Clarification added 30 November 2007; see below for details

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Supporting Online Material for

A "Silent" Polymorphism in the *MDR*1 Gene Changes Substrate Specificity

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Published 21 December 2006 on *Science* Express
DOI: 10.1126/science.1135308

This PDF file includes:

Clarification (30 November 2007):

Based on an inquiry from Jack Kornblatt, the authors wish to clarify that the protein sequence was obtained from a detailed mass spectrometric study performed at the Harvard Microchemistry Facility (HMF) by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry. HMF performed both chymotryptic and pronase digestions of the protein. In all, 82 peptides (representing 37% of the Pgp sequence by amino acid count) were identified and sequenced (see appended figure and accompanying legend, pages 16 and 17). Each of these sequences was identical to the sequence of haplotype P-glycoprotein. Moreover, several different peptides encoded by the synonymous SNP (3435C>T), which is the key polymorphism linked to the functional change in Pgp, were sequenced and found to be unchanged. In addition, the analysis of codon usage, table S1 in the original Supporting Online Material (pages 2 to 15) contains for each codon around the three polymorphisms the frequency of this codon per 1000 codons in the human genome instead of RSCU values as stated in the text. These values were obtained from the codon usage Web site at (www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). Figure 1, panels D to F, shows results in the presence of cyclosporin A (+CsA) not (+/-CsA) as indicated in the body of the figure. This is correctly stated in the legend. These clarifications do not affect the conclusions of the paper.

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Supporting Online Material

Materials and Methods

Cell Line, Cell Culture, and Propagation of the Vaccinia Virus

HeLa (cervical epidermoid carcinoma) cells, BSC-1 (epithelial cells of African green monkey kidney origin), Vero-76 (monkey kidney cells) and 12E1 (CEM human cells) were maintained in Dulbecco-modified Eagle's medium supplemented with 10% calf serum. The last three cell lines were kindly provided by Hana Golding, CBER, FDA, Bethesda, MD. Recombinant Vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3) was propagated in HeLa cells and purified as previously described (*1*). Titration of the virus was performed on HeLa infected/transfected cells using pTM1-MDR1 vector (*2*).

Vector Construction

The pTM1-MDR1 (*2*) plasmid, encoding wild-type P-gp, was used for the Vaccinia virus expression system. Three different polymorphisms in the coding region of the human *MDR*1 cDNA have been described in the literature (positions 1236, 2677, and 3435 bp). These three polymorphisms were generated in the pTM1-MDR1 vector in order to examine their impact on P-gp cell surface expression and on P-gp function. Using site-directed mutagenesis (Stratagene, La Jolla, CA) mutated sites were introduced into the *MDR*1 gene: For position 1236 the

nucleotide C was changed to T, for position 2677 the nucleotide G was changed to T, and for position 3435 the nucleotide C was changed to T and another construct was changed to A. All combinations of the double mutants as well as the haplotype C1236T-G2677T-C3435T were generated as well. All construct sequences were verified in both directions by automated sequencing with the PRISM Ready Reaction Dye Deoxy Terminator Sequencing Kit (Perkin-Elmer Corporation, Norwalk, CT).

Infection/transfection into HeLa Cells

Recombinant Vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3), which is required for the expression of a gene under the control of a T7 promoter, was obtained from Dr. B. Moss (NIAID, NIH, Bethesda, MD). Cells were infected/transfected with vTF7-3 and various plasmids as previously described (*3, 4*), and incubated for 20-24 hr at 32°C, 5% CO_2.

Determination of *MDR*1 gene expression levels by real-time quantitative RT-PCR

*MDR*1 mRNA levels were evaluated by real-time quantitative RT-PCR utilizing the LightCycler RNA Master SYBR Green Kit and LightCycler (Roche Applied Science, Indianapolis, IN) using 1 ng of total RNA. Total RNA was extracted from Vaccinia virus infected/ *MDR*1 WT or haplotype-transfected HeLa cells by the Qiagen Rneasy kit (Valencia, CA), as per the manufacturer's protocol, and then further purified using acid-phenol:chloroform 5:1 pH 4.7 extraction to ensure complete removal of plasmid DNA. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The *MDR*1 specific forward primer 5'-GCCTGGCAGCTGGAAGACAAATAC-3' and the reverse primer 5'-ATGGCCAAAATCACAAGGGTTAGC-3' were used in these studies.

