Supporting Information

Drugs Modulate Interactions Between the First Nucleotide-Binding Domain and the Fourth Cytoplasmic Loop of Human P-glycoprotein

Tip W. Loo and David M. Clarke

Department of Medicine and Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

Contents

1. Experimental Procedures.

Construction and Expression of Mutants – Cysteine mutations L443C, V472C and S909C were introduced into the human Cys-less histidine-tagged P-gp cDNA as described previously (*1*). HEK 293 cells were transfected with the mutant cDNAs by a calcium phosphate precipitation approach (*2*). The next day the medium was replaced with fresh medium containing 5 mM sodium butyrate and 5 μM cyclosporine A. The cells were incubated at 37 ^{o}C for 24 h and then incubated at 30 ^{o}C for 24 h. Expression of P-gp in the presence of drug substrates like cyclosporine A and incubation at 30 ^{o}C promotes maturation (*3, 4*). Sodium butyrate is a histone deacetylase inhibitor and has been used to increase expression of proteins in HEK 293 cells (*5*).

Assay of Drug-Stimulated ATPase Activity of Mutants – Histidine-tagged P-gp mutants were isolated by nickel-chelate chromatography as described previously (*6*). Recovery of P-gp was monitored by immunoblot analysis with rabbit anti-P-gp polyclonal antibody (*7*). A sample of the isolated histidine-tagged P-gp in 0.1% (w/v) n-dodecyl-β-D-maltoside was mixed with an equal volume of 10 mg/ml crude sheep brain phosphatidylethanolamine (Sigma, Oakville, ON) or TBS, pH 7.4. The P-gp:lipid mixture was then sonicated for 45 s at 4 ^{o}C (bath type probe,

maximum setting, Branson Sonifier 450, Branson Ultrasonic, Danbury, CT) and ATPase activity was assayed in the presence or absence of saturating levels of the drug substrates verapamil (300 μM), vinblastine (100 μM) or rhodamine B (1 mM) as described previously (*8*). Sheep brain lipids were used because P-gp basal ATPase activity is very low in these lipids (*9*).

Effect of Drug Substrates and Modulators on NBD1/ICL41 Cross-linking – To test for the effects of drug substrates or modulators on NBD1/ICL4 interactions, histidine-tagged P-gp mutants L443C, V472C, S909C, L443C/S909C, V472C/S909C or I306R/L443C/S909C were transiently expressed in HEK 293 cells and isolated by nickel-chelate chromatography as described above. Samples of the isolated mutants were added to equal volumes of TBS, pH 7.4 or 10 mg/ml sheep brain phosphatidylethanolamine (in TBS, pH 7.4). Samples were pre-incubated with or without saturating concentrations of the drug substrates verapamil (300 μM), vinblastine (100 μM) or rhodamine B (1 mM) for 30 min on ice. Stock solutions of drug substrates were prepared in DMSO and the equivalent amount of DMSO was added in the controls (final DMSO concentration was 5% (v/v)). Samples were then treated with or without oxidant (1 mM copper phenanthroline) for 3 min at 0 ^{o}C (mutants in lipids) or 5 min at 20 ^{o}C (mutants in detergent). Mutants in lipid were cross-linked at 0 ^{o}C and for a shorter incubation period to slow the reaction in order to detect any change in the presence of substrate. The reactions were stopped by addition of EDTA (10 mM final concentration). The samples were then mixed with 1 volume of 2X SDS sample buffer containing 25 mM EDTA with no thiol reducing agent and samples subjected to SDS-PAGE on 6.5% gels followed immunoblot analysis.

Modeling of human P-gp – The human P-gp structures (*11, 12*) were viewed using Pymol (*13*).

Analysis of Data – The signals from Western Blots were recorded and analyzed using ChemiDoc^{TM} XRS^{+} with Image Lab^{TM} software (Bio-Rad Lab. Inc., Mississauga, Ontario). The percent cross-linked is the amount of cross-linked protein relative to that of total (cross-linked plus mature (170 kDa) protein) and expressed as mean $\pm$ S.D. The Student T-test was used to test for statistical significance ($P < 0.001$; n=4).

