Heterologous Expression and ATPase Activity of Mutant versus Wild Type PfMDR1 Protein†

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ABSTRACT: Mutation of the *P. falciparum* chloroquine resistance transporter (PfCRT) causes resistance to chloroquine (CQ) and other antimalarial drugs. Mutation and/or overexpression of one of the multidrug resistance protein homologues found in this malarial parasite (PfMDR1) may further modify or tailor the degree of multidrug resistance. However, considerable controversy surrounds the precise contribution of PfMDR1, in part because no direct biochemical studies of PfMDR1 have yet been possible. Using codon optimization and other principles, we have designed and constructed a yeast optimized version of the wild type *pfmdr1* gene and have successfully overexpressed PfMDR1 protein in *P. pastoris* yeast. The protein is well expressed in either full length form or as two separate half transporters, is well localized to the yeast plasma membrane and is fully functional as evidenced by ATPase activity measurements. We have also expressed mutants that have previously been hypothesized to influence drug resistance in parasites. Using purified plasma membrane fractions, we have analyzed antimalarial drug effects on ATPase activity for wild type versus mutant proteins. Relative to other ABCB transporters involved in drug resistance, PfMDR1 is unusual. It has similar pH, [ATP], and Mg^{++} dependencies for ATP hydrolysis, yet relatively high K_m and V_{max} values for ATP hydrolysis, and ATPase activity is only mildly stimulated by antimalarial drugs. The largest measured drug effect is for CQ (to which PfMDR1 is not believed to confer resistance), and it is strongly inhibitory for WT PfMDR1. Drug resistance associated PfMDR1 mutants show either elevated (Dd2 allele encoded) or reduced (7G8 allele) basal ATPase activity and different patterns of drug stimulation or inhibition, relative to WT PfMDR1. The Dd2 PfMDR1 isoform also shows a slightly more alkaline pH optimum. Surprisingly, verapamil alone (1−300 μM) does not significantly affect WT ATPase activity but inhibits the Dd2 isoform at 1 μM. These data should assist ongoing analysis of the contribution of PfMDR1 to antimalarial drug resistance.

Chloroquine resistance (CQR[1]) in *P. falciparum* malaria requires mutations in the parasite PfCRT protein (*1*−*3*). A number of mutant PfCRT alleles that confer CQR are known to exist, and *P. falciparum* strains harboring these continue to both spread and evolve around the globe. These strains have different geographic origin (e.g., Africa vs South America), and their precise sensitivity to specific antimalarial drugs varies, but in general, mutation of PfCRT confers an approximately 10-fold increase in CQ IC_{50} that can be (but is not always) reversed by sublethal doses of verapamil (VPL). CQR is typically accompanied by resistance to other drugs, particularly related quinoline-based compounds, such as quinine (QN), quinidine (QD), amodiaquine (AQ), and mefloquine (MQ). That is, CQR-conferring mutations in PfCRT are likely involved in conferring some degree of these pleiotropic "quinoline resistance" phenomena (*2*). However, there is mounting evidence that mutations in and/or alternate expression of other proteins further modulate or augment CQR conferred by PfCRT mutations, particularly high level MQR and/or QNR concomitant with CQR. These additional phenomena may also contribute to multidrug resistance (MDR[2]), meaning simultaneous resistance to multiple chemical classes of drugs, such as quinolines, acridines, reactive

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[1] Abbreviations: PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; CQ, chloroquine; PfMDR1, *Plasmodium falciparum* multidrug resistance protein; WT, wild type; ABC, ATP-binding cassette; CQR, chloroquine resistance (resistant); VPL, verapamil; QN, quinine; QD, quinidine; AQ, amodiaquine; MQ, mefloquine; QR, quinoline resistance (pleiotropic quinoline resistance); MDR, multidrug resistance; Pgh1, P-glycoprotein homologue [PfMDR1]; PfNHE1, *Plasmodium falciparum* Na^+/H^+ exchanger 1; HF, halofantrine; ART, artemisinin; CQS, chloroquine sensitive; QNR, quinine resistance; PM, plasma membrane; NBD, nucleotide binding domain; TMD, transmembrane domain; ABCB, B subfamily ABC transporter; DV, digestive vacuole; Pgp, P-glycoprotein.