Real-time quantitative RT-PCR

Analysis with real-time quantitative RT-PCR revealed that *MDR*1 mRNA levels were the same in the wild type and two haplotype transfected HeLa cells, and little or no *MDR*1 mRNA was found in the pTM1 transfectants, as expected. Transfection with either the C1236T-G2677T-C3435T or C1236T-G2677T-C3435A haplotype did not change MDR1 mRNA levels as compared to wild type. The level of *MDR*1 mRNA was positively correlated with the amount of plasmid utilized in the infection-transfection protocol described above.

P-gp Expression

Levels of cell surface P-gp were detected by FACS, using monoclonal antibody MRK16 (*5*). The conformational sensitive UIC2 monoclonal antibody as well as the 17F9 monoclonal antibody were tested at three different temperatures (4°C, 25°C and 37°C) as previously described (*6*), alone or together with the following drugs: taxol, cyclosporin A, verapamil, and their combination. The monoclonal antibody 17F9 was used in a form conjugated with phycoerythrin (BD Biosciences - Immunocytometry Systems) and therefore was measured at the FL2 wavelength.

Crude membranes were prepared from vTF7-3 infected HeLa cells transfected with vector pTM1-*MDR*1 vectors as described previously (*4, 7*) and stored at -70°C. Total protein was quantified by Amido Black protein estimation method as previously described (*8*) and P-gp expression level was determined by immunoblot analysis using the monoclonal antibody C219 (*2, 9*).

Preparation of cells for confocal imaging was done as follows: Prior to immunostaining and between each immunostaining step, transduced cells were washed twice with PBS (Invitrogen, Carlsbad, CA) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, MO). Transduced cells were first fixed for 0.5 hr with 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) or with additional fixation for 0.5 hr with 70% ethanol at room temperature. The presence of P-gp was detected using MRK16. Cells were then washed once with PBS / 0.1% BSA and stained with secondary antibody, Alexa 488 (green)-conjugated donkey anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:250. In all experiments, cells were stained using a secondary antibody alone to determine non-specific staining. Antibody incubations were performed for 1 h at room temperature. After the last antibody incubation, the cells were washed with PBS/ 0.1% BSA as before, and fluorescent mounting medium (DAKO Corp., Carpinteria, CA) was then used to affix a glass coverslip to the microscope slide, and the slides were stored in the absence of light at 4°C. Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optihot microscope with a 60X planapochromat lens. Excitation at 488 nm was provided by a krypton-argon laser.

Drug Accumulation and Efflux Assays

5×10^5 cells were harvested after trypsinization by centrifugation and resuspended in 1 ml Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 5% FBS. The fluorescent substrates, Rh123 (1 μg/ml), bodipy-FL-paclitaxel (0.1 μM), bodipy-FL-verapamil (0.5 μM), daunorubicin (3 μM), bodipy-FL-vinblastine (0.5 μM), calcein-AM (0.5 μM), (Molecular Probes, Eugene, OR) were added to cells in the presence or absence of the P-gp inhibitor and incubated at 37° C for 20 min. The following inhibitors were used: cyclosporin A (5 μM) (Calbiochem, San Diego, CA), verapamil (5-20 μM), rapamycin (5-20 μM), digoxin

(250-1000 μM) and, fexofenadine (370 μM). Two different protocols were tested: pre-incubation of the inhibitor for 15 minutes prior to adding the drug, and simultaneous incubation with the drug. For daunorubicin efflux, an additional incubation with only IMDM or IMDM with cyclosporin A was performed for 40 min at 37°C. For the accumulation assay, the pellet was resuspended in 300 μl PBS prior to FACS analysis (*2*) using CellQuest software (Becton Dickinson, Mountain View, CA). For the efflux assay, the cells were resuspended with or without the inhibitors and incubated at 37°C for 40 min. Prior to FACS analysis cells were washed and resuspended as described earlier. The difference between the median value of the fluorescence substrate curve and the median value of the fluorescence substrate with an inhibitor (arbitrary units) was plotted vs. the harvesting time post infection/transfection.