2. Figures.

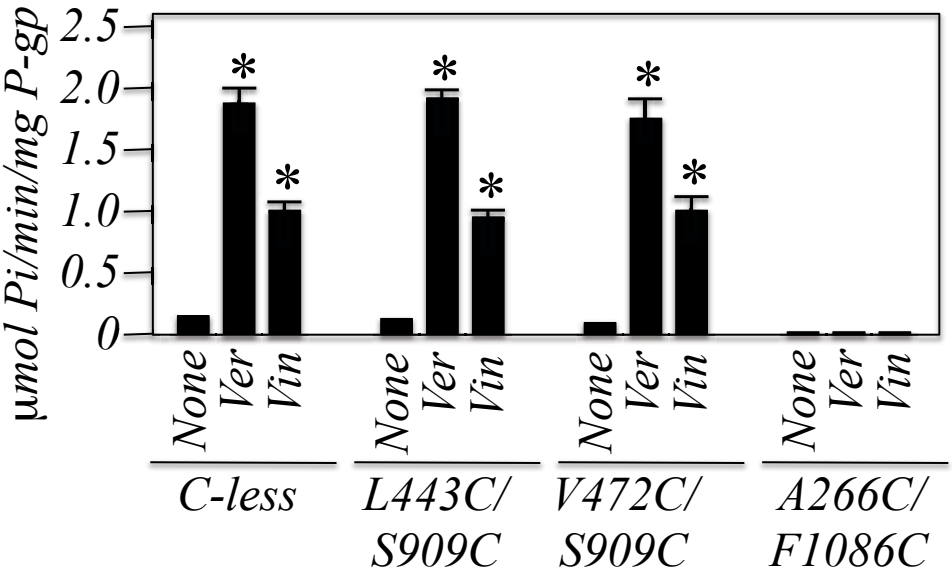

Figure S1. The ATPase activity of mutants L443C/S909C and V472C/S909C are stimulated by drug substrates. Histidine-tagged mutants were isolated by nickel-chelate chromatography, mixed with sheep brain lipid and assayed for ATPase activity in the absence (None) or presence of 0.3 mM verapamil (Ver) or 0.1 mM vinblastine (Vin). The A266C/F1086C mutant was included because it is located at the NBD2/ICL2 interface and is at an equivalent position to the L443C/S909C mutant at the NBD1/ICL4 interface. An asterisk indicates a significant difference compared to the absence of drug ($P < 0.001$, $n = 4$).

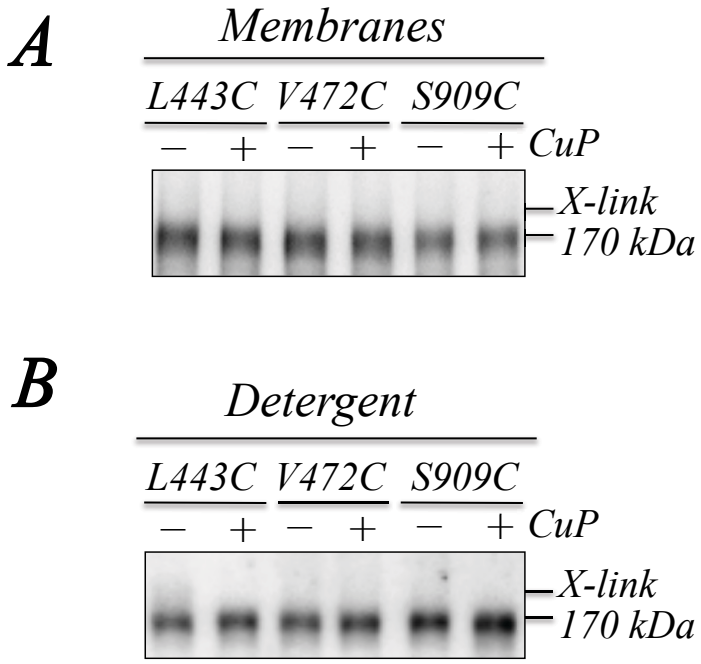

Figure S2. Single cysteine mutants do not cross-link. (A) Membranes from HEK 293 cells expressing histidine-tagged L443C, V472C or S909C were treated for 5 min at 20 ^{o}C without (-) or with (+) 1 mM copper phenanthroline (CuP). The reaction were stopped by addition of EDTA (final concentration of 10 mM) followed by one volume of 2x SDS sample buffer containing 25 mM EDTA but no thiol reducing agents. The samples were then subjected to SDS-PAGE followed by immunoblot analysis. (B) The histidine-tagged mutants were expressed in HEK 293 cells and isolated by nickel-chelate chromatography. Samples were then treated without (-) or with (+) copper phenanthroline (CuP) as in (A) and subjected to immunoblot analysis. The positions of cross-linked (X-link) and mature 170 kDa P-gp are indicated.