[2] Although used in a general sense, regardless of the system being examined (tumor cells, parasites, and bacteria), multidrug resistance refers to a spectrum of phenomena that may vary widely with regard to the drugs in question, the degree of resistance, and the number and type of genetic events linked to the resistance phenotype. The term is usually meant to indicate pleiotropic resistance to multiple chemical classes of drugs or multiple pharmacophores; thus, we use the term quinoline resistance [QR] when referring to genotypic or phenotypic features known to mediate resistance to multiple quinolines (CQ, QN, AQ, etc.) but for which relevance to MDR phenomena is less well understood.

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endoperoxides, and so forth (but neglecting antifolates, resistance to which is typically conferred by mutations in one or more pyrimidine pathway enzymes). These MDR linked phenomena include alternate expression and/or mutation of the *P. falciparum* human P-glycoprotein homologue PfMDR1 (also called Pgh1) and the *P. falciparum* sodium proton exchanger (PfNHE1 (*4*−*10*)).

A number of conflicting observations surround the putative link between PfMDR1 protein and antimalarial drug resistance. In the early 1990s, when altered expression and/or mutation of PfMDR1 was widely assumed to be the principal cause of *P. falciparum* CQR in much of the drug resistance literature, Wellems and colleagues showed that the chromosomal locus harboring PfMDR1 did not segregate with the CQR phenotype in progeny of a genetic cross (*6*, *7*). This argued for little-to-no role for PfMDR1 in CQR. Indeed, subsequently, mutations in another vacuolar membrane protein (PfCRT) were shown to be the primary cause of CQR in *P. falciparum* (*1*). However, the Cowman group published results arguing for a small, but measureable, effect on sensitivity to CQ, QN, MQ, HF, and ART due to three mutations in the C-terminal half of PfMDR1 (S1034C, N1042D, and D1246Y) (*8*). These mutations represent three of the four found in the *pfmdr1* allele for CQR strain 7G8 (South America). Reversion of the three (back to wild type S, N, and D) mildly decreased the degree of CQR for strain 7G8, whereas their introduction into a CQS strain conferred 2-fold resistance to QN and MQ, and mild increased susceptibility to HF and ART. Following this report, Sidhu et al. (*9*) performed similar experiments but noted only mild QNR upon introduction of the triple mutant and only in one of two transfected strains. Recent field data (*5*) suggest that overexpression but not necessarily the mutation of *pfmdr1* is more relevant to drug resistance observed in the field. This is consistent with some earlier results from Wirth, Cowman, and other laboratories with cultured parasite cell lines (*10*) as well as more recent analysis of drug induced effects on *pfmdr1* transcription (*11*). Perhaps multiple contributions of PfMDR1 to drug resistance are possible in a strain specific manner that depends upon the *pfcrt* allele that is present. Another hypothesis is that some mutant PfMDR1 proteins perform a function similar to that promoted by increased expression of the wild type. In sum, the role of PfMDR1 in antimalarial drug resistance is important but is likely relatively minor and critically depends on the presence of other mutations (e.g., PfCRT).

Further molecular level analysis of PfMDR1 function and of its precise contribution to antimalarial drug resistance is hampered by the protein's vacuolar membrane localization within an intracellular parasite. Clearly, further studies would benefit from the availability of more convenient experimental systems. However, there are significant challenges to high level heterologous expression of this protein that have not previously been met because of the very high AT content of the *pfmdr1* gene.

A previous study by Gros and co workers reported expression of the native *pfmdr1* cDNA in yeast on the basis of indirect evidence (*12*), but this paper was subsequently retracted (*13*). In hindsight, the very high AT content (75%) along with other features of the *pfmdr1* gene likely precluded efficient, stable expression (*14*). Indeed, no western blot confirmation of PfMDR1 expression was reported, and the published retraction (*13*) suggests that inadvertent false positive transfection with the PfMDR1 homologue STE6 was likely responsible for some, if not all, of the phenotypic features analyzed in the selected clones. Low levels of expression for some endogenous *P. falciparum* cDNAs (encoding smaller soluble proteins or soluble domains of larger proteins) have been reported, but to our knowledge, the combination of unusual gene structure, large size, and other features has prevented routine high level heterologous overexpression of large malarial parasite polytopic integral membrane proteins (*14*).