Determining the IC_{50} for trypsinization of P-gp forms

Crude membranes (1 mg/ml) of wild-type and the C1236T-G2677T-C3435T haplotype were treated with increasing concentrations of trypsin for 5 min at 37°C. The reaction was stopped by the addition of 5-fold excess soybean trypsin inhibitor. Following SDS-PAGE, and transfer to nitrocellulose paper the P-gp bands were identified by immunoblot analysis using the monoclonal antibody C219 as described previously. The X-ray film was scanned and the P-gp bands were quantified using ImageQuaNT software. The IC_{50} for the disappearance of the band corresponding to intact P-gp as a function of trypsin concentration was determined using PRIZM software.

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Supplemental Figures

Fig. S1. Drug transport by wild-type and eight *MDR*1 SNPs and haplotypes. The drug efflux of Vaccinia infected-transfected HeLa cells was determined by FACS analysis. Cells were transfected with pTM1 (control; purple), pTM1-MDR1, (wild-type P-gp; green), C1236T (pink), G2677T (light purple), C3435T (orange), pTM1-MDR1-C1236T-G2677T (blue), C1236T-

C3435T (yellow), G2677T-C3435T (light blue) and C1236T-G2677T-C3435T (red). 24 h post-infection/transfection, cells were harvested, washed and loaded for 20 min. with the following compounds, washed again and incubated for 45 min. (efflux) all at 37°C prior to FACS analysis: (**A**) 0.1 μM bodipy-FL-paclitaxel in the presence of an inhibitor, 5 μM verapamil; (**B**) 0.5 μM bodipy-FL-verapamil in the presence of an inhibitor, 5 μM CsA. (**C**) 0.5 μM Rh123 and 5 μM verapamil – SNPs only; (**D**) 0.5 μM Rh123 in the presence of an inhibitor, 5 μM verapamil; haplotypes only (**E**) 0.5 μM Rh123 and 5 μM verapamil – all vectors. (**F**) 0.5 μM Rh123 and 5 μM rapamycin – wild-type, the haplotype C1236T-G2677T-C3435T.

Fig. S2. Comparison of P-gp expression levels of HeLa infected/transfected *MDR*1 cells with those of normal human adrenal glands. Assessment of P-gp total expression using the C219 monoclonal antibody on wild-type, the haplotype C1236T-G2677T-C3435T and adrenal gland sample (Virotech International, Inc., Gaithersburg, MD). Immunoblot analysis was performed 24 h post 5 μg infection/transfection (2.5 μg protein/lane) of wild-type (lane 1) and haplotype (lane 2), 2.5 μg (lane 3) and 5 μg protein (lane 4) from protein lysates of normal human adrenal glands.