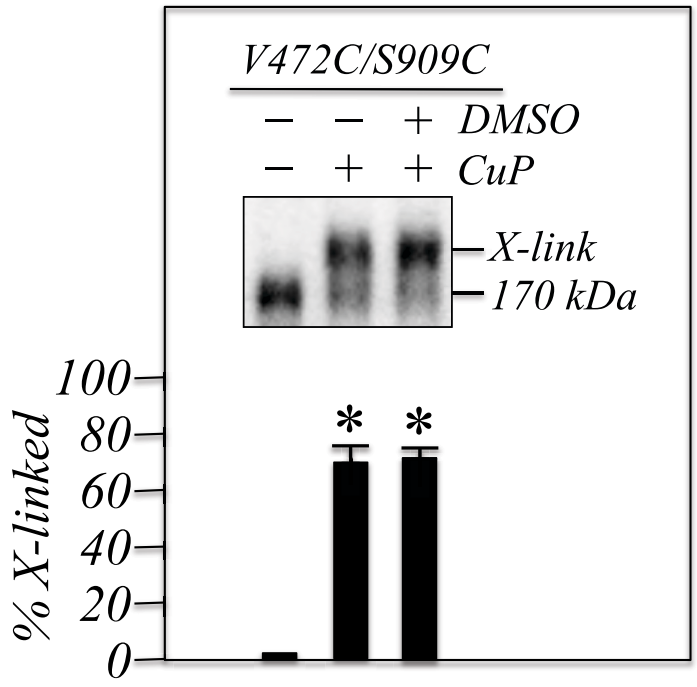

Figure S3. An amphiphile (DMSO) does not inhibit V472C/S909C cross-linking in detergent. Histidine-tagged mutant V472C/S909C was isolated by nickel-chelate chromatography and treated for 5 min at 20 ^{o}C without (-) or with (+) 1 mM copper phenanthroline (CuP) and in the absence (-) or presence (+) of DMSO (final concentration 20% (v/v)). The reactions were stopped by addition of 10 mM EDTA (final concentration) followed by one volume of 2X SDS sample buffer containing 25 mM EDTA and no thiol reducing agent. Samples were subjected SDS-PAGE followed by immunoblot analysis. An asterisk indicates a significant difference compared to the sample not treated with copper phenanthroline ($P < 0.001$, $n = 3$).

REFERENCES

1. Loo, T. W., Bartlett, M. C., Detty, M. R., and Clarke, D. M. (2012) The ATPase activity of the P-glycoprotein drug pump is highly activated when the N-terminal and central regions of the nucleotide-binding domains are linked closely together, *J Biol Chem 287*, 26806-26816.
2. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2009) Identification of residues in the drug-translocation pathway of the human multidrug resistance P-glycoprotein by arginine mutagenesis, *J. Biol. Chem. 284*, 24074-24087.
3. Loo, T. W., and Clarke, D. M. (1999) The transmembrane domains of the human multidrug resistance P-glycoprotein are sufficient to mediate drug binding and trafficking to the cell surface., *J. Biol. Chem. 274*, 24759-24765.
4. Loo, T. W., and Clarke, D. M. (1997) Correction of defective protein kinesis of human P-glycoprotein mutants by substrates and modulators, *J. Biol. Chem. 272*, 709-712.
5. Gorman, C. M., Howard, B. H., and Reeves, R. (1983) Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate, *Nucleic acids research 11*, 7631-7648.
6. Loo, T. W., and Clarke, D. M. (1995) Rapid purification of human P-glycoprotein mutants expressed transiently in HEK 293 cells by nickel-chelate chromatography and characterization of their drug-stimulated ATPase activities, *J. Biol. Chem. 270*, 21449-21452.
7. Loo, T. W., and Clarke, D. M. (1995) P-glycoprotein. Associations between domains and between domains and molecular chaperones, *J. Biol. Chem. 270*, 21839-21844.
8. Loo, T. W., and Clarke, D. M. (2015) The Transmission Interfaces Contribute Asymmetrically to the Assembly and Activity of Human P-glycoprotein, *J. Biol. Chem. 290*, 16954-16963.
9. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003) Drug binding in human P-glycoprotein causes conformational changes in both nucleotide-binding domains, *J. Biol. Chem. 278*, 1575-1578.
10. Urbatsch, I. L., and Senior, A. E. (1995) Effects of lipids on ATPase activity of purified Chinese hamster P-glycoprotein, *Arch. Biochem. Biophys. 316*, 135-140.
11. Bikadi, Z., Hazai, I., Malik, D., Jemnitz, K., Veres, Z., Hari, P., Ni, Z., Loo, T. W., Clarke, D. M., Hazai, E., and Mao, Q. (2011) Predicting P-glycoprotein-mediated drug transport based on support vector machine and three-dimensional crystal structure of P-glycoprotein, *PLoS One 6*, e25815.
12. Jin, M. S., Oldham, M. L., Zhang, Q., and Chen, J. (2012) Crystal structure of the multidrug transporter P-glycoprotein from Caenorhabditis elegans, *Nature 490*, 566-569.
13. DeLano, W. L. (2002) The PyMol Molecular Graphics System *DeLano Scientific, Palo Alto, CA. USA.* , <http://www.pymol.org>