Therefore, similar to earlier work with PfCRT (*14*), we have back translated the PfMDR1 protein sequence and have designed a yeast optimized synthetic *pfmdr1* gene. We have fused this to poly His and biotin acceptor-encoding domains, and report stable high level, inducible overexpression of WT PfMDR1 protein in *P. pastoris* yeast. We are able to express the two half transporters as well as the full length version of the protein in biotinylated form and report similarly efficient expression of 3D7, Dd2, and 7G8 isoforms of PfMDR1. Because drug stimulation of ATPase activity is considered to be a hallmark of ABCB protein-mediated drug resistance phenomena, we compare the ATPase activities of these isoforms under various conditions to test hypotheses for their role in antimalarial drug resistance phenomena.

MATERIALS AND METHODS

Materials. *Pfu*Ultra was from Stratagene (La Jolla, CA). Streptavidin HRP and ECL detection reagents were from Amersham Biosciences (Piscataway, NJ). Prestained SDS−PAGE molecular markers were from Bio-Rad (Hercules, CA). The PentaHis HRP detection kit, mini-elute spin columns, and miniprep spin columns were from Qiagen (Valencia, CA). *Pichia* plasmids and expression reagents were from Invitrogen (Carlsbad, CA). Oligonucleotides were from MWG Biotech (High Point, NC) and Genscript (Piscataway, NJ). All other reagents were reagent grade or better and were purchased from Sigma (St. Louis, MO).

Strains and Growth Conditions. The *Escherichia coli* strain DH5 α (F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) was used for all routine subcloning work. *Pichia pastoris* strains KM71 and X-33 from Invitrogen were used for heterologous expression of PfMDR1 isoforms. Yeast strain X-33 harboring pPICZc/3′Pfmdr16HB, KM71 harboring pPIC3.5/3′Pfmdr16HB, and KM71 harboring pPICZc/5′Pfmdr16HB or pPICZc/Pfmdr16HB were selected for growth on YPD medium + 100 mM zeocin, minimal glycerol medium lacking histidine, and minimal glycerol medium supplemented with 100 mM zeocin, respectively.

Design and Synthesis of the Synthetic (Yeast Optimized) WT pfmdr1 Gene. Design and synthesis of the yeast optimized WT *pfmdr1* gene followed procedures outlined previously (*14*) with a few modifications. Because of the very large size of *pfmdr1* and previous observations that suggest that 12 helix-2 NBD ABC transporters can be well expressed as half transporter cassettes (N- or C-terminal half 6 helix-1 NBD polypeptides; see ref *15*), we first constructed the yeast optimized gene as two cassettes (*pfmdr5′* and *pfmdr3′*). The first 2250 nucleotides of the *pfmdr1* gene sequence were obtained from GenBank (www.ncbi.nlm.ni-

h.gov) and translated. A total of 114 40-mers were constructed that encoded both strands of the theoretically optimized gene (*14*). Equal volumes of all 40-mers were combined (1.5 μM each), and the resultant mixture was diluted 25-fold in *Pfu*Ultra buffer supplemented with 0.5 mM each dNTP, 2 mM Mg^{2+}, and 1.25 U *Pfu*Ultra. The initial assembly PCR program was one denaturation step at 95 °C for 1 min, followed by 40 cycles of 95 °C (45 s), 52 °C (45 s), 72 °C (3 min), and finally a 10 min incubation at 72 °C. One microliter of this assembly solution was diluted 50-fold in similar buffer but with 1 μM of oligonucleotides #1 and #58 (the 5′- and 3′-flanking primers). The amplification PCR program was one denaturation step at 95 °C for 1 min, followed by 30 cycles of 95 °C (1 min), 60 °C (1 min), 72 °C (5 min), and a final 10 min incubation at 72 °C. After subcloning and propogation in *E. coli*, several PCR product clones were fully sequenced, and the one with the fewest errors was selected for further work. All spurious PCR errors were corrected using the multisite directed mutagenesis kit (Stratagene) and the completed gene confirmed by sequencing in both directions. Similar procedures were followed to synthesize the last 2115 nucleotides of the yeast optimized *pfmdr1* sequence (*pfmdr3′*), using the custom gene synthesis services of the Genscript corporation (Piscataway, NJ). Yeast expression vectors based on pPIC3.5 or pPICz from Invitrogen (see above) and containing either *pfmdr5′*, *pfmdr3′*, or the fused full length *pfmdr1*, an inducible alcohol oxidase promoter, and other convenient added features as described previously (*14*) were used to express WT and mutant (see below) PfMDR1 proteins.