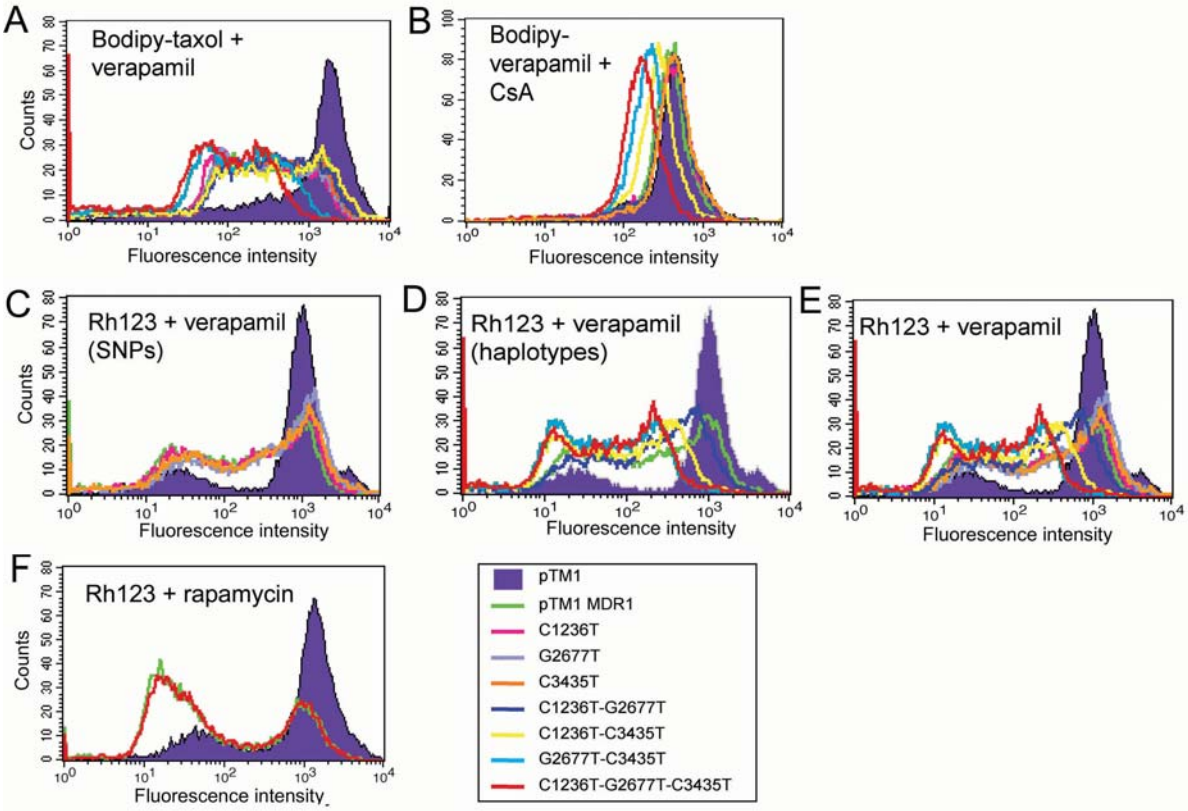
Fig. S3. Drug accumulation of HeLa cells expressing wild-type and *MDR*1 haplotype C1236T-G2677T-C3435T. The drug accumulation in Vaccinia virus infected-transfected HeLa cells was determined by FACS analysis. Cells were transfected with pTM1 (control; purple), pTM1-MDR1, (wild-type P-gp; green), and C1236T-G2677T-C3435T (red). 24 h post-infection/transfection, cells were harvested, washed and incubated for 20 min. with 2 mM bodipy-FL-verapamil at 37°C and subjected to FACS analysis.

Fig. S4. Expression and function of infected/transfected *MDR*1in BSC1 monkey cells. (**A**) Assessment of cell surface expression using MRK16 monoclonal antibody pTM1 (control; purple), pTM1-MDR1, (wild-type P-gp; green), C1236T-G2677T-C3435T (red); (**B**) Efflux of Rh123 in the presence of 150 mM digoxin of Vaccinia virus infected-transfected BSC1 cells as determined by FACS analysis: pTM1 (control; purple), pTM1-MDR1, (wild-type P-gp; green), C1236T-G2677T-C3435T (red).

Fig. S5. Expression studies using UIC2 conformational sensitive monoclonal antibody, MRK16 and 17F9 monoclonal antibodies (A) Assessment of cell surface expression using UIC2 conformational sensitive monoclonal antibody pTM1 (control; purple), pTM1-MDR1, (wild-type

P-gp; green), C1236T-G2677T-C3435T (red) of Vaccinia virus infected-transfected HeLa cells as determined by FACS analysis; (**B**) Infected/transfected cells in the presence of MRK16 specific monoclonal antibody (same constructs as in **A**). (**C**) Infected/transfected cells in the presence of 17F9 monoclonal antibody (same constructs as in **A**).

