et al.</em>, 1996</td>
</tr>
</tbody>
</table>
selectivity, in that certain mutations can affect transport of LTC₄ but not E₂₁₇βG, and vice-versa, suggesting these organic anions bind to distinct yet overlapping binding sites (Haimeur et al., 2002; Ito et al., 2001a). However, the potencies of several non-GSH-containing modulators including MK571, BAY u9773, and LY171883 are not affected by either type of mutation (Maeno et al., 2009). Taken together, these studies suggest that MRP1 has at least three substrate/modulator binding sites: one that interacts with E₂₁₇βG, one that interacts with LTC₄, and one that interacts with neither E₂₁₇βG nor LTC₄.

Another piece of evidence which supports the hypothesis that MRP1 has multiple binding sites is that NNAL-O-glucuronide can inhibit both LTC₄ and GSH transport, but not E₂₁₇βG transport, suggesting that the chemical structure of the parent compound (and not the glucuronide moiety) determine where the metabolite binds (Leslie et al., 2001b; Zelcer et al., 2003).

MRP1 also mediates the transport both GSH and GSSG, although the affinity ($K_m$) for GSSG is reportedly ~40 times higher than for GSH (Salerno et al., 2001; Leier et al., 1996; Heijn et al., 1997). Lorico et al. (1997) generated Mrp1 knockout mice and found that GSH levels were increased (by 25-90%) in tissues known to express Mrp1 compared to wild-type mice. Similarly, Rappa et al. (1997) generated an Mrp1 knockout embryonic stem cell line and found that GSH levels were increased compared to wild-type cells, in this case two-fold. Efflux of both GSH and GSSG is relevant, since it implies a role for MRP1 in controlling the intracellular GSH:GSSG ratio, an indicator of oxidative stress (Cole and Deeley, 2006; Ballatori et al., 2009). Thus, MRP1 may efflux GSSG in order to maintain an optimal GSH:GSSG ratio, so that oxidative stress can be controlled during cytotoxic conditions (e.g. during the intracellular accumulation of
reactive metabolites) (Mueller et al., 2005). In addition, it has been shown that MRP1-mediated transport of vincristine, aflatoxin B1, and daunorubicin can be enhanced in the presence of GSH (Loe et al., 1996b; Loe et al., 1997; Renes et al., 1999). Inhibition of MRP1 by certain modulators, including several bioflavonoids (e.g. apigenin), agosterol A, and several tricyclic isoxazole derivatives (e.g. LY475776), has also been shown to be dependent on, or stimulated by, GSH (Loe et al., 2000; Leslie et al., 2003a; Ren et al., 2001; Mao et al., 2002). Furthermore, MRP1-mediated efflux of GSH potentiates oxidative stress levels in H69AR-drug selected cells, which can then lead to tumour cell death by induction of apoptosis (Laberge et al., 2007).

Additionally, MRP1 can protect against the accumulation of endogenous toxins. Thus, MRP1 can mediate the removal of unconjugated bilirubin from the brain (Rigato et al., 2004). This is important because high bilirubin levels are toxic to many tissues, and prolonged exposure (e.g. in newborns with jaundice) can cause irreversible damage (Chang et al., 2009). Furthermore, although Mrp1 knockout mice are viable and fertile, they display an impaired inflammatory response, which likely is associated with defective transport of LTC4 from leukotriene-synthesizing mast cells (Wijnholds et al., 1997). These in vivo studies corroborate earlier in vitro studies reporting the importance of MRP1 in mediating LTC4 transport (Leier et al., 1994; Loe et al., 1996).

### 1.6 Structure of MRP1

Based on both biochemical evidence and protein folding algorithms, it has been determined that MRP1 is likely comprised of seventeen transmembrane (TM) α-helices arranged into three MSDs (MSD0, MSD1 and MSD2), and two intracellular NBDs
(Figure 1.1) (Bakos et al., 1996; Hipfner et al., 1997). MSD0 contains five TM helices (TMs 1-5), while MSD1 and MSD2 each contain six TM helices, TMs 6-11 and 12-17, respectively (Hipfner et al., 1997). As mentioned previously, MRP1 is considered a ‘long’ ABCC transporter (along with MRP2, 3, 6, and 7), due to the presence of MSD0 at the NH2-proximal end of these transporters (Bakos et al., 1998; Borst et al., 2000).

Current evidence strongly supports a model where MSD1 and MSD2 provide the translocation pathway for substrates, and the two NBDs form a ‘sandwich’ dimer capable of binding and hydrolyzing ATP (Figure 1.4) (Higgins et al., 2004).

As mentioned earlier, the function of MSD0 is not entirely understood. Firstly, this domain (usually defined as amino acids 1-203) does not appear to directly participate in substrate translocation, since its deletion up to amino acid 203 does not substantially affect LTC4 transport (Bakos et al., 1998). Some evidence suggests that the linker region (cytoplasmic loop 3, CL3; residues 208-270) joining MSD0 to MSD1 contains a redundant trafficking signal, such that when removed, the MSD1-NBD1/MSD2-NBD2 ‘core’ is still trafficked to the plasma membrane correctly and retains transport activity, but when both the CL3 and the COOH-proximal trafficking signals (located within the COOH-terminal 30 residues) are removed, MRP1 is not expressed on the plasma membrane (Westlake et al., 2005; Deeley et al., 2006). However, mutations of individual amino acids in this region do adversely affect the transport of LTC4 (Deeley et al., 2006).

Post-translational modifications of MRP1 include glycosylation and phosphorylation. MRP1 is glycosylated at Asn19, Asn23, and Asn1006, and this information was useful in establishing that the NH2 terminus as well as the loop connecting TM12
Figure 1.4: An atomic homology model of MRP1 based on the crystal structure of Sav1866 from *S. aureus*.

The homology model of MRP1 shown was generated using the crystal structure of Sav1866 from *S. aureus* as template (DeGorter et al., 2008). MSD1, MSD2, NBD1, and NBD2 are coloured green, blue, yellow, and magenta, respectively. TM17 and the TM17-proximal region are coloured red and orange, respectively. The thick horizontal lines represent the putative location of the lipid bilayer. Note that MSD0 is absent from this homology model. When rotated 90° and viewed from the extracellular side, a putative translocation pathway can be observed. Images were generated using PyMOL v1.1.
and TM13 are extracellular (Figure 1.1) (Hipfner et al., 1997). In contrast, the extent and sites of phosphorylation are currently unknown, although preliminary evidence suggests that Ser and Thr, but not Tyr residues are phosphorylated (Ma et al., 1995; Almquist et al., 1995; Almquist, Loe, and Cole, unpublished). A recent inspection of the MRP1 protein sequence using current phosphorylation site prediction software (NetPhos 2.0, http://www.cbs.dtu.dk/services/NetPhos; KinasePhos 2.0, http://kinasephos2.mbc.nctu.edu.tw) indicates the presence of 40 possible serine phosphorylation sites, and 7 threonine sites.

Although electron microscopy structural data of MRP1 are of low resolution (~22 Å), they indicate that MRP1 contains a “pore” in the centre of the molecule (Rosenberg et al., 2001). Additionally, based on crystal structures of both the bacterial ABC transporter Sav1866 and murine P-gp, the core structure of MRP1 (i.e. MSD1-NBD1/MSD2-NBD2) is believed to be comprised of TM helices that are substantially twisted and somewhat flexible, allowing for each MSD to interact with the same and opposite NBD (DeGorter et al., 2008; Dawson and Locher, 2006; Aller et al., 2009). Twisting of the TMs is thought to promote communication between MSD1 and NBD1/2, as well as between MSD2 and NBD1/2, and thus allowing for substrate(s) to be appropriately accommodated during the various conformations adopted by MRP1 throughout the active transport process (Dawson and Locher, 2006; DeGorter et al., 2008).

1.7 MRP1 Transport Mechanism
The ATPase activity of ABC transporters including MRP1 requires three highly conserved motifs found within each NBD – the Walker A, Walker B, and ‘C’ active
transport signature motifs. The Walker A and Walker B motifs of NBD1, in cooperation with the ‘C’ motif of NBD2, form a composite nucleotide binding site (NBS) which acts as a pocket to bind and hydrolyze ATP, as well as release ADP/Pi (Dawson and Locher, 2006). Similarly, the Walker A and Walker B motifs of NBD2 interact with the ‘C’ signature motif from NBD1 to form a second NBS.

The NBDs of ABCC proteins, including MRP1, are unlike most other ABC transporters, since they are functionally and structurally non-equivalent (Deeley et al., 2006). In MRP1 (and presumably other MRPs which share similar distinctive structural features), NBD1 has a higher affinity for ATP than NBD2, while NBD2 has a higher hydrolytic activity (Gao et al., 2000; Hou et al., 2000). The ATPase activity of purified MRP1 is ~100-fold lower than that of some ABC transporters which have functionally equivalent NBDs (e.g. prokaryotic ABC transporters and mammalian P-gp) and the non-equivalency of the NBDs of MRP1 may contribute to this (Mao et al., 1999; Mao et al., 2000).

Using azido-derivatized nucleotides as photoactive cross-linking agents, it has been demonstrated that ATP binding by NBD1 of MRP1 can occur independently of NBD2 in vitro, while the binding of ATP by NBD2 appears dependent on the binding of ATP to NBD1 (Gao et al., 2000; Qin et al., 2008). A significant structural difference between NBD1 and NBD2 is the absence of 13 amino acids between the Walker A and ‘C’ signature motifs in NBD1 that are present in NBD1 of P-gp (Cole et al., 1992; Hung et al., 1998; Yuan et al., 2001). In addition, the higher hydrolytic activity of NBD2 has been attributed to the presence of a Glu residue immediately following the Walker B motif, which enhances the cleavage of the high energy β-γ phosphodiester bond of ATP,
while an Asp residue at this same position in NBD1 is believed to be responsible for the enhanced ATP binding (Qin et al., 2008). The latter has been suggested due to the fact that mutation of Glu to Asp in NBD2 significantly enhanced azido-ATP binding and decreased azido-ATP hydrolysis (Payen et al., 2003).

The current models of the transport cycle of MRP1 have many similarities regarding the steps which must occur for substrate translocation, but the order in which these events take place has not yet been established (Deeley et al., 2006; Rothnie et al., 2006). In one simplified model, substrate first binds to a high-affinity site(s) on the cytoplasmic side of MRP1 in its inward facing conformation, which in turn induces conformational changes that enhances the binding of ATP by NBS1, as it facilitates the formation of a ‘sandwich’ dimer with both NBDs (Figure 1.5) (Deeley et al., 2006; Szakács et al., 2008). The binding of a second molecule of ATP by NBS2 is facilitated after binding of ATP by NBS1, which alters the conformation of the NBD ‘sandwich’ dimer, thus signaling to the MSDs to ‘open’ the transporter ‘outward’ and decrease its affinity for its substrate. The release of ADP/Pi from one or both of the NBSs induces changes in the structure of MRP1, resulting in the resetting of the transporter to its original high-affinity conformation so that it may undergo another cycle of transport (Chang, 2007).

1.8 Atomic Homology Models of MRP1
X-ray crystallography, electron microscopy, and nuclear magnetic resonance imaging are currently the most common methods used to determine the structure of proteins, but these methods remain limited in their ability to elucidate the structure of large hydrophobic
Figure 1.5: A hypothetical transport cycle of MRP1-mediated substrate efflux.

In this model of substrate translocation: **1-2**, substrate is recognized by MRP1 and binds to an intracellular site, which in turn induces conformational changes that enhance the binding of ATP by NBS1 and NBS2; **3-5**, ATP binding and hydrolysis induce changes in the conformation of the NBD ‘sandwich’ dimer, and these changes are transmitted to the MSDs causing the transporter to ‘open’ and the affinity of MRP1 for substrate to decrease (substrate is subsequently released into the extracellular space), while release of ADP/Pi from one or both of the NBSs resets MRP1 back to its original high-affinity conformation so that it may undergo another cycle of transport. Modified from Deeley et al. (2006), DeGorter et al. (2008), and Aller et al. (2009).
mammalian proteins (Chayen and Saridakis, 2008; Rosenberg et al., 2001; Hollenstein et al., 2007). Thus, solved structures of membrane proteins currently represent <1% of all structures available in the Research Collaboratory for Structural Bioinformatics’ Protein Data Bank (Hurwitz et al., 2006; http://www.rcsb.org/pdb/home/home.do).

As mentioned before, existing electron microscopy structural data of MRP1 are of low resolution, and thus are unable to provide information on the precise arrangement of the TM helices and NBDs in the functional protein (Rosenberg et al., 2001; Higgins et al., 2004). Crystallization of mammalian MRP1 (and other human ABC proteins) poses a significant challenge, mainly due to their large size and polytopic nature, as well as the difficulty of isolating large amounts of purified active protein (Wu et al., 2005; Chayen and Saridakis, 2008; Dawson and Locher, 2006). For this reason, structural information on MRP1 and other human ABCs is primarily inferred from models based on solved structures of bacterial ABC transporters (Dawson and Locher, 2006).