Synthesis of Mutant pfmdr1 Genes. Three of the PfMDR1 mutations that have been proposed to play a role in antimalarial drug resistance are found in the C-terminal cassette (*pfmdr3′*), whereas two others are found in the N-terminal cassette. Three primers were designed to create the common S1034C, N1042D, and D1246Y mutations. The multisite directed mutagenesis kit (Stratagene) was used according to manufacturer's instructions. pPICZc/*pfmdr3′* was used as template with the following primers:

5′-CGCTGCACTTTGGGGATTC***T***GCCAATCG-GCACAACTGTTC-3′,

5′-AATCGGCACAACTGTTCATC***G***ACTCGT-TCGCGTACTGGTT-3′, and

5′-TGCGACTACAACCTTAGG***T***ACCTAC-GAAACCTCTTCTCAA-3′, respectively.

To create mutations in the 5′ cassette, found in PfMDR1 Dd2 (N86Y) or 7G8 (Y184F) isoforms, we used the following oligos:

5′-GGTTATCTTGAAGAACATG***T***ACTTGGGG-GACGATATCAAC-3′, and

5′-GTCTTTCCTTGGGCTGT***T***CATCTGGTC-CCTGATCAAGAAC-3′, respectively.

Yeast Transformations. Yeast were transformed with either the LiCl method using 50 μg of salmon sperm DNA as the carrier or via the *Pichia* transformation kit (for the pPICZc plasmids). Both methods used 3 μg of linearized target DNA. Transformants were plated on either minimal dextrose or YPD supplemented with 100 mM zeocin.

Isolation of Yeast Crude Membranes. Yeast cells were grown to midlog phase and induced with minimal methanol medium. Cells were harvested 18−24 h post induction, and crude cellular membranes were isolated via a glass bead protocol (*16*) and stored at −80 °C.

Purification of Yeast Plasma Membranes Harboring PfMDR1 Proteins. We followed the procedures in ref *16* with some modifications as in refs *17* and *18*. Freshly prepared crude membranes were diluted to 3.0 mg/mL in precipitation buffer (10 mM imidazole/1 mM $MgCl_2$/pH 5.20), adjusted to pH 5.20 with 100 mM HCl, and centrifuged at 7500*g* for 5 min. The supernatant was adjusted to pH 7.50 with 100 mM NaOH and centrifuged at 100,000*g* for 1 h at 4 °C. The resulting PM was resuspended in suspension buffer (10 mM imidazole/1 mM $MgCl_2$/pH 7.5), protein was quantified by amido black and densitometry (see below), and PM was aliquoted and stored at −80 °C.

Biotin and Polyhistidine Detection. SDS−PAGE gels (7.5%) were run for 100 min at 110 V, and protein was transferred onto polyvinylidene difluoride membranes at 40 mA for 16 h at 4 °C. For biotin detection, the membranes were washed once for 5 min with PBS-T (20 mM PO_4^{-3}/150 mM NaCl/pH 7.4), incubated for 1 h with 10% dried milk in PBS-T, washed again for 5 min in PBS-T, and then incubated in PBS-T supplemented with streptavidin-HRP following the manufacturer's instructions. For the poly histidine blots, the PentaHis detection kit from Qiagen was used according to manufacturer's instructions.

ATPase Activity Assays. The ATPase activity of purified PM fractions was measured using the colorimetric determination of orthophosphate released from ATP as described previously (*16*, *17*), but performed on a microliter scale in 96-well plates. Briefly, plates were set up on ice; assay buffer (180 mM NH_4Cl/100 mM Mes-Tris/10 mM $MgCl_2$/0.01% NaN_3, at various pH (see Results)) was added to each well followed by relevant drug solutions and finally membrane samples, to a total volume of 100 μL. The plate was shaken at 650 rpm on MixMate for 1 min and warmed to 37 °C using a water bath for exactly 1 min, and ATP was then added to all wells. After the plate was shaken (165 rpm) at 37 °C using a shaking incubator, stopping and stabilizing solutions were added at indicated times. (These are made weekly and stored at 4 °C.) Typically, 100 μL of the stopping/ascorbate mixture was added to each well followed 10 min later by 100 μL of stabilizing solution. Absorbance at 720 nm was read exactly 30 min after stabilizer was added, using a Victor 3V plate reader. Fresh solutions of 6% sodium ascorbate in 1 N HCl and assay buffer at various pH were made daily. Stocks solutions of ATP and drugs, and stock suspensions of PMs were thawed on ice, used once, and then discarded.