Figure S1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Fluorescence intensity
B
Bodipy-verapamil + CsA
Fluorescence intensity
C
Rh123 + verapamil (SNPs)
Counts
Fluorescence intensity
D
Rh123 + verapamil (haplotypes)
Fluorescence intensity
E
Rh123 + verapamil
Fluorescence intensity
F
Rh123 + rapamycin
Counts
Fluorescence intensity
pTM1
pTM1 MDR1
C1236T
G2677T
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Figure S2

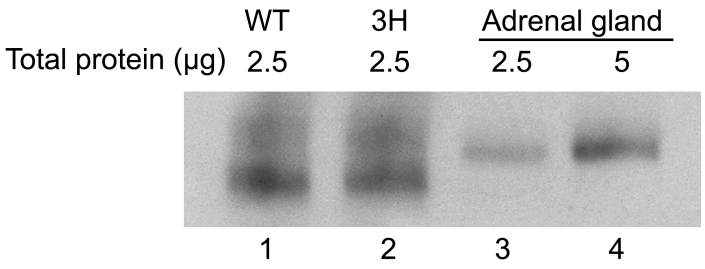
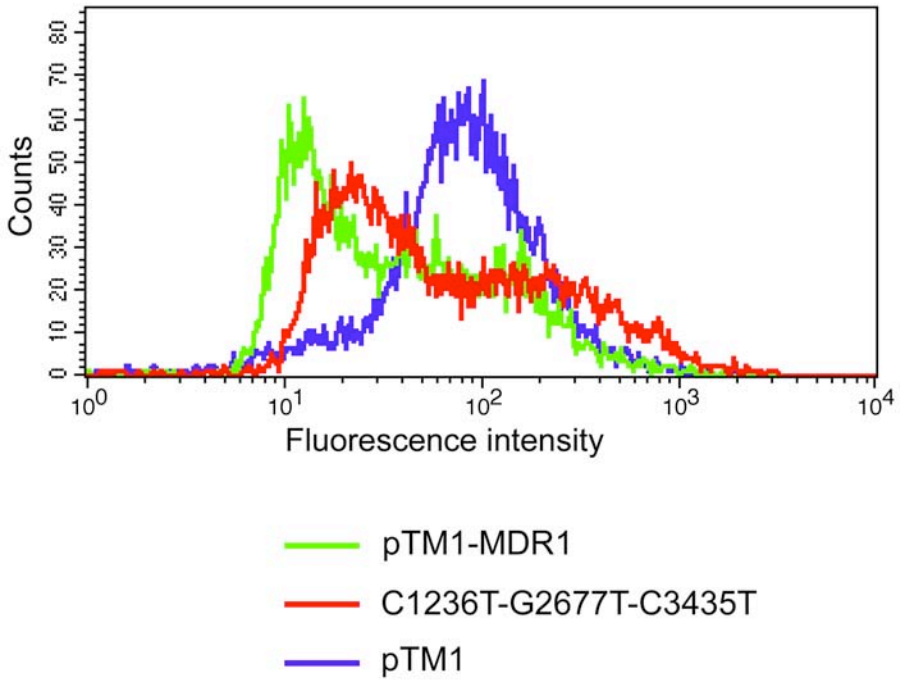