The first atomic homology models of human ABC transporters were based on the crystal structures of the *E. coli* and *V. cholera* lipid A transporter MsbA (Chang et al., 2001; Chang, 2003a). MsbA is a homodimeric transporter with each monomer consisting of one MSD (containing six TMs), as well as one NBD (Chang, 2003a). In an initial study, two MsbA monomers constituting the active transporter were aligned to MSD1-NBD1 and MSD2-NBD2 of MRP1, and the 4-domain core structure of MRP1 was modeled using computational software (Campbell et al., 2004). However, in 2006 it became evident that the structures of the *E. coli* and *V. cholera* MsbA did not agree with the structure of another homologous bacterial ABC transporter from *S. aureus*, Sav1866 (Dawson and Locher, 2006). Errors in the structures of *E. coli* and *V. cholera* MsbA were
due in part to mistaken interpretations of the handedness, caused by inversions in the diffraction data, which affected the topology of both structures (Chang, 2003b). In light of these mistakes, the MsbA structural data were retracted, and the validity of MsbA-based models of human ABCs including MRP1 discredited (Miller, 2006). Structures of the homodimeric ABC transporter from S. aureus, Sav1866, in both ADP- and ATP-bound states have been solved at high resolutions (3.0 and 3.4 Å, respectively) (Dawson and Locher, 2006; 2007). Subsequently, the crystal structure of Sav1866 was used as a template to develop new atomic homology models of mammalian ABC proteins including MRP1 (DeGorter et al., 2008) and P-gp (Zolnerciks et al., 2007; O’Mara and Tieleman, 2007).

Recently, a crystal structure of nucleotide-free mouse P-gp has been reported to a resolution of 3.8 Å (Aller et al., 2009). In this study, P-gp was crystallized into identical forms in the presence and absence of two recently identified inhibitors, QZ59-SSS and QZ59-RRR (Aller et al., 2009). This is unexpected because it might have been anticipated that P-gp would be in different conformations when an inhibitor is bound or absent. Another unexpected observation was the estimated distance between the NBDs, which at ~30 Å represents a substantial amount of space for the NBDs to move through to come together to form a ‘sandwich’ dimer as required during the transport process. This large distance is energetically unfavorable and not easily explained. Indeed, a recent review commenting on the study by Aller et al. (2009) suggests that this form of P-gp is unlikely to be physiologically relevant (Gottesman et al., 2009). Nevertheless, the discovery of crystallization conditions by this study at least provides important new
information that may be useful to those investigating crystal structures of other ABC transporters, including MRP1.

In the meantime, as mentioned, the structure of Sav1866 has been used as a template to model the core 4-domain structure of MRP1 and other ABCs (e.g. P-gp, MRP4, MRP5) (O’Mara and Tieleman, 2007; El-Sheikh et al., 2008; Ravna et al., 2007; DeGorter et al., 2008). Each monomer of Sav1866 has moderate sequence similarity to MRP1, in that Sav1866 is approximately ~22% identical to each ‘half’ (MSD1-NBD1 and MSD2-NBD2) of MRP1, although most of this is in the NBDs (DeGorter et al., 2008). These Sav1866-based models of MRP1 were derived so that they could be used to guide the design and interpretation of biochemical studies (DeGorter et al., 2008). However, it should be remembered that these models represent only a ‘snapshot’ of just one (i.e. low-affinity state; nucleotide-bound, substrate-free) of the many conformations assumed by MRP1 (and other ABCCs) during the complex transport process, and thus models the positions of amino acids in only a single conformation.

1.9 MRP1 Substrate Specificity and Structure-Function Studies of MRP1

As described above in Sections 1.3 and 1.5, MRP1 mediates the transport of a wide range of structurally diverse conjugated and unconjugated organic anions, and drugs and toxins. Other MRPs share the ability to transport some of these organic anions, with MRP2 having a substrate transport profile most similar to MRP1, although the affinity of the two homologs for a given organic anion can differ substantially (Cole and Deeley, 2006). Extensive biochemical analyses have been very useful in identifying specific domains and amino acids that are important for the structure and function of these transporters.
Using site-directed mutagenesis, a large number of mutation-sensitive residues have been identified that are critical for overall transport activity, substrate specificity, and/or stable expression of MRP1 in the plasma membrane (Deeley and Cole, 2006). In addition, this approach has uncovered specific regions of MRP1 which are critical for helix packing (using ‘reciprocal’ or ‘double exchange’ mutagenesis) (Haimeur et al., unpublished), inter-domain communication (Koike et al., 2004; Conseil et al., 2009), and ATPase activity (Letourneau et al., 2007; Qin et al., 2008).

Some mutagenesis studies have been based on testing the assumption that amino acids conserved among MRP1 and its orthologs are likely to be functionally important. They have also targeted specific residues that have distinctive biophysical properties (e.g. polarity, ionizability, aromaticity, helix breaking) (Deeley and Cole 2006). For example, it has been shown that mutation of certain ionizable residues in TM6 (i.e. Lys$^{332}$, His$^{335}$) causes substrate selective changes, such that LTC$_4$ transport is eliminated or reduced while E$_2$17βG transport remains intact (Haimeur et al., 2002). In contrast, mutation of certain polar or aromatic residues in TM17 (i.e. Thr$^{1242}$, Tyr$^{1243}$, Asn$^{1245}$, Trp$^{1246}$) mainly reduces just E$_2$17βG transport, and not LTC$_4$ (Ito et al., 2001; Zhang et al., 2001, 2002). Consistent with the importance of residues in TM6 and TM17 for substrate specificity, Bao et al. (2005) reported that LTC$_4$ transport was eliminated by poly-Ala substitution of TM6, whereas LTC$_4$ transport was not affected by poly-Ala substitution of TM17. Unfortunately, this study did not determine the transport of substrates other than LTC$_4$, and so it is not clear how the TM helix substitution will affect the transport of other substrates by MRP1.
Using a combination of photolabeling and mass spectrometry techniques, Wu et al. (2005) identified regions of MRP1 that interact with LTC4. Thus, after photolabeling purified MRP1 with LTC4, MALDI-TOF mass spectrometry identified short sequences in TM6, TM7, TM10, TM17, and part of CL3 as regions of modification by LTC4 (Wu et al., 2005). These results are somewhat inconsistent with previous site-directed mutagenesis studies, in that, for example, residues in TM17 have been shown to be less important for LTC4 transport (Ito et al., 2001a; Zhang et al., 2001; Zhang et al., 2002). Further studies involving tandem mass spectrometry (i.e. MS/MS) are needed to identify the specific residues that interact with LTC4, and whether they differ at the different stages of the transport cycle of MRP1.

1.10 Rationale, Hypotheses, and Objectives

As described above, previous studies of MRP1 have demonstrated that many charged, aromatic, and polar residues within the TMs of MSD1 and MSD2 are mutation sensitive, and thus may contribute directly or indirectly to one or more of the substrate binding sites, proper folding of the protein, and/or the ATPase activity of this transporter. Therefore, amino acids in the core structure of MRP1 play multiple roles in structure and function.

The COOH-terminal TM of MRP1, TM17 (which we have defined here as residues 1228-1248), is very amphipathic (Ito et al., 2001). Substrate selective transport and/or binding activities become apparent when certain TM17 residues with aromatic or polar properties are replaced (Ito et al., 2001a; 2001b; Zhang et al., 2001, 2002; Situ et al., 2004; Ren et al., 2002). On the other hand, both LTC4 and E217βG transport were eliminated when the TM17-proximal residue Arg1249 was mutated (including replacement
with the same charge amino acid Lys) (Ren et al., 2002; Situ et al., 2004). This adjacent Arg1249 may affect overall activity by affecting structure rather than just a substrate binding site. Thus, Arg1249 may assist in anchoring this TM within the plasma membrane by interacting with the negatively charged head groups of the phospholipids in the inner leaflet of the bilayer, and possibly by forming a cation-π bond with nearby Trp1246. Thus, the role of residues in the intracellular α-helical extension of TM17 may differ from residues within TM17 itself, in that residues in TM17 may be part of a substrate binding site, while the TM17-proximal region may be more involved in overall MRP1 structure and thus overall function.

Accordingly, we first hypothesized that the helical extension of TM17 (residues 1249-1269), which is predicted to be located in the cytoplasm where it continues into NBD2, is important for MRP1 structure and function. To test this hypothesis, four highly conserved acidic residues (Glu1253, Glu1255, Glu1262, and Glu1266) and three other highly conserved residues (Val1261, Arg1263, and Tyr1267) in this region of MRP1 were chosen for investigation by site-directed mutagenesis (Figures 1.1 and 1.6).

Current homology models suggest that the TM17-proximal region of MRP1 may be in close proximity to the cytoplasmic helical extensions from TM14 and TM15, raising the possibility of interhelical bonding interactions between the side-chains of these helices with those of the TM17-proximal region. Consequently, we also hypothesized that such interhelical side-chain interactions may play a role in the stable expression and/or function of MRP1. To test this hypothesis, reciprocal (exchange) mutations of selected amino acids in the TM17-proximal region with amino acids in the cytoplasmic extensions of TM14 or TM15 were generated and functionally characterized.
Figure 1.6: Helical projection of amino acids in the TM17-proximal region.

The TM17-proximal region (residues 1249-1269) is shown as a linear sequence and as a helical projection. The helical projection, or helical ‘wheel’, begins at Arg$^{1249}$ and continues as a right-handed $\alpha$-helix ‘down’ the z-axis ‘into’ the page. Non-polar, polar uncharged, acidic, and basic residues are coloured orange, green, pink, and blue, respectively. This image was generated using the Helical Wheel Applet (http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html) and Adobe Photoshop v5.0.
Thus, to test the hypotheses that (i) conserved residues found in the TM17-proximal region of MRP1 are important for its expression and/or function, and (ii) at least some of these residues participate in bonding interactions with the side-chains of residues in the cytoplasmic extensions of TM14 and/or TM15, the following objectives were pursued:

1. Glu residues at positions 1253, 1255, 1262 and 1266, as well as Val^{1261}, Arg^{1263} and Tyr^{1267}, were replaced with Ala, and the effects on MRP1 expression in HEK293T cells and transport activity in inside-out membrane vesicles were determined.

2. For those Glu mutants exhibiting an altered phenotype, same charge and opposite charge mutations were also created to determine whether the charge or other characteristics (e.g. side-chain volume) of the Glu residue were important for MRP1 function.

3. Based on in silico analyses of a homology model of MRP1 which identified potential interhelical bonding partners of the above seven conserved residues, single and double reciprocal mutants of two pairs of amino acids, Glu^{1253} and Lys^{1141}, and Glu^{1262} and Arg^{1142}, were created, and MRP1 expression and transport activity determined as before.

In this thesis, the materials and methods used for all of the studies listed in the objectives above are described in Chapter II. The results obtained from the characterization of the four Glu mutants, and the Glu^{1253}/Lys^{1141} and Glu^{1262}/Arg^{1142} double exchange mutants are presented and discussed in Chapter III, while the results obtained from the characterization of the Val^{1261}, Arg^{1263} and Tyr^{1267} single mutants are
presented and discussed in Chapter IV. Finally, Chapter V includes an overall discussion of the results described in Chapters III and IV, and proposes future directions of research.
CHAPTER II: MATERIALS AND METHODS

2.1 Materials

[14,15,19,20-\textsuperscript{3}H(n)]LTC\textsubscript{4} (160 Ci mmol\textsuperscript{-1}), [6,7-\textsuperscript{3}H(n)]E\textsubscript{2}17\beta G (45 Ci mmol\textsuperscript{-1}), and Western Lightning Plus-Enhanced Chemiluminescence (ECL) blotting substrate were from Perkin Elmer Life Sciences (Woodbridge, ON). E\textsubscript{2}17\beta G, AMP, ATP, benzamidine, bovine serum albumin (BSA), Dulbecco’s Modified Eagle’s Medium (DMEM), GenElute\textsuperscript{TM} Plasmid Miniprep kit, Kodak X-Omat LS film for autoradiography, and 2-mercaptoethanol were from Sigma-Aldrich (Oakville, ON). Creatine kinase, creatine phosphate, and protease inhibitors (EDTA-free) were from Roche (Mississauga, ON). LTC\textsubscript{4} was purchased from Calbiochem (La Jolla, CA) and Amplify\textsuperscript{TM} was from GE Healthcare (formerly Amersham; Milwaukee, WI). Film for immunoblots was purchased from Ultident Inc. (St. Laurent, QC) and Bradford protein assay kit was from Bio-Rad Laboratories (Hercules, CA). Fetal bovine serum was purchased from Gibco-Invitrogen (Grand Island, NY). Lipofectamine 2000\textsuperscript{TM} transfection reagent was from Invitrogen (Burlington, ON). Mouse anti-human MRP1 MAb QCRL-1 was previously generated by Hipfner \textit{et al.} (1994) and its epitope determined as MRP1 amino acids 918-924 (Hipfner \textit{et al.}, 1996). Rabbit anti-Na\textsuperscript{+}/K\textsuperscript{+} ATPase antibody (sc-28800) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies were from Pierce Biotechnology (Rockford, IL). PageRuler Plus protein ladder was from Fermentas International Inc. (Burlington, ON) and polyvinylidene fluoride (PVDF) membranes were from Pall Corporation (East Hills, NY).
2.2 Secondary Structure Predictions

Algorithms used for secondary structure predictions of MRP1 included DSC, MLRC, PHD, and Predator (default parameters, performed May 2008), and can be found at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html.

2.3 Sequence Alignments and In Silico Illustrations

ClustalW 1.0 (default parameters, performed May 2008; http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) was used to perform sequence alignments. PyMOL 1.1 (DeLano Scientific, Palo Alto, CA; http://www.pymol.org) was used to view the homology model of MRP1 (DeGorter et al., 2008), and to estimate distances between the side-chains of amino acids.