Negative control membranes from zeocin-resistant yeast transformed with empty vector (pPICzc) were included in all assays. Absorbance readings were converted to nmol Pi using a standard curve generated with K_2HPO_4 samples. Values for PM samples were then also scaled for total amount of protein according to densitometry results for silver stained gels as described in Results. Values for different PfMDR1 samples were normalized versus relative PfMDR1 content as described in Results. Replicates (at least three for each condition in each assay) were averaged, and the average control value under each assay condition was subtracted from the average for each PfMDR1 sample. All data shown are the results from multiple assays using at least two independent PM preparations for each PfMDR1 isoform, ±SD.

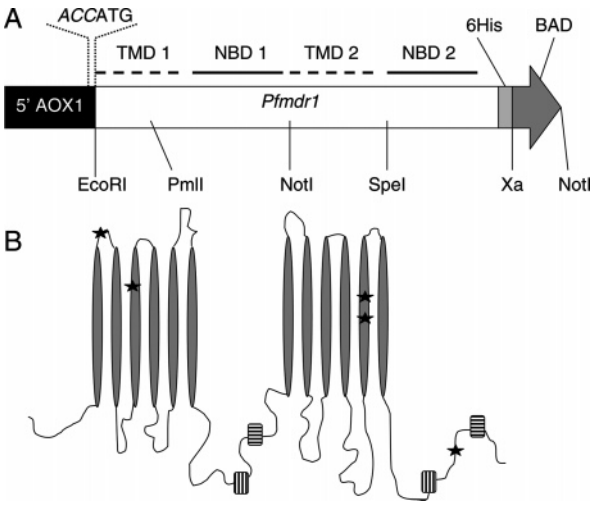

FIGURE 1: (A) Schematic of the yeast optimized PfMDR1 gene created for this work. The position of the alcohol oxidase promoter (AOX), an engineered Kozak sequence, restriction sites unique to the ORF, predicted 6 transmembrane helix domains (TMDs), nucleotide binding domains (NBDs), and fused hexa histidine (6His) as well as biotin acceptor domain (BAD) are indicated. (B) Cartoon of the predicted PfMDR1 secondary structure indicating helices (gray), loops (lines), and Walker A (vertical stripes)/Walker B (horizontal stripes) motifs within the NBD. The stars indicate the relative location of PfMDR1 point mutations previously associated with drug resistance (residues 84, 184, 1034, 1042, and 1246) found in the Dd2 and 7G8 isoforms vs 3D7 (see also Table 2).

When comparing the activity of the half transporter constructs to those of full length WT PfMDR1, data were normalized versus relative protein and relative hexa His label reactivity as above, and then also normalized versus relative number of NBD. Although activity of Pgp half transporters have been analyzed in a qualitative sense (*15*), to our knowledge, turnover quantification following this degree of densitometry-based normalization (see Results) has not previously been done.

RESULTS

We have previously overexpressed the *P. falciparum* membrane transporter PfCRT in *P. pastoris* and *S. cerevisiae* yeast (*14*). In those studies, it was readily apparent that the native cDNA did not express in yeast because of high AT content characteristic of *P. falciparum* genes, which manifests a large number of unpreferred codons, premature truncation signals, and other unfavorable features (*14*). Similarly, early attempts to reproducibly express native *pfmdr1* cDNA either failed or proved unreliable and at best led to vanishingly low levels of expression that could not be directly verified by western blot or other measurements (*13*). We therefore back translated the PfMDR1 amino acid sequence using a yeast preferred codon table and random codon seeding values as described (*14*). Upon inspecting numerous hypothetical genes for repeats and other potentially deleterious features, one gene was chosen and further tailored to remove poly A regions, insert convenient restrictions sites, and fuse C-terminally disposed biotin acceptor and hexa histidine tags (Figure 1). The final base and codon composition of this fully synthetic 4.59 kbp gene and comparison to average *P. falciparum* versus *P. pastoris* codon usage are shown in Table 1. The linear sequence is available in Supporting Information.