Figure S3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30
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50
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70
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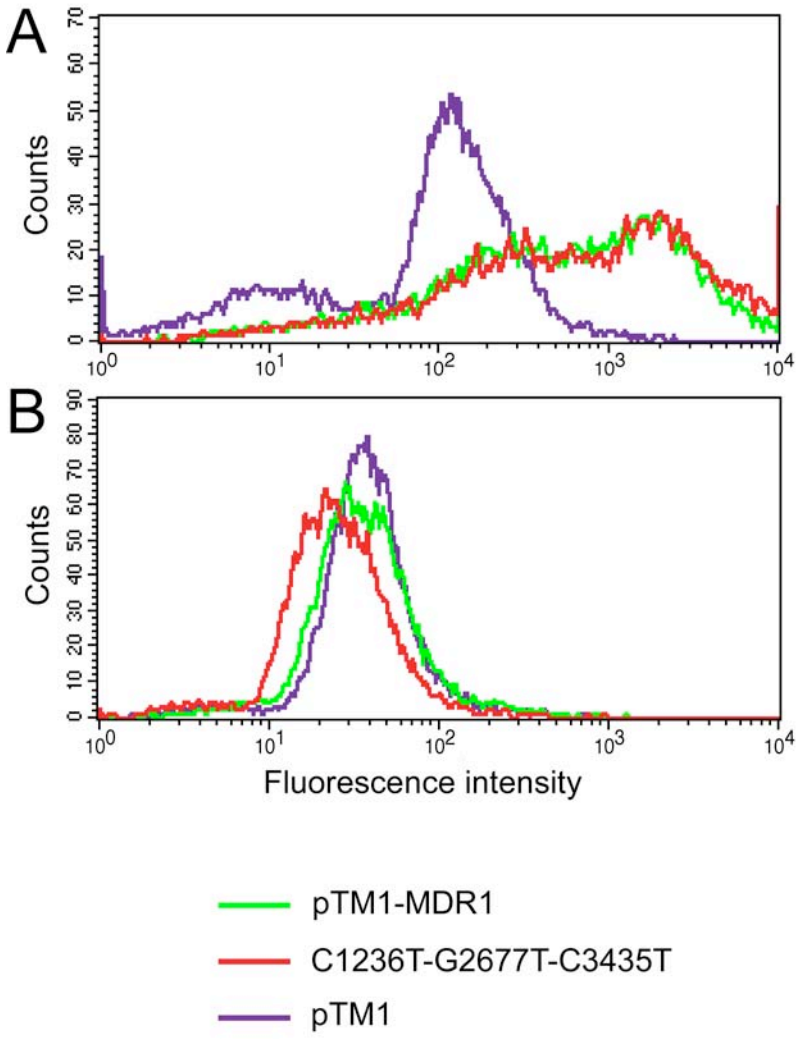
Fluorescence intensity
pTM1-MDR1
C1236T-G2677T-C3435T
pTM1

Figure S4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Fluorescence intensity
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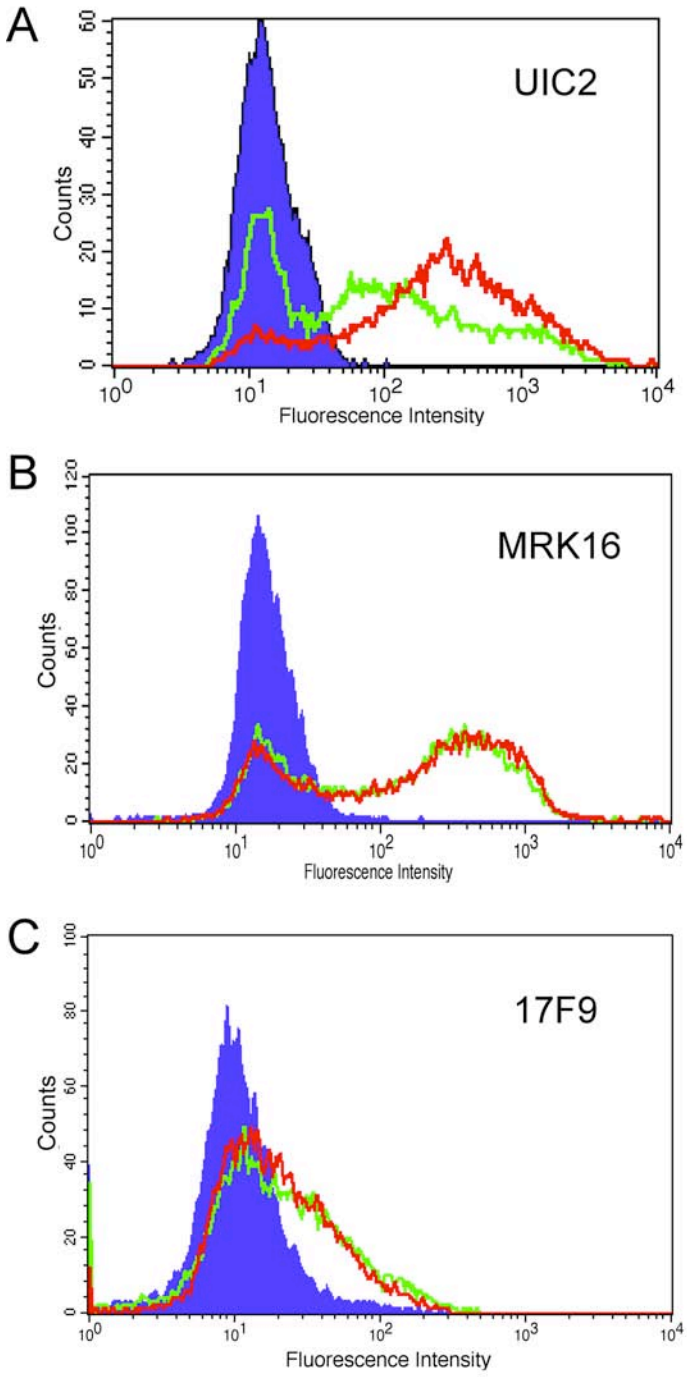
C1236T-G2677T-C3435T
pTM1