2.4 Vector Construction and Site-directed Mutagenesis

The template for mutagenesis was prepared by cloning a 2.0 kb XmaI fragment from pcDNA3.1(−)-MRP1K (containing nucleotides 2337-4322 of the MRP1 sequence encoding amino acids 780-1440) into pGEM3Z (Promega, Madison, WI) (Ito et al., 2001a). Mutations were generated using the following sense primers, with wild-type MRP1 pGEM3Z plasmid (nucleotides 2337-4322) as template (Integrated DNA Technologies Inc., Coralville, IA) (substituted nucleotides are underlined, and silent mutations used to introduce restriction sites to facilitate characterization are italicized):

E1253A, 5’-CGG ATG TCA TCT GCC ATG GAA ACC-3’; E1253D, 5’-CGG ATG TCA TCT GAC ATG GAA ACC-3’; E1253K, 5’-CGG ATG TCA TCT GAA ATG GAA ACC-3’; E1255A, 5’-CTG GAA ATG GCC ACC AAC ATC GTG-3’; V1261A,
5'-C ATC GTG GCC GCG GAG AGG CTC-3'; E1262A, 5'-C GTG GCC GTG GCC AGG CTC AAG G-3'; E1262D, 5'-GTG GCC GTG GAT AGG CTC AAG GAG-3'; E(D)1262E, 5'-GTG GCC GTG GAG AGG CTC AAG GAG-3'; E1262R, 5'-GTG GCC GTG AGA AGG CTC AAG GAG-3'; E1266A, 5'-G GCC GTG GA GC G CTC AAG GAG-3'; Y1267A, 5'-GG CTC AAG GA GC T TCA GAG ACT GAG-3'. Generation of MRP1 mutants K1141E and R1142E has been previously described (Conseil et al., 2006). Sense primers included: K1141E, 5'-CGG CAG CTG GAG CGC CT GAG TCG GTG AGC-3'; R1142E, 5'-CCC GGC AGC TGA AGG AG C TCG AGT CGG TCA GCC G-3'. K1141E/E1253K and R1142E/E1262R double mutant constructs were generated by site-directed mutagenesis using E1253K and E1262R sense/antisense primers, with K1141E and R1142E mutant constructs as template, respectively.

Mutations were generated using polymerase chain reaction (PCR). PCR was performed in a PTC-100 Programmable Thermo Controller (MJ Research Inc., Watertown, MA), and for each substitution the following components were used: 1 µl (100 ng) of MRP1 pGEM3Z plasmid DNA (nucleotides 2337-4322) as template, 1.25 µl (125 ng) of both sense and antisense mutant DNA primers, 5 µl of 2.5 mM dNTPs, 1 µl (2.5U) of *Pfu* Turbo reaction buffer (Stratagene, La Jolla, CA), 4 µl of 2.5 mM dNTPs, 1 µl (2.5U) of *Pfu* Turbo DNA polymerase (Stratagene), and 36.5 µl of Milli-Q water (total reaction volume of 50 µl). PCR was carried out under the following conditions: step 1, 94 °C for 45 sec; step 2, (primer $T_{melt}$)-5 °C for 1 min, step 3, 72 °C for 10 min; step 4, repeat steps
1-3 for 18 cycles. Reactions were stopped by incubating tubes at 4 °C, and then 1 µl (10U) of DpnI was added to each reaction tube for 60 min at 37 °C to remove methylated parental template DNA. XL1-Blue competent (E. coli) cells (50 µl) were transformed with MRP1 mutant constructs (40 µl PCR volume) by first using a 30 min incubation at 4 °C, followed by a 90 sec ‘heat shock’ at 42 °C. Transformed cells were then incubated at 4 °C for 2 min, shaken at 37 °C for 30 min, plated on LB-Ampicillin selective agar plates, and then incubated at 37 °C overnight. Colonies were picked after 16-18 h, and plasmid DNA was prepared using the GenElute™ Plasmid Miniprep kit (Sigma).

The presence of Ala substitutions in the pGEM3Z plasmids was initially confirmed by appropriate digests using NcoI, MscI, SacII, MscI, Eco47I, and HindIII for E1253A, E1255A, V1261A, E1262A, R1263A and Y1267A mutant constructs, respectively. The presence of mutation(s) in pGEM3Z constructs, including those already determined by diagnostic digest, were further confirmed by DNA sequencing (TCAG Inc., Toronto, ON). A 1.5 kb BsmBI/ClaI-fragment containing each mutation(s) in pGEM3Z was subcloned back into the pcDNA3.1(−)-MRP1k expression vector, and this 1.5 kb fragment was again sequenced to confirm the integrity of the cloned region.

2.5 Cell Culture

HEK293T cells (ATCC CRL-11268) were maintained in DMEM supplemented with 4 mM L-glutamine and 7.5% FBS in 175-cm² flasks by Kathy Sparks. All cultures were grown at 37 °C in 5% CO₂/95% air in a Series II water-jacketed CO₂ incubator with HEPA filtration (Thermo Scientific, Waltham, MA).
2.6 Transfection of Human Embryonic Kidney Cells

Transient transfections were performed as previously described, with small modifications to cell seeding density and the transfection reagent used (Ito et al., 2001a). Briefly, wild-type pcDNA3.1(−)-MRP1K and mutant MRP1 expression vectors were transfected into HEK293T cells that had been seeded at 20 x 10^6 cells per 20 ml in 150 mm plates. Twenty-four h later (at 80-90% confluence), MRP1 cDNA expression vectors (20 μg in 40 μl, 260/280 nm ratio ~1.8), purified using GenElute™ Plasmid Miniprep kits (Sigma), were incubated with 75 μl Lipofectamine 2000™ reagent (Invitrogen) at a ratio of 1:3.75 (w:v) in 4 ml DMEM for 30 min, and then added to the cells. Six h later, the media was replaced with fresh DMEM/7.5% FBS. After 48 h, cells were pelleted by centrifugation at 650 x g for 5 min at 4°C, washed with 10 ml homogenization buffer consisting of 250 mM sucrose/50 mM Tris pH 7.4/0.25 mM CaCl2, 10 μl benzamidine (200 mg/ml) and protease inhibitors, and then cells were collected by centrifugation at 800 x g for 5 min at 4°C. Collected cell pellets were layered with 10 ml homogenization buffer consisting of 250 mM sucrose/50 mM Tris pH 7.4/0.25 mM CaCl2, 10 μl benzamidine (200 mg/ml), and protease inhibitors, and kept at -80°C until required for membrane vesicle preparation (Section 2.7).

2.7 Preparation of Membrane Vesicles from MRP1 Transfectants

Membrane vesicles were prepared from transfected HEK293T cells as previously described (Loe et al., 1996a; Letourneau et al., 2005). Briefly, cell pellets were thawed in warm water for 5 min, and while on ice, resuspended in homogenization buffer (described in Section 2.6). Cells were exploded by argon cavitation (300 psi, 4°C, 5 min).
and the exploded cell suspension was centrifuged at 800 x g at 4 °C for 10 min in an IEC CentraGP8R centrifuge (DJB Labcare Ltd., Buckinghamshire, UK). The supernatant (15 ml) was layered onto an equal volume of a 35% (w/v) sucrose/1 mM EDTA/50 mM Tris, pH 7.4 cushion. After centrifugation at 100,000 x g at 4 °C for 60 min in an Optima™ L-90K Ultracentrifuge (Beckman Coulter, Fullerton, CA), the interface was removed and placed in a 25 mM sucrose/50 mM Tris, pH 7.4 buffer solution and centrifuged at 100,000 x g at 4 °C for 30 min again. The membranes were washed with 1 ml Tris-Sucrose Buffer (TSB) (250 mM sucrose, 50 mM Tris, pH 7.4), and centrifuged at 68,000 x g at 4 °C for 20 min in an TL-100 Ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet was resuspended in 50-100 μl TSB by passage through a 1 ml syringe 12 times with a 27-gauge needle, aliquoted (14 μg protein per aliquot), and stored at -80°C until required. Vesicular protein concentrations were determined using the Bradford protein assay (Bio-Rad) and a standard curve prepared with BSA.

2.8 Determination of MRP1 Protein Levels in Transfected Cells

The expression levels of wild-type and MRP1 mutants (and untransfected HEK293T controls) were determined by immunoblot analysis of membrane vesicle proteins from transfected cells as described previously (Ito et al., 2001a). Proteins were loaded onto a 1.5 mm thick SDS-polyacrylamide gel (7% acrylamide resolving, 4% acrylamide stacking) with a 15-well comb using Laemmli buffer containing 14 mM 2-mercaptoethanol, separated by electrophoresis at 75-150 V for 1.5-2 h, and then electrotransferred (100 V, 1 h) to a PVDF membrane using a BioRad Mini-Gel apparatus. The PVDF membrane was washed (3 times, 5 min per wash) at room temperature with
TBS-T, and blocked with 4% (w/v) skim milk in TBS-T for 30 min at room temperature, followed by incubation with the human MRP1-specific murine MAb QCRL-1 (1:10000 in 4% skim milk/TBS-T, 4 °C, overnight) (Hipfner et al., 1996). Immunoblots were washed (3 times, 5 min per wash) with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10000 in 4% skim milk/TBS-T, room temperature, 1 h). Blots were washed (5 times, 5 min per wash) with TBS-T, followed by application of equal volumes (1.5 ml each) of Western Lightning Plus-Enhanced Chemiluminescence (ECL) blotting substrates, and then the membrane was exposed to film within 15 min of ECL addition, for 1-5 s exposures. Relative levels of MRP1 expression were measured by densitometric analysis of the blots using ImageJ 1.36b software (http://rsb.info.nih.gov/ij/). Binding of rabbit anti-Na+/K+ ATPase (diluted 1:5000 in 4% skim milk/TBS-T, and incubated at 4 °C overnight) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:10000 in 4% skim milk/TBS-T at room temperature for 1 h) was used to control for protein loading. Washes and ECL substrate were applied as described for MRP1 protein detection. After densitometry of the Na+/K+ ATPase bands, relative MRP1 expression levels were determined to take into account differences in total protein loaded.

2.9 MRP1-mediated Transport of ³H-Labeled Substrates by Membrane Vesicles

ATP-dependent vesicular transport of ³H-labeled substrates by membrane vesicles was measured using a microplate rapid filtration technique as previously described (Letourneau et al., 2005), with the following modification: 20 nCi of [³H]LTC₄ was used per reaction instead of 10 nCi. For LTC₄ transport assays, reactions were performed in
96-well round bottom plates (Sarstedt, Newton, NC) at 23 °C (water bath) in a 30 µl reaction volume containing 50 nM [3H]LTC4 (20 nCi), 4 mM AMP or ATP, 10 mM MgCl2, 10 mM creatine phosphate, 100 µg/ml creatine kinase, and 2 µg of vesicle protein. Creatine phosphate and creatine kinase were omitted from reactions containing AMP, and volumes were made up with TSB. Reaction mixtures (24 µl) and membrane vesicles (2 µg in 6 µl) were allowed to acclimate (3 min) to the reaction temperature separately prior to transport. Uptake was stopped after 1 min by addition to a Dynablock 2000™ 96-deep-well plate (VWR International, Mississauga, ON) containing ice-cold TSB (800 µl), and the diluted incubation mixture was filtered through a UniFilter GF/B 96-well filter plate (Packard, Meriden, CT). Radioactivity was quantified using liquid scintillation counting by a TopCountNXT™ Microplate Scintillation and Luminescence Counter (Perkin-Elmer). Transport in the presence of AMP was subtracted from transport in the presence of ATP to determine ATP-dependent [3H]LTC4 uptake. Uptake of E217βG was similarly measured, except that membrane vesicles (2 µg of protein) were incubated at 37 °C (water bath) in a total reaction volume of 30 µl containing [3H]E217βG (400 nM; 20 nCi) and components as described for [3H]LTC4 transport.

2.10 Kinetic Analysis of [3H]E217βG Transport

Kinetic analyses were performed as previously described (Letourneau et al., 2005), with some modifications. \(K_m\) and \(V_{max}\) values for [3H]E217βG transport were determined by measuring uptake at eight different E217βG concentrations (0.25-25 µM) and either 2 or 4 µg protein for 1 min at 37 °C in a 30 µl reaction volume of transport buffer containing components as described above. For the three highest E217βG concentrations (5, 10, 25
µM), 25 nCi [3H]E217βG was used. Data were analyzed, and kinetic parameters were calculated by nonlinear regression using GraphPad Prism™ 3.0 (GraphPad Software, La Jolla, CA).