Our initial expression attempts focused on half transporter or cassette constructs as described in Materials and Methods. Upon successful, inducible, and approximately equal expression of yeast optimized 5′ and 3′ cassettes (see below), genes encoding the cassettes were fused together, and full length wild type (strain 3D7) PfMDR1 was successfully expressed to high levels (Figure 2A , lane 3) in inducible fashion (Figure 2C). The protein was found to be very well localized to the PM (Figure 2B, compare lanes 1−6). The initial assembly of the full length gene construct using introduced unique restriction sites created three additional alanine codons at the half transporter junction site (codon 749 in the primary amino acid sequence). These Ala codons did not affect expression levels, cellular localization, or measured ATPase activity (not shown, see below).

After optimizing the expression of the full length 3D7 isoform, point mutations were introduced as described in Materials and Methods to create genes encoding full length Dd2 and 7G8 PfMDR1 isoforms (see Table 2 and footnote). These full length proteins were also inducibly expressed to similar levels in *P. pastoris* (Figure 2A, lanes 4 and 5). We note that of the three isoforms, 7G8 PfMDR1 appears to express and insert into the PM to a higher extent (Figure 2A, compare lane 5 to lanes 3 and 4). We analyzed the kinetics of expression and PM insertion after MeOH induction (see Materials and Methods), and noted that the relative rate of PM integration for all isoforms is similar (not shown; representative membrane insertion kinetics shown in Figure 2C).

Expression of the full length PfMDR1 proteins in yeast was stable, as little obvious evidence of degradation was seen in hexa His gel analysis of purified PM fractions (e.g., Figure 2A), upon gel analysis of isolated cell fractions (e.g., Figure 2B) or upon analysis of expression kinetics (Figure 2C). No PfMDR1 was found in the cytosolic fraction 24 h post induction (Figure 2B, lane 1), and crude membrane fractions (Figure 2B, lane 3) or purified PM (lane 5) did not release PfMDR1 upon washing with high salt or chaotrope (e.g., 4 M NaCl wash, compare lane 3 vs 4 and 5 vs 6). However, we note that occasionally, biotin blots of full length PfMDR1 (e.g., Figure 3, discussed below) showed two minor protease−inhibitor sensitive bands that may represent minor degradation products produced during PM isolation.

A number of important ABC protein structure−function issues revolve around communication between the two homologous 6 helix TMD/1 NBD halves that are arranged in tandem fashion in full length ABCBs (Figure 1). We therefore also engineered expression vectors for the half transporters as previously described for human P-glycoprotein (Pgp) (*15*). Expression levels of all three constructs (5′ half transporter, 3′ half transporter, and full length) are comparable by either biotin or hexa His detection (Figure 3A and B respectively), and proceed via similar kinetics (not shown); however, expression and PM insertion of the N-terminally disposed half transporter (5′ encoded) is measureably higher than those of the C-terminal (3′ encoded) (Figure 3B; compare lane 7 vs 8). The full length Dd2 and 7G8 resistance-associated isoforms could also be stably expressed as half transporters, either individually or together (data not shown), similar to the case for the 3D7 isoform (Figure 3).

Table 1: Codon Usage and Base Composition for the Synthetic PfMDR1 Gene Used in This Work