Figure S5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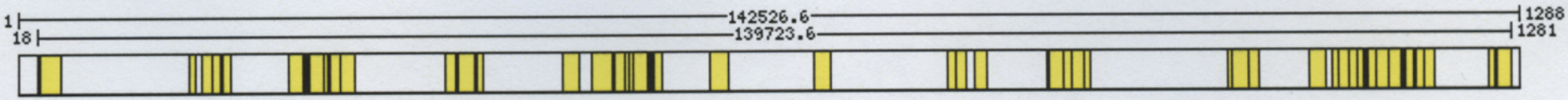
UIC2
Counts
Fluorescence Intensity
B
MRK16
Counts
Fluorescence Intensity
C
17F9
Counts
Fluorescence Intensity

Table S1. RSCU values for 15 codons surrounding the 1236, 2677 and 3435 SNPs

								SNP							
1236	**AAA**	**GAA**	**GTT**	**AAG**	**ATC**	**TTG**	**AAG**	**GGT**	**CTG**	**AAC**	**CTG**	**AAG**	**GTG**	**CAG**	**AGT**
Max	32.0	39.6	28.3	32.0	22.1	39.9	32.0	22.4	39.9	19.1	39.9	32.0	28.3	34.2	19.4
Act	24.2	28.7	11.0	32.0	22.1	12.8	32.0	10.8	39.9	19.1	39.9	32.0	28.3	34.2	12.1
2677	**GAT**	**AAG**	**AAA**	**GAA**	**CTA**	**GAA**	**GGT**	**TCT**	**GGG**	**AAG**	**ATC**	**GCT**	**ACT**	**GAA**	**GCA**
Max	25.2	32.0	32.0	39.6	39.9	39.6	22.4	17.7	22.4	32.0	20.9	28.5	19.0	39.6	28.0
Act	21.7	32.0	24.0	28.7	7.1	28.7	10.2	15.1	16.5	32.0	20.9	18.5	13.0	28.7	15.9
3435	**CGG**	**GTG**	**GTG**	**TCA**	**CAG**	**GAA**	**GAG**	**ATT**	**GTG**	**AGG**	**GCA**	**GCA**	**AAG**	**GAG**	**GCC**
Max	11.5	28.3	28.3	17.7	34.2	39.6	39.6	20.9	28.3	12.0	28.0	28.0	32.0	39.6	28.0
Act	11.5	28.3	28.3	12.2	34.2	28.7	39.6	15.8	28.3	11.9	15.9	15.9	32.0	39.6	28.0

RSCU values of the 15-codon neighborhood surrounding each of the 3 SNPs: 1236, 2677 and 3435 (in red). For each codon, the maximal RSCU ("Max"; i.e., the most common codon usage for this amino acid), and the actual ("Act") RSCU are shown. The cells are shaded yellow where the actual codons are not the most common ones.