2.11 Photolabeling of MRP1 by [3H]LTC4

Photolabeling of MRP1 was performed as previously described (Koike et al., 2004, Letourneau et al., 2008). Briefly, membrane vesicles (50 µg protein in 40 µl TSB) were incubated with [3H]LTC4 (200 nM; 100 nCi) in a 96-well flat bottom flexible plate (BD Biosciences, Durham, NC) at room temperature for 30 min and then snap-frozen by placing into liquid nitrogen for 10 sec. Membrane vesicles (1.5 µg protein) were retained prior to cross-linking, and immunoblotted to quantify relative MRP1 expression. [3H]LTC4 was cross-linked to MRP1 by irradiating (1100 µW, 302 nm) for ten 1 min exposures using a CL-1000 Ultraviolet Crosslinker (DiaMed, Mississauga, ON), with snap-freezing in liquid nitrogen for 10 sec between exposures. Radiolabeled proteins (40 µg) were loaded on a 1.5 mm thick SDS-polyacrylamide gel (7% acrylamide resolving, 4% acrylamide stacking) with a 10-well comb using Laemmli buffer containing 14 nM 2-mercaptoethanol, and separated by electrophoresis at 75-150 V (1.5-2 h). The gel was processed for autoradiography by first incubating in a fixing solution (65:25:10; water, isopropanol, acetic acid) for 30 min, and then incubation in Amplify™ solution for 30 min. The gel was dried on a Model 583 Gel Dryer (Bio-Rad) in a vacuum at 80 °C for 2-4 h. After drying, the gel was exposed to Kodak X-Omat LS autoradiography film at -80 °C for 5 days or longer, as required. Cassettes containing film were allowed to
acclimate to room temperature (3 h) prior to processing. Signal intensities on the film were analyzed by densitometry using ImageJ 1.36b software as described above.

### 2.12 Statistical Analyses

Differences in $[^3\text{H}]E_217\beta\text{G}$ and $[^3\text{H}]\text{LTC}_4$ transport activities of wild-type and mutant MRP1 were analyzed for statistical significance using a 2-tailed Student’s t-test (GraphPad Prism 3.0). Differences were considered significant when $p < 0.05$. 
CHAPTER III: FUNCTIONAL ANALYSIS OF
GLU\textsuperscript{1253}, GLU\textsuperscript{1255}, GLU\textsuperscript{1262} AND GLU\textsuperscript{1266} MRP1 MUTANTS

3.1 Introduction

Ionizable amino acids (Glu, Asp, Arg, and Lys) are present within internal regions of hydrophobic proteins as a result of evolutionary processes (e.g. variation and selection), and it appears that these amino acids randomly accumulated (through mutations) within the protein’s enzymatic or active site, in order to develop the ability to perform a specific task (Isom et al., 2008). This suggests that ionizable amino acids are likely to have an important role in modulating the function of such proteins. Consistent with this notion, previous studies on MRP1 have demonstrated that many ionizable amino acids are sensitive to mutation when present in or proximal to TMs of the core structure (MSD1 and MSD2) of the transporter (Deeley and Cole, 2006; Conseil et al., 2007, 2009; Maeno et al., 2009). For example, it has been shown that conservative and non-conservative substitutions of Lys\textsuperscript{332} and His\textsuperscript{335} in TM6 cause substrate selective changes in activity, such that LTC\textsubscript{4} transport is eliminated or reduced while E\textsubscript{2}17\betaG transport remains intact (Haimeur et al., 2002, 2004). On the other hand, even conservative mutations of Asp\textsuperscript{336} in TM6, and Arg\textsuperscript{1249} at the TM17-cytoplasmic interface completely eliminate activity (Haimeur et al., 2004; Situ et al., 2004). Non-conservative mutation of another ionizable amino acid, Asp\textsuperscript{430} in the cytoplasmic loop connecting TM7 to TM8, sharply reduces expression of MRP1 (Haimeur et al., 2004).

In the present study, acidic amino acids were identified in the TM17-proximal region of MRP1, and their role in expression and transport investigated (Figure 3.1).
Figure 3.1: Secondary structure, and *in silico* illustration of the TM17-proximal region of MRP1.

**A**, a secondary structure of MRP1 showing the predicted locations of TM17 (black cylinder) and its COOH-proximal region (black star), defined in this study as amino acids 1228-1248 and 1249-1269, respectively. Key conserved residues are shown in bold. **B**, the predicted locations of TM17 (dark gray) and its COOH-proximal region (black) spanning amino acids 1228 to 1269 are shown in the three-dimensional homology model of MRP1 (lacking MSD0) generated using the crystal structure of Sav1866 from *S. aureus* as template (DeGorter *et al.*, 2008). The lipid bilayer is represented by dotted lines.
Because of its proximity to the functionally important TM17, and because it links TM17 to NBD2, it was hypothesized that ionizable residues in this region could be important for MRP1 function. In addition, since current Sav1866-based models of MRP1 predict that this α-helical region extending from TM17 is likely to participate in interhelical interactions, it was further hypothesized that amino acids in the TM17-proximal helix might form electrostatic interactions with residues extending from TM14 and TM15. In this chapter, the results of experiments designed to test these hypotheses are described.

3.2 Results

3.2.1 Secondary Structure Predictions, Sequence Alignments, and In Silico Illustrations of the region COOH-proximal to TM17

To begin this study (and those described in Chapter IV), several algorithms (DSC, MLRC, PHD, Predator) were used to determine a consensus secondary structure of the region COOH-proximal to TM17 (defined in this study as amino acids 1249-1269) (Figure 3.1) as described in Chapter II (Section 2.2). The consensus structure obtained identifies residues 1249-1269 as being α-helical in nature, which is in agreement with the Sav1866-based homology model of the core structure of MRP1 (DeGorter et al., 2008) (Figure 3.2A). Next, a multiple sequence alignment of TM17 and the COOH-proximal region of MRP1 (residues 1228-1269) with the sequences of its eleven mammalian homologs was performed using ClustalW v1.0 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). As illustrated in Figure 3.2B, TM17 and its COOH-proximal region are well conserved, and four acidic residues (Glu1253, Glu1255, Glu1262, and Glu1266) within the COOH-proximal region exhibit a high
Figure 3.2: Structural predictions, and sequence alignments of the TM17-proximal region of MRP1.

- h, helical;  
- c, coiled;  
- e, extension.

B, sequence alignment of human MRP1 (residues 1227-1269) and its eleven homologs, as well as the sequence of Sav1866, generated using ClustalW 1.0.

Amino acids which are identical in a majority of homologs are shown on a black background, while those which are conserved are shown on a gray background. G1228 and V1248 define the boundaries of TM17, while residues E1253, E1255, E1262, and E1266 are of key interest in this study.  

SUR, sulfonylurea receptor; CFTR, cystic fibrosis transmembrane conductance regulator.
degree of sequence identity and/or similarity (Glu$^{1253}$, >90% similarity; Glu$^{1255}$, >80% identity, >90% similarity; Glu$^{1262}$, 75% identity, 75% similarity; and Glu$^{1266}$, >55% similarity).

As described in Chapter I (Section 1.6), atomic homology models of the four-domain core structure of nucleotide-bound MRP1 (MRP1.B99990092.pdb) were recently generated in our lab using the crystal structure of Sav1866 from *S. aureus* as template (DeGorter *et al.*, 2008). Using a model based on a multiple sequence alignment of MRP1 homologs, the locations and side-chain positions of the four conserved Glu residues relative to a putative substrate translocation pathway formed by MSD1 and MSD2 were examined. According to this model, the side-chain of Glu$^{1253}$ projects into the putative translocation pathway, while the side-chains of Glu$^{1255}$, Glu$^{1262}$, and Glu$^{1266}$ project away from it (Figure 3.3).

When the potential for interhelical electrostatic, H-bond, and hydrophobic interactions between TM17-proximal amino acids and possible partner residues in adjacent α-helices was examined, four amino acids complementary (i.e. oppositely charged or polar, and capable of forming electrostatic and hydrogen bonds) to the side-chains of the four Glu residues were found, with each pair estimated to be within 3.4-9.7 Å of each other’s side-chains (discussed below in Section 3.3) (Table 3.1). Thus, the four pairs of potentially interacting amino acids identified were: Glu$^{1253}$ and Lys$^{1141}$, Glu$^{1255}$ and Arg$^{1138}$, Glu$^{1262}$ and Arg$^{1142}$ and Glu$^{1266}$ and Arg$^{1075}$ (Figure 3.4), of which two were investigated experimentally as described below (Section 3.2.6).
Figure 3.3: Putative relative locations and side-chain positions of Glu$^{1253}$, Glu$^{1255}$, Glu$^{1262}$ and Glu$^{1266}$ in a homology model of MRP1.

The three-dimensional homology model of MRP1 (lacking MSD0) of DeGorter et al. (2008) described in Chapter I (Section 1.6) showing the predicted location of TM17-proximal region (dark gray), as well as the predicted locations and side-chain positions of Glu$^{1253}$, Glu$^{1255}$, Glu$^{1262}$ and Glu$^{1266}$ (in black) (amino acids are shown as ‘spheres’ in the full model, and as ‘sticks’ in the inset). E1253 projects into the translocation pathway, while E1255, E1262, and E1266 project away from it.
<table>
<thead>
<tr>
<th>TM17-Proximal Residue</th>
<th>Potential Partner Residue (Position in MRP1)</th>
<th>Predicted Distance Between Closest Side-Chain Atoms (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1253</td>
<td>K1141 (TM15-proximal)</td>
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</tr>
<tr>
<td>E1255</td>
<td>R1138 (TM15-proximal)</td>
<td>6.1</td>
</tr>
<tr>
<td>E1262</td>
<td>R1142 (TM15-proximal)</td>
<td>3.4</td>
</tr>
<tr>
<td>E1266</td>
<td>R1075 (TM14-proximal)</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 3.1: Estimated distances between side-chains of Glu$^{1253}$, Glu$^{1255}$, Glu$^{1262}$ and Glu$^{1266}$, and other amino acids which potentially form interhelical bonds in MRP1.

Predicted distances between the four conserved Glu residues in the TM17-proximal helix and complementary amino acids in the TM14- and TM15-proximal helices were estimated from a Sav1866-based atomic homology model of MRP1 (DeGorter et al., 2008), as described in the text.
Figure 3.4: *In silico* illustrations of potential paired interhelical interactions involving side-chains of Glu$^{1253}$ and Lys$^{1141}$, Glu$^{1255}$ and Arg$^{1138}$, Glu$^{1262}$ and Arg$^{1142}$, and Glu$^{1266}$ and Arg$^{1075}$.

Using a homology model of MRP1 (lacking MSD0) (DeGorter *et al.*, 2008), the predicted locations of TM17-proximal residues (black) and their potential interhelical partners (dark gray) are shown. Also shown are potential electrostatic interactions between Glu$^{1253}$ and Lys$^{1141}$ (*A*), Glu$^{1255}$ and Arg$^{1138}$ (*B*), Glu$^{1262}$ and Arg$^{1142}$ (*C*), and Glu$^{1266}$ and Arg$^{1075}$ (*D*). Dotted lines represent distances between side-chains, and these values are listed in Table 3.1.
3.2.2 Expression, and E$_2$17βG and LTC$_4$ Transport Activities of Ala-Substituted Glu$^{1253}$, Glu$^{1255}$, Glu$^{1262}$, and Glu$^{1266}$ MRP1 Mutant Proteins

To begin analysis of the functional importance of these four Glu residues, single Ala-substituted mutant constructs were created by site-directed mutagenesis and transfected into HEK293T cells. Immunoblot analyses, first of whole cell lysates to confirm transfection efficiency (not shown), and subsequently, of membrane vesicles prepared from the transfected HEK293T cells were performed. Wild-type and mutant MRP1 proteins were detected using the human MRP1-specific MAb (QCRL-1) (Hipfner et al., 1994; Ito et al., 2001a). As shown in Figure 3.5A, all four mutants were expressed at levels comparable to wild-type MRP1 in membrane vesicles, demonstrating that Ala-substitution of these residues does not affect the biosynthesis of MRP1.

Next, the ability of the four Ala-substituted Glu MRP1 mutants to mediate ATP-dependent transport of E$_2$17βG and LTC$_4$ was examined using a microplate in vitro vesicular uptake assay (Loe et al., 1996; Letourneau et al., 2005). As shown in Figure 3.5B&C, the E1253A MRP1 mutant showed a statistically significant loss of both E$_2$17βG and LTC$_4$ transport activity (p < 0.05). However, the loss of E$_2$17βG transport (by 75%) was substantially more than the loss of LTC$_4$ transport (by 30%), suggesting some substrate selective effects of this mutation. The E1262A mutant also showed a statistically significant loss of both E$_2$17βG and LTC$_4$ transport activities (p < 0.05); however, the loss of LTC$_4$ transport (by 75%) was greater than the loss of E$_2$17βG transport (by 50%). In contrast to E1253A and E1262A LTC$_4$ transport activities, LTC$_4$ transport by E1255A was similar to that of wild-type MRP1, with only a small but significant (p < 0.05) increase in E$_2$17βG uptake (by 20%), while LTC$_4$ transport by
Figure 3.5: Expression levels and vesicular uptake of $^3$H-labeled organic anions by Ala-substituted Glu$^{1253}$, Glu$^{1255}$, Glu$^{1262}$ and Glu$^{1266}$ MRP1 mutants.