amino acid	codon	*P. falciparum* usage (% codon used per amino acid)	*P. pastoris* usage (% per amino acid)	ncbi *pfmdr1* gene (exact number used)	synthetic 5′*pfmdr1* gene (exact number used)	synthetic 3′*pfmdr1* gene (exact number used)
Ala	GCA	43	26	30	6	1
	GCC	11	25	5	4	6
	GCG	4	5	0	5	11
	GCT	42	44	25	13	8
Arg	AGA	62	47	41	3	2
	AGG	15	17	0	7	3
	CGA	9	10	3	1	9
	CGC	2	6	0	2	7
	CGG	1	5	0	1	4
	CGT	12	16	7	6	5
Asn	AAC	15	50	16	90	57
	AAT	85	50	129	1	2
Asp	GAC	13	42	5	32	31
	GAT	87	58	75	7	11
Cys	TGC	14	35	1	7	5
	TGT	86	65	13	4	0
Stop	TAA	73	48	0	0	0
	TAG	11	30	0	0	0
	TGA	17	22	1	0	0
Gln	CAA	88	61	24	2	5
	CAG	12	39	4	12	9
Glu	GAA	87	59	65	11	10
	GAG	13	41	12	31	28
Gly	GGA	44	32	35	10	8
	GGC	4	14	0	6	5
	GGG	7	10	2	6	4
	GGT	44	44	31	17	17
His	CAC	17	44	3	6	7
	CAT	83	56	15	4	7
Ile	ATA	53	20	58	1	2
	ATC	7	31	6	71	55
	ATT	40	49	74	3	8
Leu	CTA	7	12	5	0	11
	CTC	2	8	4	13	18
	CTG	2	16	1	27	19
	CTT	11	17	10	4	7
	TTA	64	15	107	0	0
	TTG	13	32	10	34	5
Lys	AAA	82	48	119	1	2
	AAG	18	52	10	68	61
Met	ATG	100	100	34	16	19
Phe	TTC	18	43	11	32	50
	TTT	82	57	72	0	2
Pro	CCA	51	39	15	5	4
	CCC	10	16	113	2	3
	CCG	4	10	2	5	0
	CCT	35	35	8	3	7
Ser	AGC	6	9	57	7	9
	AGT	32	15	33	17	17
	TCA	27	19	45	11	7
	TCC	8	20	14	24	17
	TCG	4	8	2	11	12
	TCT	23	29	23	16	4
Thr	ACA	51	26	29	3	5
	ACC	12	23	8	24	12
	ACG	8	11	3	9	9
	ACT	28	40	25	4	3
Trp	TGG	100	100	6	5	2
Tyr	TAC	11	52	1	30	29
	TAT	89	48	63	2	3
Val	GTA	42	16	33	2	8
	GTC	6	22	2	18	11
	GTG	11	20	5	2	13
	GTT	41	41	31	13	5

To test whether the expressed proteins were functional, we analyzed ATPase activity versus time, [ATP], [Mg^{++}], pH, and several ATPase inhibitors. Using the plate-based assay described in Materials and Methods, activity exhibited inhibition at high [ATP] with an optimum near 5 mM (Figure 4A), was linear with time for at least 15 min (Figure 4B), was clearly Mg^{++} dependent (Figure 4C), had high activity at alkaline pH (Figure 4D), and had relatively low sensitivity