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81    NAGNLEDLMS NITNRSDIND TGFFMNLEED MTRYAYYYSG IGAGVLVAAY IQVSFWCLAA GRQIHKIRKQ FFHAIMRQEI
161   GWFDVHDVGE LNTRLTDDVS KINEVIGDKI GMFFQSMATF FTGFIVGFTR GWKLTLVILA ISPVLGLSAA VWAKILSSFT
241   DKELLAYAKA GAVAEEVLAA IRTVIAFGGQ KKELERYNKN LEEAKRIGIK KAITANISIG AAFLLIYASY ALAFWYGTTL
321   VLSGEYSIGQ VLTVFFSVLI GAFSVGQASP SIEAFANARG AAYEIFKIID NKPSIDSYSK SGHKPDNIKG NLEFRNVHFS
401   YPSRKEVKIL KGLNLKVQSG QTVALVGNSG CGKSTTVQLM QRLYDPTEGM VSVDGQDIRT INVRFLREII GVVSQEPVLF
481   ATTIAENIRY GRENVTMDEI EKAVKEANAY DFIMKLPHKF DTLVGERGAQ LSGGQKQRIA IARALVRNPK ILLLDEATSA
561   LDTESEAVVQ VALDKARKGR TTIVIAHRLS TVRNADVIAG FDDGVIVEKG NHDELMKEKG IYFKLVTMQT AGNEVELENA
641   ADESKSEIDA LEMSSNDSRS SLIRKRSTRR SVRGSQAQDR KLSTKEALDE SIPPVSFWRI MKLNLTEWPY FVVGVFCAII
721   NGGLQPAFAI IFSKIIGVFT RIDDPETKRQ NSNLFSLLFL ALGIISFITF FLQGFTFGKA GEILTKRLRY MVFRSMLRQD
801   VSWFDDPKNT TGALTTRLAN DAAQVKGAIG SRLAVITQNI ANLGTGIIIS FIYGWQLTLL LLAIVPIIAI AGVVEMKMLS
881   GQALKDKKEL EGSGKIATEA IENFRTVVSL TQEQKFEHMY AQSLQVPYRN SLRKAHIFGI TFSFTQAMMY FSYAGCFRFG
961   AYLVAHKLMS FEDVLLVFSA VVFGAMAVGQ VSSFAPDYAK AKISAAHIIM IIEKTPLIDS YSTEGLMPNT LEGNVTFGEV
1041  VFNYPTRPDI PVLQGLSLEV KKGQTLALVG SSGCGKSTVV QLLERFYDPL AGKVLLDGKE IKRLNVQWLR AHLGIVSQEP
1121  ILFDCSIAEN IAYGDNSRVV SQEEIVRAAK EANIHAFIES LPNKYSTKVG DKGTQLSGGQ KQRIAIARAL VRQPHILLLD
1201  EATSALDTES EKVVQEALDK AREGRTCIVI AHRLSTIQNA DLIVVFQNGR VKEHGTHQQL LAQKGIYFSM VSVQAGTKRQ
1281  LAHHHHHH
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Appended Figure, added on 11/30/07

Legend to Appended Figure: Identification and sequencing of chymotryptic and pronase digestions of the protein P-gp. Detailed mass spectrometric studies were performed at the Harvard Microchemistry Facility (HMF) by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry. Both chymotryptic and pronase digestions of the protein were performed. The yellow highlights represent the coverage of the sequence and the green lines the individual peptides that were identified. In all, 82 peptides (representing 37% of the P-gp sequence by amino acid count) were identified and sequenced. Each of these sequences was identical to the sequence of haplotype P-gp. Amino acids corresponding to the SNPs were Gly412 (not identified), Ala893→Ser (change identified) and Ile1145 (6 peptides identified and sequenced). Note however that the methodology does not distinguish the isobaric residue pair Leu/IIe.