A. Shown is a representative immunoblot of membrane vesicles (0.5 and 1.0 μg protein) prepared from HEK293T cells transfected with E1253A, E1255A, E1262A, E1266A, and wild-type (WT) MRP1 cDNA expression vectors. Untransfected cells were used as a negative control (control). MRP1 was detected with MAb QCRL-1, and the relative (corrected based on Na$^+$/K$^+$ ATPase loading controls) protein expression levels are shown below the blot and were determined by densitometry as described in Chapter II (Section 2.8). B and C, ATP-dependent uptake of $[^{3}\text{H}]$E217βG (B) and $[^{3}\text{H}]$LTC$_4$ (C) was measured in the membrane vesicles shown in panel A. Vesicles prepared from untransfected cells were used as a negative control (control). Uptake values were normalized based on mutant MRP1 levels relative to WT-MRP1 levels (according to panel A), and uptake by the mutants was expressed as a percent of uptake by WT-MRP1. The results shown are the means (+S.D.) of three or more independent experiments; n values are shown above each bar. Similar results were obtained in at least one additional experiment using vesicles prepared from an independent transfection. MV, membrane vesicles; *, significantly different from wild-type MRP1 activity (p < 0.05).
A

<table>
<thead>
<tr>
<th>MV (μg)</th>
<th>Control</th>
<th>WT-MRP1</th>
<th>E1253A</th>
<th>E1255A</th>
<th>E1262A</th>
<th>E1266A</th>
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</table>

Relative expression (corrected)

B

E217/G Uptake (% WT-MRP1)

C

LTC4 Uptake (% WT-MRP1)
the E1266A mutant was slightly (20%) but significantly decreased (p < 0.05) and E$_{217}$βG uptake was comparable to wild-type MRP1. Since the changes in the transport activities of the E1255A and E1266A mutants were so small as to be unlikely biochemically significant, these mutants were not investigated further.

3.2.3 Expression and Transport Activities of MRP1 Mutants with Same- and Opposite-Charge Substitutions of Glu$_{1253}$ and Glu$_{1262}$

To determine if it is the negative charge at positions 1253 and 1262 that is important for supporting MRP1 transport, and if a larger positively charged side-chain would cause a greater disruption of transport activity, same and opposite charge substitutions of Glu$_{1253}$ (E1253D and E1253K) and Glu$_{1262}$ (E1262D and E1262R) were generated and expressed in HEK293T cells. Immunoblotting experiments revealed that MRP1 protein expression levels of these mutants in both whole cell lysates (not shown) and membrane vesicles (Figures 3.6A&B) were similar to wild-type MRP1. The same-charge E1253D MRP1 mutant displayed E$_{217}$βG and LTC$_4$ transport activities comparable to those of wild-type MRP1, while the opposite-charge E1253K mutant exhibited substantially and significantly reduced E$_{217}$βG and LTC$_4$ uptake (decreased by 85% and 75%, respectively; p < 0.05) (Figures 3.6C&D). In contrast, the same-charge E1262D MRP1 mutant exhibited significantly reduced E$_{217}$βG and LTC$_4$ uptake (decreased by 50% and 80%, respectively) (p < 0.05) (Figures 3.6C&D). Transport by the opposite-charge E1262R mutant was also significantly reduced to a similar degree, by 75% for E$_{217}$βG, and by 90% for LTC$_4$ uptake (p < 0.05) (Figures 3.6C&D).
Figure 3.6: Expression levels and vesicular uptake of $^3$H-labeled organic anions by same- and opposite-charge substitutions of Glu$^{1253}$ and Glu$^{1262}$ MRP1 mutants.

A and B, Shown are representative immunoblots of membrane vesicles (0.5 and 1.0 μg protein) prepared from HEK293T cells transfected with wild-type (WT) MRP1, as well as E1253A/D/K (A) and E1262A/D/R (B) mutant MRP1 expression vectors. MRP1 was detected with MAb QCRL-1, and an antibody against Na$^+$/K$^+$ ATPase was used as a loading control, as described in the legend of Figure 3.5. C and D, ATP-dependent uptake of $[^3]$H]E217βG (C) and $[^3]$H]LTC$_4$ (D) was measured in membrane vesicles prepared from HEK293T cells transfected with WT-MRP1, and Glu$^{1253}$ and Glu$^{1262}$ mutant MRP1 (E1253A/D/K, E1262A/D/R) cDNAs, as described in the legend to Figure 3.5. The results shown are the means (+S.D.) of three or more independent experiments; n values are shown above each bar. Similar results were obtained in at least one additional experiment using vesicles prepared from an independent transfection. MV, membrane vesicles; *, significantly different from wild-type MRP1 activity (p < 0.05).
### A

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**MRP1**

**Na⁺/K⁺ ATPase**

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### B

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**MRP1**

**Na⁺/K⁺ ATPase**

<table>
<thead>
<tr>
<th>Relative expression (corrected)</th>
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<tr>
<td>Control</td>
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### C

**E-17βG Uptake (% WT-MRP1)**

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<th>E1253K</th>
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### D

**LTC4 Uptake (% WT-MRP1)**

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<th>E1253D</th>
<th>E1253K</th>
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<td>(5)</td>
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</table>
A ‘revertant’ construct "E(D)1262E", in which the E1262D construct was mutated back to the wild-type sequence, was generated and characterized to rule out the possibility that the phenotype of E1262D mutant may be due to mutations introduced inadvertently outside of the cloning region during mutagenesis. However, this was not the case as expression levels of E(D)1262E in membrane vesicles were comparable to wild-type MRP1 (not shown), and LTC₄ transport activity of this mutant was also comparable to wild-type MRP1 (not shown).

### 3.2.4 Kinetic Analysis of [³H]E₂₁⁷βG Uptake by E₁₂₅₃A, E₁₂₆₂A and E₁₂₆₂D MRP1 Mutants

To determine whether reduced E₂₁⁷βG uptake by the Ala-substituted Glu₁₂₅₃ and Glu₁₂₆₂, and Asp-substituted Glu₁₂₆₂ MRP1 mutants was caused by differences in apparent uptake affinity (\(K_m\)) or changes in the maximum transport velocity (\(V_{max}\)), kinetic parameters were determined by measuring uptake for a fixed time period (1 min) over a range of E₂₁⁷βG concentrations (0.25-25 µM). Experiments using 4 µg protein per point were conducted as previously described (Conseil et al., 2006), while experiments using 2 µg protein per point were included since it was determined that this amount of protein would be sufficient for these analyses (Letourneau et al., 2005). Kinetic parameters were then determined by non-linear regression analysis of the data, and the variability of \(K_m\) and \(V_{max}\) values between experiments may be due to the range in the date of preparation (or ‘freshness’) of membrane vesicles used to analyze E₂₁⁷βG kinetics.

While there were differences in the absolute \(K_m\) values for E₂₁⁷βG uptake by the E₁₂₅₃A and E₁₂₆₂A mutants, in that each was substantially increased (3.1–14.3-fold and
ATP-dependent [3H]E217βG uptake by membrane vesicles prepared from HEK293T cells transfected with wild-type MRP1, Ala-substituted Glu1253 (A), and Ala- (B) and Asp-substituted Glu1262 (C) MRP1 mutants were measured at the indicated E217βG concentrations (0.25-25 μM) for 1 min at 37 °C, as previously described (Conseil et al., 2006). Values shown are duplicate determinations in a single representative experiment (2 μg protein/point); 2-3 independent experiments were performed for each mutant (see Table 3.2). $V_{max}$ values are corrected for any differences in expression of the mutant proteins relative to wild-type MRP1. Kinetic parameters for E217βG transport were determined from non-linear regression analysis and are summarized in Table 3.2.
Table 3.2: Summary of kinetic parameters of [3H]E217G uptake by Ala-substituted Glu\textsuperscript{1253} and Glu\textsuperscript{1262}, and Asp-substituted Glu\textsuperscript{1262} MRP1 mutants.

Kinetic analyses were performed by measuring E217βG uptake (initial concentration range of 0.25-25 µM) for 1 min at 37 °C using either 2 or 4 µg vesicle protein per point as explained in text. Each $K_m$ and $V_{max}$ value shown is from a single independently prepared batch of vesicles and is the mean of duplicate determinations. $V_{max}$ values have been corrected for differences in protein expression relative to WT-MRP1. $V_{max}/K_m \times 10^3$ ratios were calculated and indicate the relative E217βG uptake efficiency of the MRP1 mutants. N/D, not determined.
1.8–3.0-fold, respectively) relative to wild-type MRP1, the $V_{max}$ values of both mutants were comparable to wild-type MRP1 (Figure 3.7 & Table 3.2). On the other hand, while the $K_m$ (E$_{217}\beta$G) of E1262D was similar to wild-type MRP1, the $V_{max}$ value was reduced (by 55-70%) (Figure 3.7 & Table 3.2). Thus, this indicates that charge is not important for E$_{217}\beta$G uptake affinity in this case, but instead for uptake efficiency (as reflected by the 3–6-fold difference in the $V_{max}/K_m \times 10^3$ ratio). As reported in Table 3.2, the observed uptake efficiencies of E1253A, E1262A, and E1262D were reduced by 65-85%, 65-75%, and 65%, respectively, compared to wild-type MRP1.

The kinetic data correlate, reasonably well with the E$_{217}\beta$G transport data. E$_{217}\beta$G transport by the E1253A mutant was decreased by 75%, while the apparent $K_m$ (E$_{217}\beta$G) was 3.1–14.3-fold higher than wild-type MRP1. Transport by the E1262A mutant was reduced by 50%, and the apparent $K_m$ (E$_{217}\beta$G) was 1.8–3.0-fold higher than wild-type MRP1. Finally, E$_{217}\beta$G transport by the E1262D mutant was reduced by 50% compared with wild-type MRP1, and this was associated with a 55-70% reduction in $V_{max}$. Thus, reductions in $K_m$ (E$_{217}\beta$G) of E1253A and E1262A explain their decreased E$_{217}\beta$G transport, while the reduced $V_{max}$ of E1262D explains its decreased E$_{217}\beta$G transport.

### 3.2.5 Photolabeling of E1253A, E1262A and E1262D MRP1 Mutants by $[^3H]LTC_4$

To determine if the reduced LTC$_4$ uptake by E1253A, E1262A, and E1262D MRP1 mutants was associated with changes in substrate binding, $[^3H]LTC_4$ photolabeling experiments were conducted. As shown in Figure 3.8, E1253A and E1262A were photolabeled by $[^3H]LTC_4$ at levels comparable to wild-type MRP1, this is somewhat
unexpected since the apparent $K_m$ ($E_{217\beta G}$) was reduced and it was anticipated that binding of LTC$_4$ would also be reduced in concordance with the reduced LTC$_4$ transport by these mutants. On the other hand, photolabeling of the E1262D MRP1 mutant was approximately 40% lower (Figure 3.8), which was unexpected since the $K_m$ ($E_{217\beta G}$) was comparable to wild-type MRP1, and thus it was anticipated that $[^3H]$LTC$_4$ labeling by this mutant would be similar to wild-type MRP1.

### 3.2.6 Expression and $E_{217\beta G}$ and LTC$_4$ Transport Activities of K1141E/E1253K and R1142E/E1262R Double Reciprocal MRP1 Mutants

One way to explore the possibility that two residues might form an interhelical bond is to determine whether double exchange (reciprocal) mutants of the paired amino acids would restore wild-type activity. To do this, single TM15-proximal K1141E and R1142E, as well as the double mutants K1141E/E1253K and R1142E/E1262R were needed. The K1141E and R1142E mutant constructs were available from a previous study (Conseil et al., 2006), while the double exchange mutants were generated as described in Chapter II (Section 2.4).

Immunoblot analyses demonstrated that the expression levels of the K1141E and R1142E MRP1 mutants in whole cell lysates (not shown) and membrane vesicles (Figures 3.9A & 3.10A, respectively) were similar to wild-type MRP1. Expression levels of K1141E observed in this study were substantially higher (by 3.3–4.8-fold) than previously reported (Conseil et al., 2006), a difference that cannot presently be explained.
Figure 3.8: Photolabeling of Ala-substituted Glu\textsuperscript{1253} and Glu\textsuperscript{1262}, and Asp-substituted Glu\textsuperscript{1262} MRP1 mutant proteins by \([^{3}H]LTC_4\).

A. Membrane vesicles (50 µg protein) were incubated with \([^{3}H]LTC_4\) and irradiated (1100 µW, 302 nm). After resolving vesicles (40 µg protein) by SDS-PAGE, the gel was processed for autoradiography and exposed to film (7 day exposure). Signal intensities on the film were analyzed by densitometry. Relative levels of \([^{3}H]LTC_4\) photolabeling are indicated below the image, and have been corrected for any differences in protein expression levels relative to wild-type MRP1, as shown by the immunoblot in panel B. The \([^{3}H]LTC_4\) photolabeling results shown are from a single experiment. Similar results were observed in a second independent experiment. B. Shown is an immunoblot which quantifies the relative protein expression levels of the MRP1 mutants used for the photolabeling in Panel A. MRP1 was detected with MAb QCRL-1, and an antibody against Na\textsuperscript{+}/K\textsuperscript{+} ATPase was used as a loading control, as described in the legend to Figure 3.5. The mean MRP1 expression levels from 0.5 and 1.0 µg protein/lane were used to correct for differences in photolabeling. HEK293T cells were used as a control in all photolabeling and immunoblot experiments.
Figure 3.9: Expression levels and vesicular uptake of $^3$H-labeled organic anions by MRP1 mutants containing single and exchange substitutions of Lys$^{1141}$ and Glu$^{1253}$.

A. Shown is a representative immunoblot of membrane vesicles (0.5 and 1.0 μg protein) prepared from HEK293T cells transfected with K1141E, E1253K, K1141E/E1253K, and wild-type (WT) MRP1 cDNA expression vectors. MRP1 was detected with MAb QCRL-1, and an antibody against Na$^+/K^+$ ATPase was used as a loading control, as described in the legend to Figure 3.5. B and C, ATP-dependent uptake of $[^3]$H]E$_2$17βG (B) and $[^3]$H]LTC$_4$ (C) was measured in membrane vesicles prepared from HEK293T cells transfected with WT-MRP1, and single and exchange substitutions of Lys$^{1141}$/Glu$^{1253}$. The results shown are the means of two independent experiments. Untransfected cells were used as a control in all experiments. MV, membrane vesicles.
A

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<th>E1253K</th>
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Relative expression (corrected) - - 1.0 1.0 1.3 1.9 2.0 1.3 1.6 1.3

MRP1

Na+/K+ ATPase

B

E\textsubscript{2} 17\beta G Uptake (% WT-MRP1)

C

LTC\textsubscript{4} Uptake (% WT-MRP1)
Figure 3.10: Expression levels and vesicular uptake of E217βG and LTC4 by MRP1 mutants containing single and double exchange substitutions of Arg1142 and Glu1262.

A. Shown is a representative immunoblot of membrane vesicles (0.5 and 1.0 μg protein) prepared from HEK293T cells transfected with R1142E, E1262R, R1142E/E1262R, and wild-type (WT) MRP1 cDNA expression vectors. MRP1 was detected with MAb QCRL-1, and an antibody against Na⁺/K⁺ ATPase was used as a loading control, as described in the legend to Figure 3.5. B and C, ATP-dependent uptake of [³H]E217βG (B) and [³H]LTC4 (C) was measured in membrane vesicles prepared from HEK293T cells transfected with WT-MRP1, and single and exchange substitutions of Arg1142/Glu1262. The results shown are the means (+S.D.) of 3-4 independent experiments. Similar results were obtained in at least one additional experiment using vesicles prepared from an independent transfection. Untransfected cells were used as a control in all experiments. *MV, membrane vesicles; *, significantly different from wild-type MRP1 activity (p < 0.05).
A

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<tr>
<th>MV (μg)</th>
<th>Control</th>
<th>WT-MRP1</th>
<th>R1142E</th>
<th>E1262R</th>
<th>R1142E/E1262R</th>
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Relative expression (corrected)

MRP1

Na⁺/K⁺ ATPase

B

E₂¹十七G Uptake (% WT-MRP1)

Control

WT-MRP1

R1142E

E1262R

R1142E/E1262R

C

LTC₄ Uptake (% WT-MRP1)

Control

WT-MRP1

R1142E

E1262R

R1142E/E1262R
When assessed using a vesicular uptake assay, E$_{217}\beta$G uptake by both the K1141E (Fig. 3.9B) and R1142E (Fig. 3.10B) mutants was reduced (by 80% and 40%, respectively) (p < 0.05), as was uptake of LTC$_4$ (by 80% each) (p < 0.05) (Figures 3.9C & 3.10C, respectively). These uptake levels were comparable to those previously reported (Conseil et al., 2006), and confirm that Lys$^{1141}$ and Arg$^{1142}$ are important for MRP1 function.

Double exchange (reciprocal) mutations (K1141E/E1253K and R1142E/E1262R) were then generated as described in Chapter II (Section 2.4). Expression levels of the K1141E/E1253K mutant in whole cell lysates (not shown) and membrane vesicles (Figure 3.9A) were comparable to wild-type MRP1. However, when the ability of K1141E/E1253K reciprocal MRP1 mutant to mediate ATP-dependent vesicular uptake of E$_{217}\beta$G and LTC$_4$ was examined, only a modest increase (by 20-30%) in E$_{217}\beta$G uptake was observed compared to the single K1141E and E1253K mutants, and they still remained substantially lower than that of wild-type MRP1 (Figure 3.9B). LTC$_4$ uptake by the double reciprocal mutant was also comparable to that of the two single mutants (Figures 3.9C).

Expression levels of the R1142E/E1262R reciprocal mutant were also similar to that of wild-type MRP1 in whole cell lysates (not shown) as well as membrane vesicles (Figure 3.10A). However, E$_{217}\beta$G uptake by R1142E/E1262R was increased by only 35-45% relative to uptake by E1262R, and unchanged relative to R1142E (Figure 3.10B), and remained significantly (p < 0.05) decreased (by 65-75%) relative to wild-type MRP1. LTC$_4$ uptake by this reciprocal MRP1 mutant was improved (by 15-25%) (p < 0.05) compared to R1142E and E1262R (Figure 3.10C), although it remained significantly reduced (by 60%) (p < 0.05) relative to wild-type MRP1 (Figure 3.10C).
3.3 Discussion

In this study, Ala substitutions were initially made to determine which of the four Glu residues in the TM17-proximal cytoplasmic region of MRP1 were important for expression and substrate transport. Replacing Glu with Ala is a so-called ‘cavity-creating’ mutation due to the small volume of the Ala side-chain. The side-chain of Ala is also incapable of H-bonding due to its lack of hydrogen donor or acceptor electrons. Thus, Ala provides a non-conservative substitution for polar, aromatic, and ionizable amino acids, as well as a conservative substitution (with decreased steric volume) for non-polar amino acids. For these reasons, Ala-substitution is commonly used in structure-function studies utilizing site-directed mutagenesis.

From the biochemical analysis described in Section 3.2.2, it is clear that Ala-substitution of the four Glu residues does not affect MRP1 levels, suggesting that these acidic amino acids are not critical for proper folding and assembly during its biosynthesis in HEK293T cells (Figure 3.5A). However, Ala-substitution of Glu\textsuperscript{1253} and Glu\textsuperscript{1262} significantly reduced transport of both E\textsubscript{217}\textbeta G and LTC\textsubscript{4} (Figure 3.5B&C). E\textsubscript{217}\textbeta G kinetic analyses suggest that decreased apparent uptake affinities (increases in apparent $K_m$) by both E1253A and E1262A contribute to the reduction in E\textsubscript{217}\textbeta G transport activity (Table 3.2), while in seeming contrast $[^3H]LTC_4$ photolabeling experiments showed that the decreases in LTC\textsubscript{4} transport by the E1253A and E1262A mutants were not due to detectable differences in MRP1 labeling by this substrate (Figure 3.8A). The similarity of the E1253A and E1262A phenotypes could suggest that the two Glu residues play a similar role in substrate translocation. However, if this was true, then one would expect that same charge substitutions of the two residues would have the same effect, but this
was not the case. Thus, substitution of Glu\textsuperscript{1253} with Asp had no affect on expression or transport activity, indicating the role of Glu\textsuperscript{1253} can be retained by Asp, despite its shorter side-chain length (Figure 3.6C&D). In contrast, however, transport activity was not retained with an Asp-substitution at position 1262 (Figure 3.6C&D). Thus, the roles of these two amino acids must differ. Finally, substitution of an oppositely (positively) charged amino acid at both positions (1253 and 1262) significantly decreased transport activities, a result that might have been expected because such non-conservative substitutions of Glu\textsuperscript{1253} and Glu\textsuperscript{1262} are much more likely to disrupt MRP1 structure and thus its function (Figure 3.6C&D).

Although the apparent $K_m$ (E217\textbeta G) of the E1262D mutant was similar to wild-type MRP1, the $V_{max}$ was reduced by approximately 55-70\% (Table 3.2). However, $[^3]$H\textsubscript{LTC}\textsubscript{4} labeling of E1262D was also reduced (by 40\%) (Figure 3.8A). This suggests that although binding of E217\textbeta G by E1262D is intact, the transport efficiency was impaired, whereas reductions in LTC\textsubscript{4} uptake (by 80\%) by E1262D appear to be explained, at least in part, by a decreased apparent association with this substrate. Thus, the volume of the Glu\textsuperscript{1262} side-chain seems important for MRP1 function, but in different ways for E217\textbeta G and LTC\textsubscript{4} transport. Similar findings have been reported for the human concentrative nucleoside transporter 3 (hCNT3), in which Asp-substitution of Glu\textsuperscript{519} was able to restore functionality of Na\textsuperscript{+} but not H\textsuperscript{+} coupling compared with non-conservative mutations, suggesting subtly different positional requirements for this negative charge (Slugoski \textit{et al.}, 2009). Therefore, it may be concluded that MRP1-Glu\textsuperscript{1262} plays a complex role in substrate binding/efflux, and could contribute to a highly structured local environment.
It is of interest to note that several of the residues chosen for functional analysis in this study are of clinical relevance in homologous ABCC proteins, including CFTR (ABCC7) and MRP6 (ABCC6). Mutation of residues in CFTR analogous to Glu\textsuperscript{1253} and Glu\textsuperscript{1255} of MRP1 (Asp\textsuperscript{1152} and Asp\textsuperscript{1154}, respectively) have been linked to cystic fibrosis, and like the Glu residues in MRP1, are located in the cytoplasmic extension connecting the last TM to NBD2 (Mussaffi \textit{et al.}, 2006; Vankeerberghen \textit{et al.}, 1998). The CFTR mutation of D1152H has been shown to cause a mild disease state, possibly by interference with chloride channel gating (Mussaffi \textit{et al.}, 2006). In addition, although the CFTR mutation D1154G has been found in a patient with cystic fibrosis, interpretation of the phenotype is somewhat confounded by the fact that the patient also has the well-characterized and disease-causing ΔF508 mutation on the other allele (Vankeerberghen \textit{et al.}, 1998).

These observations together with the present MRP1 data indicate that mutation of an amino acid in the region connecting the most COOH-proximal TM to NBD2 in at least one other ABCC protein, namely TM12 in CFTR, which is analogous to one of the TM17-proximal residues of MRP1 sensitive to mutation (i.e. Glu\textsuperscript{1253}), is associated with disease. Based on these findings, the functional importance of this conserved amino acid may suggest that the structure of this region is conserved as well.

In contrast to CFTR, only one mutation in MRP6/ABCC6 (analogous to Glu\textsuperscript{1266} in MRP1) is associated with \textit{Pseudoxanthoma elasticum}, and was found by searching the literature, including the PXE international foundation website (http://www.pxe.org), and the \textit{ABCC6} human gene mutation database (http://www.hgmd.cf.ac.uk/ac/all.php). Mutation of Asp\textsuperscript{1238} to His was found in one individual with \textit{Pseudoxanthoma elasticum},
but this mutation has not yet been well characterized (Ladewig et al., 2006). In addition, it must be noted that it is currently unclear how mutations in MRP6 cause PXE, which is in contrast to mutations in MRP1 and CFTR, where the physiological substrates of these ABCC proteins are well known.

Charged residues within hydrophobic regions of soluble proteins have been shown to interact with oppositely charged residues in order to neutralize charge and minimize energetic constraints (Anderson et al., 1990; Tissot et al., 1996). This charge stabilization also holds true for membrane proteins, and this phenomenon can substantially contribute to the global functionality of such proteins (Zhou et al., 1994).

Pairs of residues that may potentially interact via ionic bonding (i.e. salt bridge formation) and H-bonding were identified (Table 3.1), and since substitution of Glu$^{1253}$ and Glu$^{1262}$ with Ala had the greatest effects on transport activity, the potential interhelical pairs involving these two residues were investigated by reciprocal mutagenesis.

The reported maximum distance between two residues involved in a salt bridge (ionic bond) is ~3.5 Å (Chang et al., 2008; Sackin et al., 2009). However, a previous study in our lab (Haimeur and Cole, unpublished) obtained biochemical evidence suggesting that an interhelical bond occurs via an electrostatic interaction between Lys$^{396}$ and Asp$^{436}$, despite the fact that these two residues are estimated to be 10.5 Å apart in current MRP1 models. Thus, reciprocal mutagenesis experiments showed that the double mutant K396D/D436K was a fully functional transporter, even though the single mutants K396D and D436K exhibited substantially reduced (by >70%) E$_2$17βG and LTC$_4$ transport activities. These observations suggest a relatively exclusive or dominant
interaction between these two amino acids. Therefore, in the present study it seemed reasonable to set a cutoff value, with respect to the maximal distance between two residues, that is higher than that which may be theoretically achievable. In this way, imprecision in the model with regard to placement of the side-chains can be allowed for. Thus, reciprocal mutagenesis experiments were undertaken despite the fact that Glu\textsuperscript{1253} is predicted to be within 9.7 Å of its potential bonding partner Lys\textsuperscript{1141} (Table 3.1).

The E1253K MRP1 mutant exhibited significant reductions (>75%) in both E\textsubscript{2}17βG and LTC\textsubscript{4} transport (Figure 3.9B&C), and so by mutating the nearby Lys\textsuperscript{1141} to Glu (K1141E), it was hoped that transport activity might be restored as a result of acid/base pairing. However, this was not the case since only a slight increase in E\textsubscript{2}17βG uptake (to 40% of wild-type MRP1 activity) was observed (Figure 3.9B&C). These observations do not exclude the possibility that Glu\textsuperscript{1253} interacts with Lys\textsuperscript{1141}, but do indicate that the interaction is not exclusive, or is somewhat weak.

With respect to Glu\textsuperscript{1262}, it is interesting to note that the amino acid corresponding to this residue in the distantly related ABCC protein SUR2 (ABCC9) is a Lys (1280) residue, and the amino acid in SUR2 at the same position as Arg\textsuperscript{1142} in MRP1 (its potential bonding partner) is a Glu (1162) residue. The presence of oppositely charged residues at the same two positions of these proteins in which an interhelical interaction has been predicted to occur in MRP1 is intriguing and could suggest that an evolutionarily conserved interhelical salt bridge might exist in both proteins (i.e. Arg\textsuperscript{1142} and Glu\textsuperscript{1262} in MRP1, and Glu\textsuperscript{1162} and Lys\textsuperscript{1280} in SUR2). However, the positioning of these charged residues could just be a coincidence, since one might expect that if a conserved salt bridge existed, it would be present in more than just one homolog.
The E1262R MRP1 mutant exhibited substantial reductions in both E217βG and LTC₄ transport (Figure 3.10B&C), and so by mutating the nearby Arg¹¹⁴² to Glu (R1142E), and restoring the possibility of acid/base pairing by creating R1142E/E1262R, it was thought that transport function could be enhanced. However, this was not the case, suggesting Glu¹²⁶² likely does not interact exclusively with Arg¹¹⁴² (Figure 3.10B&C).

Together, the data presented here indicate that the side-chain positions of Glu¹²⁵³ and Lys¹¹⁴¹, and Glu¹²⁶² and Arg¹¹⁴² may be accurately predicted in the homology model of MRP1, and thus are indeed too far apart to provide a substantial interhelical bond, and/or that interhelical bonding interactions are more complex than hypothesized.
CHAPTER IV: FUNCTIONAL ANALYSIS OF ALA-SUBSTITUTED VAL\textsuperscript{1261}, ARG\textsuperscript{1263} AND TYR\textsuperscript{1267} MRP1 MUTANTS

4.1 Introduction

As described and discussed in Chapters I and III, many functionally important amino acids in the TM helices of MRP1 are ionizable and/or polar (Haimeur et al., 2002; Situ et al., 2004; Ren et al., 2002). Aromatic residues in the TM helices of MRP1 also play a role in MRP1 function, such that the transport, and in some cases, the binding of substrates is altered when these residues are mutated (Ito et al., 2001a; Koike et al., 2002; Zhang et al., 2002; Campbell et al., 2004). For example, Trp\textsubscript{1246} in TM17 is critical for E\textsubscript{2}17βG transport, in that both conservative and non-conservative substitutions of this residue abrogate E\textsubscript{2}17βG uptake, while LTC\textsubscript{4} transport remains intact (Ito et al., 2001a). In contrast, the functional importance of aliphatic amino acids located within or proximal to TM helices has not yet been explored. In the present study, the aim was to investigate the importance of three highly conserved aliphatic, basic, and aromatic residues (Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267}, respectively) in the α-helical region COOH-proximal to TM17, in determining the expression and transport activity of MRP1 (Figure 3.1). Because of its proximity to the functionally important TM17, and because it links TM17 to NBD2, it was hypothesized that conserved amino acids in this region could be important for MRP1 function. In addition, since current Sav1866-based models of MRP1 predict that this α-helical region extending from TM17 is likely to participate in interhelical interactions, it was further hypothesized that these amino acids in the TM17-proximal helix might form electrostatic interactions with residues extending from TM14, as well as those within the
cytoplasmic loop (CL6) connecting TM13 to TM14. In this chapter, the results of experiments designed to test the importance of individual amino acids are described, and residues possibly involved in interhelical bonds are identified.

4.2 Results

4.2.1 Secondary Structure Predictions, Sequence Alignments, and In Silico Illustrations of the Region COOH-Proximal to TM17

As described in Chapter III (Section 3.2) and shown in Figure 4.1, secondary structure predictions and multiple sequence alignments of the cytoplasmic TM17-proximal region of MRP1 and its eleven homologs revealed the presence of a significant number of highly conserved amino acids. In addition to the 4 Glu residues investigated in Chapter III, three other amino acids (Val^{1261}, Arg^{1263}, and Tyr^{1267}) within the COOH-proximal region of TM17 of MRP1 exhibited a high degree of sequence identity and/or similarity (Val^{1261}, 100% identity; Arg^{1263}, >80% identity, >90 similarity; and Tyr^{1267}, 75% identity, >90% identity), and these were targeted for investigation.

According to a model of the four-domain core structure of MRP1 (DeGorter et al., 2008), the side-chains of Val^{1261} and Arg^{1263} project toward the cytoplasmic α-helical extension of TM14, while the side-chain of Tyr^{1267} projects toward CL6 (Figure 4.2). Thus, none of these three amino acids are predicted to project directly into the putative translocation pathway. When Val^{1261}, Arg^{1263}, and Tyr^{1267}, and amino acids in adjacent α-helices were examined in the MRP1 homology model, the following pairs of potential bonding interactions were identified: Val^{1261} with Val^{1083} (van der Waals), Arg^{1263} with
Figure 4.1: Structural predictions and sequence alignments of Val$^{1261}$, Arg$^{1263}$, and Tyr$^{1267}$ of MRP1.

A, secondary structure predictions of amino acids 1249-1280 of human MRP1. \( H \), helical; \( C \), coiled; \( E \), extension. B, sequence alignment of human MRP1 (residues 1227-1269) and its eleven homologs, as well as the sequence of Sav1866, generated using ClustalW 1.0. Amino acids which are identical in a majority of homologs are shown on a black background, while those which are conserved are shown on a gray background. G1228 and V1248 define the boundaries of TM17, while residues V1261, R1263, and Y1267 were examined in this study. \( SUR \), sulfonylurea receptor; \( CFTR \), cystic fibrosis transmembrane conductance regulator.
Figure 4.2: Putative relative locations and side-chain positions of Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267} in a homology model of MRP1.

A three-dimensional homology model of the four-domain core structure of MRP1 shows the predicted location of TM17-proximal region (dark gray) spanning amino acids 1249 to 1269, as well as the predicted locations and side-chain positions of Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267} (in black) (amino acids are shown as ‘spheres’ in the full model, and as ‘sticks’ in the inset). The side-chains of Val\textsuperscript{1261} and Arg\textsuperscript{1263} project toward the TM14-proximal $\alpha$-helix, while the side-chain of Tyr\textsuperscript{1267} projects toward CL6.
Glu^{1079} (ionic), and Tyr^{1267} with Phe^{1063} (π-π/aromatic stacking) (Figure 4.3 and Table 4.1). It should be noted that the predicted distance (5.3 Å) between the side-chains of Tyr^{1267} and Phe^{1063} is larger than that likely to make the above bonding interaction plausible (Chang et al., 2008; Sackin et al., 2009). However, this potential interaction was still investigated, taking into account the notion that there may be imprecision in MRP1 models with respect to placement of the side-chains of these amino acids.

### 4.2.2 Expression and E217βG and LTC₄ Transport Activities of Ala-Substituted Val^{1261}, Arg^{1263}, and Tyr^{1267} MRP1 Mutant Proteins

To begin analysis of the functional importance of Val^{1261}, Arg^{1263}, and Tyr^{1267}, single Ala-substitutions of these residues were made, and after transfection in HEK293T cells immunoblot analysis of whole cell lysates (not shown) and membrane vesicles was performed using the human MRP1-specific MAb (QCRL-1) as before (Hipfner et al., 1994; Ito et al., 2001a). As shown in Figure 4.4A, all three mutants were expressed at levels comparable to wild-type MRP1 in membrane vesicles, demonstrating that Ala-substitution of these residues did not affect the biosynthesis of MRP1 in HEK293T cells.

Next, the ability of these three MRP1 mutants to mediate ATP-dependent transport of E₂₁₇βG and LTC₄ was examined using a microplate *in vitro* vesicular uptake assay (Loe et al., 1996; Letourneau et al., 2005). As before, uptake levels were normalized to take into account differences in expression of the mutant proteins relative to wild-type MRP1, and activity was calculated as a percent of the activity of vesicles enriched for wild-type MRP1.
Figure 4.3: *In silico* illustrations of potential interhelical interactions involving side-chains of Val^{1261} and Val^{1083}, Arg^{1263} and Glu^{1079}, and Tyr^{1267} and Phe^{1063}.

A homology model of the core structure (MSD1 and MSD2) of MRP1 shows the predicted location of the cytoplasmic TM17-proximal amino acids (black) and their potential interhelical bonding partners (dark gray). Shown are potential interactions between the side-chains of Val^{1261} and Val^{1083} (A), Arg^{1263} and Glu^{1079} (B), and Tyr^{1267} and Phe^{1063} (C). Dotted lines represent the distances between the side-chains, which are listed in Table 4.1.
<table>
<thead>
<tr>
<th>TM17-Proximal Residue</th>
<th>Potential Partner Residue (Position in MRP1)</th>
<th>Predicted Distance Between Closest Side-Chain Atoms (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1261</td>
<td>V1083 (TM14-proximal)</td>
<td>2.9</td>
</tr>
<tr>
<td>R1263</td>
<td>E1079 (TM14-proximal)</td>
<td>3.2</td>
</tr>
<tr>
<td>Y1267</td>
<td>F1063 (CL6)</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table 4.1: Estimated distances between side-chains of Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267}, and other amino acids in other helices which might form bonding interactions.

The distances between the side-chains of the indicated pairs of amino acids were estimated based on a Sav1866-based atomic homology model of MRP1 (DeGorter et al., 2008) using PyMOL (DeLano Scientific; http://www.pymol.org).
Figure 4.4: Expression levels and vesicular uptake of E217βG and LTC₄ by Ala-substituted Val₁₂₆¹, Arg₁₂₆³, and Tyr₁₂₆⁷ MRP1 mutants.

A. Shown is a representative immunoblot of membrane vesicles (0.5 and 1.0 μg protein) prepared from HEK293T cells transfected with V1261A, R1263A, Y1267A, and wild-type (WT) MRP1 cDNA expression vectors. Untransfected cells were used as a negative control (control). MRP1 was detected with MAb QCRL-1, and relative MRP1 expression levels were adjusted to take into account differences in total protein loaded using an antibody against Na⁺/K⁺ ATPase. Relative MRP1 levels (corrected for total protein loaded) are shown below the blot, and were determined by densitometry as described in Chapter II (Section 2.8). B and C, ATP-dependent uptake of [³H]E217βG (B) and [³H]LTC₄ (C) was measured in membrane vesicles prepared from HEK293T cells transfected with WT-MRP1, and Ala-substituted mutant MRP1 cDNA expression vectors. Vesicles prepared from untransfected cells were used as a negative control (control). Uptake values were normalized based on mutant MRP1 levels relative to WT-MRP1 levels (according to panel A), and uptake by the mutants was expressed as a percentage of uptake by WT-MRP1. The results shown are the means (+S.D.) of at least three independent experiments (numbers in parentheses above the bars). Similar results were in at least one additional experiment using vesicles prepared from an independent transfection (not shown). MV, membrane vesicles; *, significantly different from wild-type MRP1 activity (p < 0.05).
A

<table>
<thead>
<tr>
<th>Control</th>
<th>WT-MRP1</th>
<th>V1261A</th>
<th>R1263A</th>
<th>Y1267A</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV (μg)</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Relative expression (corrected)

B

E217βG Uptake (% WT-MRP1)

C

LTC4 Uptake (% WT-MRP1)
As shown in Figures 4.4B&C, and in contrast to the Ala-substituted Glu\textsuperscript{1253} and Glu\textsuperscript{1262} mutants described in Chapter III (Figure 3.5), the Ala-substituted Val\textsuperscript{1261}, Arg\textsuperscript{1263} and Tyr\textsuperscript{1267} mutants showed only modest reductions (by 25-55\%) in transport activity, although these were statistically significant (p < 0.05). However, the loss of E\textsubscript{2}17\(\beta\)G activity (by 30\%) by the R1263A mutant was somewhat less than the loss of LTC\(_4\) transport (by 55\%), suggesting some substrate selective effect of this mutation, since these activities were statistically different from each other (p < 0.05). For the V1261A and 1267A mutants, the reduction in transport of LTC\(_4\) and E\textsubscript{2}17\(\beta\)G was \(\lesssim\) 35\%.

4.3 Discussion

A Sav\textsubscript{1866}-based MRP1 homology model was initially used to predict the putative locations of the highly conserved Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267} in the TM17-proximal region of MRP1. According to this model, the side-chains of Val\textsuperscript{1261} and Arg\textsuperscript{1263} project toward the TM14-proximal \(\alpha\)-helix, while the side-chain of Tyr\textsuperscript{1267} projects toward CL6. Their putative orientations projecting away from the predicted translocation pore could suggest that any changes in activity observed after Ala-substitution of these residues would likely not be due to direct interaction of substrate with these amino acids.

When tested experimentally, Ala-substitution of Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267} did not affect MRP1 biosynthesis (Figure 4.4A), indicating that these amino acids are not critical for proper folding and stable expression of MRP1 in HEK293T cells. Vesicular uptake assays showed that Ala-substitution of Val\textsuperscript{1261}, Arg\textsuperscript{1263}, and Tyr\textsuperscript{1267} reduced both the E\textsubscript{2}17\(\beta\)G and LTC\(_4\) transport activities by a modest (by 25-55\%), but statistically significant degree (Figure 4.4B&C). These findings suggest that the side-chain
orientations of these amino acids may in fact be accurately predicted, since mutations did not substantially affect transport of either E217βG or LTC₄, thus indicating that these residues likely do not directly interact with substrates.

In the homologous CFTR, several mutations of the amino acid analogous to Arg₁²⁶³ of MRP1 have been reported (http://www.hgmd.cf.ac.uk/ac/all.php). Thus, the CFTR mutation R1162L (located in the cytoplasmic extension connecting TM12 to NBD2) was found in one individual with cystic fibrosis, although this patient also had another disease-causing mutation, G542X (Groman et al., 2002). In addition, a R1162Q mutation was found in another individual with a mild disease state (i.e. having normal sweat chloride levels and pancreatic sufficiency) (Strandvik et al., 2001). Unfortunately, these findings do not indicate whether or not mutation of CFTR-Arg¹¹⁶² alone is detrimental to the function of this protein.

Mutations of residues in CFTR analogous to MRP1-Tyr¹²⁶⁷ have not been reported. On the other hand, mutations of the analogous MRP6-Tyr¹²³⁹ were found in the ABCC6/MRP6 human gene mutation database (http://www.hgmd.cf.ac.uk/ac/all.php). The Y1239H mutation in TM17 of MRP6 was detected in an individual diagnosed late (at 32 years of age) with mild PXE (Schulz et al., 2005).

In summary, mutation of amino acids analogous to MRP1-Arg¹²⁶³ and Tyr¹²³⁹ in the region connecting the most COOH-proximal TM to NBD2 in the ABCC proteins CFTR and MRP6 are associated with disease, although mild in some cases. Furthermore, it is clear the prevalence of these mutations in patients with cystic fibrosis and PXE is low.
CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

Homology models of MRP1 based on the crystal structure of Sav1866 have been useful in guiding the design and aiding in the interpretation of biochemical analyses of this transporter. In this thesis, MRP1 homology models were used to aid in the interpretation of biochemical data for single mutants of seven conserved residues in the region COOH-proximal to TM17 of MRP1, and assisted in the design of experiments involving double exchange mutants of several of these TM17-proximal amino acids. Implications of these data with regard to MRP1 function and structure, as well as conclusions and future directions are discussed in this chapter.

5.1 Implications for MRP1 Structure

As discussed in Chapter III, it has been shown that Ala-substitution of Glu\textsuperscript{1255} and Glu\textsuperscript{1266} had little effect on the transport activity of MRP1, which is consistent with a model in which both residues are predicted to project away from the translocation pathway of MRP1. On the other hand, Glu\textsuperscript{1253} was found to be important for the translocation of both E\textsubscript{217}\beta G and LTC\textsubscript{4}, although to varying degrees (i.e. more so for E\textsubscript{217}\beta G). These observations together with its predicted projection into the translocation pathway are compatible with, but do not prove the idea that this amino acid directly interacts with at least E\textsubscript{217}\beta G. These findings are in agreement with several previous observations in which mutation of Arg\textsuperscript{1249} and Met\textsuperscript{1250} in the TM17-proximal region also greatly reduced transport of both organic anions (Situ et al., 2004; Haimeur et al., unpublished), while mutation of Thr\textsuperscript{1242}, Asn\textsuperscript{1245}, and Trp\textsuperscript{1246} within TM17 selectively affect the transport of
glucuronide conjugates (Zhang et al., 2001; Zhang et al., 2002; Ito et al., 2001a; Leslie et al., 2001a).

In contrast, although Glu$^{1262}$ is predicted to project away from the putative translocation pathway, Ala- and Asp-substitutions significantly reduced transport of both E$_2$17$\beta$G and LTC$_4$, and Ala-substitution of Glu$^{1262}$ reduced the apparent uptake affinity ($K_m$) of E$_2$17$\beta$G. These data may indicate that the homology model does not sufficiently predict the true side-chain conformation of Glu$^{1262}$, since this amino acid has demonstrated its importance for apparent uptake affinity with E$_2$17$\beta$G, suggesting the side-chain may in fact project into the pore. Therefore, the side-chain orientation of Glu$^{1262}$ in the atomic homology model of MRP1 used in this study may need to be refined as a result of these findings. A direct interaction with LTC$_4$ has not yet been thoroughly investigated for the Ala- and Asp-substituted Glu$^{1262}$ mutants, and thus kinetic analyses of LTC$_4$ uptake are needed to support the notion that the true side-chain orientation projects into the translocation pathway. Furthermore, the side-chain orientation of this amino acid may differ during the various conformations of MRP1 during its catalytic cycle, and thus indirect effects may contribute to the observed phenotypes (e.g. apparent reductions in E$_2$17$\beta$G affinity), and therefore the model may in fact be accurate to some extent.

In addition, these findings may suggest that Glu$^{1262}$ is important for transducing signals between MSD2 and NBD2, since mutations caused an overall reduction in transport activity. One possibility is that mutation of Glu$^{1262}$ affects the geometry of NBD2 to which TM17 is connected, and in this way, affects the ATPase activity of the transporter. This has been suggested for certain amino acids in TM12 of P-gp (Crowley et
Thus, the mutations V988C and Q990C in TM12 of P-gp appears to reduce ATP hydrolysis by perturbing the geometry of the helical extension which is connected to NBD2, as demonstrated by in silico characterization using the crystal structure of murine P-gp (Aller et al., 2009). Similarly, TM17-proximal mutations that change NBD2 of MRP1 might alter dimerization of its NBDs, as well as the subsequent interaction of ATP at the NBSs. In this way, the coupling of ATP binding and hydrolysis with transport would be affected, and thus an overall reduction in activity might be expected. Further experiments using azio-derivatives of $^{32}$P-ATP to determine the ability of E1253A, E1262A, and E1262D mutants to bind and hydrolyze ATP are needed to test this idea (Letourneau et al., 2008).

The reciprocal mutagenesis studies described in Chapter III indicate that Glu$^{1253}$ and Lys$^{1141}$, and Glu$^{1262}$ and Arg$^{1142}$ do not interact exclusively with each other, since double exchange mutations were unable to enhance MRP1 transport activity. It is quite possible that the mutations disrupted other important bonding interactions, and therefore these data should be considered inconclusive with respect to the hypothesized interhelical interactions. Therefore, the side-chain orientations of these residues may in fact be accurately predicted in the homology model of MRP1, and thus the cutoff distance of 9.7 Å set in this study likely overestimated the maximum interhelical distance in which an interaction can occur, and so a shorter cutoff (e.g. 3-5 Å) would have been sufficient.

Based on the conserved nature of Val$^{1261}$, Arg$^{1263}$ and Tyr$^{1267}$, it was initially thought that each residue may play a role in MRP1 structure and function. However, data described in Chapter IV demonstrate that these amino acids are not critical for plasma membrane expression, and had little influence on MRP1 function compared to Glu$^{1253}$
and Glu\textsuperscript{1262}. On the other hand, Ala-substitution of Arg\textsuperscript{1263} did have some effect on transport, which is in agreement with all other studies to date that indicate a functional role for most ionizable residues in MRP1. Overall, these observations are thus far consistent with current MRP1 models, which predict the side-chain orientations of Val\textsuperscript{1261}, Arg\textsuperscript{1263} and Tyr\textsuperscript{1267} away from the translocation pore.

5.2 Limitations of MRP1 Homology Models and Reciprocal Mutagenesis

Although much of the data described in this thesis are compatible with the predicted projections of the seven amino acids studied by current models of MRP1 (DeGorter \textit{et al.}, 2008), the use of homology models is still limited by the fact that the template used to generate them is a homodimeric bacterial ABC transporter structure (Dawson and Locher, 2006). Differences in membrane composition of eukaryotic and prokaryotic cells have been established, and thus may pose some challenges with respect to modeling eukaryotic transporters from prokaryotic structures (Costerton \textit{et al.}, 1974).

A limitation of reciprocal mutagenesis is that this technique relies on amino acids in a potential interacting pair to interact essentially exclusively with each other (Zhou \textit{et al.}, 1994; Chang \textit{et al.}, 2008). Although this type of interaction is possible, it is more likely that the side-chains of residues which assist in maintaining the structure of polytopic membrane proteins, including MRP1, interact with multiple atoms in nearby amino acids (Pace, 2009). An alternative and relatively common approach to studying the proximity between TM helices, and thus potentially interacting amino acid pairs, is to use a Cys-less mutant, as has been done for P-gp and other membrane proteins (Loo and Clarke, 1995; Taylor \textit{et al.}, 2001). By using a mutant protein that lacks Cys residues, Cys...
residues can be introduced at positions which are thought to be involved in interhelical bonding interactions, and following treatment with a sulfide crosslinking agent, pairs of amino acids that form exclusive bonds in the native protein can be identified by electrophoretic methods (Loo et al., 2004). However, this approach may be problematic for MRP1, since Cys substitutions of several of the 25 Cys residues it contains have significantly affected the structural and functional properties of this transporter (Yang et al., 2002; Leslie et al., 2003b).

5.3 Concluding Remarks

In summary, the data presented in this thesis suggest the functional importance of several conserved amino acids (mainly Glu\textsuperscript{1253} and Glu\textsuperscript{1262}) in the TM17-proximal region of MRP1, although none of the seven residues investigated are critical for expression. Thus, Glu\textsuperscript{1253} and Glu\textsuperscript{1262} have different and complex roles in substrate recognition and translocation, while no functional importance can be ascribed to Glu\textsuperscript{1255} and Glu\textsuperscript{1266}. The data suggest that Val\textsuperscript{1261}, Arg\textsuperscript{1263} and Tyr\textsuperscript{1267} may be important for MRP1 function as well.

Glu\textsuperscript{1262} could be further investigated by analyses of E1262D, to determine whether its reduced photolabeling of [\textsuperscript{3}H]LTC\textsubscript{4} is due to changes in apparent LTC\textsubscript{4} uptake affinity ($K_m$). Investigations involving the mutation-sensitive Glu\textsuperscript{1253} and Glu\textsuperscript{1262} could include determining if the interactions of E1253A, E1262A and E1262D mutants with ATP have changed. One approach would require purifying the protein, and measuring ATP binding and hydrolysis, and/or ADP release. Alternately, measurement of ATP binding has commonly been measured by photolabeling with 8-azido-[$\gamma$-\textsuperscript{32}P]ATP
under non-hydrolytic conditions. Similarly, “trapping” experiments using 8-azido-[α-\(^{32}\)P]ATP at 37°C (which permits ATP hydrolysis and trapping of 8-azido-[α-\(^{32}\)P]ADP in the presence of sodium orthovanadate) could be carried out to determine if changes in transport activities are associated with changes in ATP hydrolysis or ADP release (Koike et al., 2004; Letourneau et al., 2007). However, given the differences in the structures of azido-ATP and ATP, these experiments may have some limitations.

Suggested future studies of Arg\(^{1263}\) could involve the generation of the same charge mutation (i.e. R1263K) to determine if the positive charge at this position is the determining factor for maintaining MRP1 transport function. In addition, kinetic analyses of LTC\(_4\) uptake of Arg\(^{1263}\) mutants would help determine whether its reduced LTC\(_4\) transport is caused by changes in apparent \(K_m\) and/or \(V_{max}\), and furthermore, photolabelling of these mutants by \([^3\text{H}]\)LTC\(_4\) would assist in supporting the findings from such kinetic analyses.

In conclusion, a better understanding of how MRP1 structure relates to its function is essential to understanding the basis of its diverse substrate specificity. Such information could be used to aid in the rational design of selective inhibitors to counteract MDR in tumours overexpressing MRP1. It could also be used to help in the design of new drugs that could avoid MRP1-mediated efflux, so that under certain conditions, exposure to therapeutics at tissues which are normally protected by MRP1 activity can be prolonged. In addition, determination of MRP1 structure would be useful in predicting drug-drug interactions with respect to the normal physiological roles of this transporter. Thus, structure-function analyses of previously unexplored regions of MRP1 are of current interest, since such studies could aid in elucidating the complex molecular
mechanisms of MRP1 activity. Since the cytoplasmic α-helical region COOH-proximal to TM17 has not been previously characterized, the studies described in this thesis have provided the first evidence of the functional importance of charged residues in this region. These studies have also assisted in validating the accuracy of the current Sav1866-based structural models of MRP1, and may one day contribute to the mapping of the multiple substrate binding sites of MRP1.
REFERENCES


APPENDIX

A1.1 Uncharacterized MRP1 Mutants E1079A, R1263K, and E1263E Generated and Related to this Study

Several Arg$^{1263}$ mutants in pcDNA3.1 vectors were generated (see Chapter II, Section 2.4) (glycerol stocks of these vectors are stored at -80ºC). These mutants (and the primers used) include: R1263K (5’-GTG GCC GTG GAG AA\_G CTC AAG GAG-3’), R1263E (5’-GTG GCC GTG GAG GAA\_G CTC AAG GAG-3’), and E1079A (5’-GCT TCT CCA AGG CC\_GC TGG ACA CAG TG-3’).

A1.2 MRP1 Mutants R1202E, R1202E/E1204R, and R1202D/E1204K Generated During the Course of this Master's Thesis in Collaboration with Marina Chan

Several Arg$^{1202}$ mutants in pcDNA3.1 vectors were generated (see Chapter II, Section 2.4) (glycerol stocks of these vectors are stored at -80ºC). These mutants (and the primers used) include: R1202E (5’-GGC TGG CCG TGG AG\_GC TGG AGT GTG-3’), R1202E/E1204R (5’-CTG GCC GTG GA\_G CTG AG\_G TGT GTG GG-3’), and R1202D/E1204K (sense DNA primer sequence of E1204R, to PCR ‘onto’ R1202D: 5’-GGC CGT GGA T\[CT TA\_A G\]TG TGT GGG C-3’; AfIII restriction site is in square brackets).