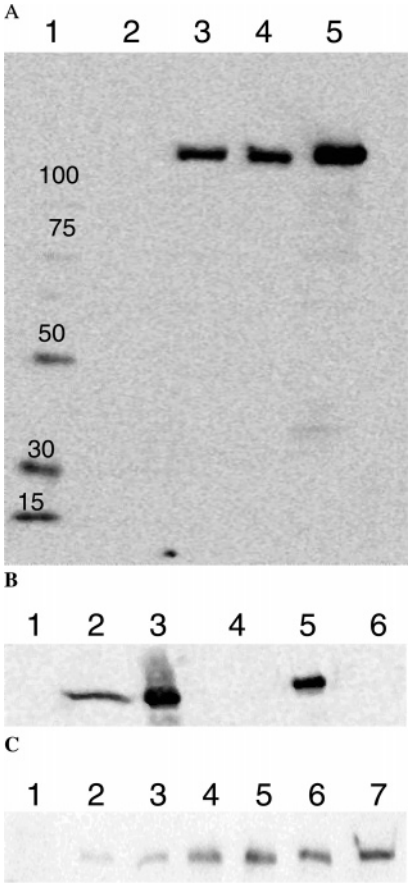

FIGURE 2: (A) Approximately equal expression of PfMDR1 isoform 3D7 (lane 3) Dd2 (lane 4), and 7G8 (lane 5) in purified *P. pastoris* PM as described in the text. Lane 1 is hexa His mole mass standards, and lane 2 is purified PM for similarly MeOH induced *P. pastoris* harboring empty pPICzc vector (Materials and Methods). The full length PfMDR1 migrates at 161.69 kDa (predicted mass = 171.81 kDa). Each lane harbors 15 μg of protein. (B) Analysis of relative PfMDR1 abundance in various cell fractions and assessment of membrane integration. Lane 1, cytosolic fraction; lane 2, 18000*g* pellet after cell rupture; lane 3, isolated crude membrane (CM) fraction; lane 4, 3.8 M NaCl wash of CM; lane 5, purified PM fraction; lane 6, 3.8 M NaCl wash of PM. Each lane harbors 40 μg of protein. Greater than 80% of expressed full length PfMDR1 was found in the crude membrane fraction (lane 3), and >60% of that was recovered in highly purified plasma membrane fractions (e.g., lane 5). (C) Time course of MeOH induction of 3D7 PfMDR1 in *P. pastoris*. Lane 1, 0 h; lane 2, 3 h; lane 3, 6 h; lane 4, 9 h; lane 5, 12 h; lane 6, 18 h; lane 7, 24 h. Each lane harbors 20 μg of protein.

Table 2: PfMDR1 Isoforms[a]

allele	N86Y	Y184F	S1034C	N1042D	D1246Y
3D7 (wild type)	N	Y	S	N	D
Dd2	Y	Y	S	N	D
7G8	N	F	C	D	Y

[a] The amino acid substitutions at codon positons 86, 184, 1034, 1042, and 1246 (denoted by stars in Figure 1B) for the different PfMDR1 isoforms analyzed in this study.

to vanadate (Figure 4E) but high sensitivity to concanamycin (Figure 4F). Interestingly, the Dd2 PfMDR1 isoform was considerably more active under basal conditions and showed a higher pH optimum relative to 3D7 (Figure 4D triangles/dashed line vs squares/solid line), yet the other drug resistance-associated isoform (7G8; circles/dotted line) was

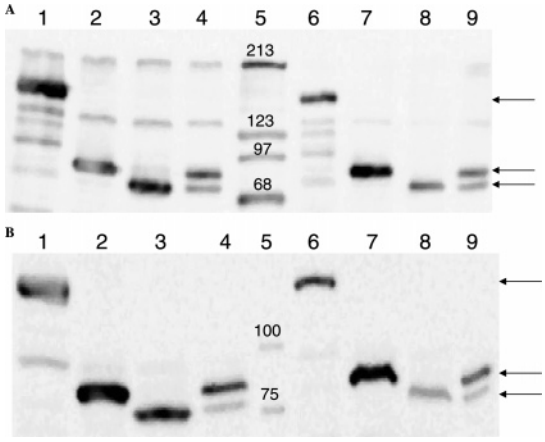

FIGURE 3: (A) Biotin detection blot of WT full length (lanes 1 and 6) vs N- (lanes 2 and 7) and C-terminal (lanes 3 and 8) half transporters. Each lane harbors 10 μg of protein. The full length PfMDR1 migrates at 161.69 kDa (first arrow right-hand side; predicted mass = 171.81 kDa), N-terminal polypeptide migrates at 87.44 kDa (second arrow; predicted mass = 94.69 kDa), and the C-terminal migrates at 77.03 kDa (third arrow; predicted mass = 86.94 kDa). Lanes 1−4 are crude yeast membrane fractions, lane 5 is biotinylated MW standards, and lanes 6−9 are purified PM fractions. Lanes 1 and 6, membranes expressing full length PfMDR1; lanes 2 and 7, N-terminal half transporter; lanes 3 and 8, C-terminal; lanes 4 and 9, both N-terminal and C-terminal half transporters coexpressed. (B) Parallel hexa His blot of the same samples as in A, except each lane harbors 40 μg of protein.

less active relative to 3D7. Although a range of characteristics can be noted in the literature for ABC proteins, in general, this ATPase activity is similar to that of other 12 helix ABCB transporters. However, we note that at high [ATP], the ATPase activity of PfMDR1 is particularly robust relative to human or mouse Pgp, yeast PDR5, and other ABCB involved in drug resistance phenomena, and is instead more similar to the basal activity previously measured for prokaryotic ABC transporters (see Discussion). Using densitometry to rigorously quantify PfMDR1 in the yeast PM (Figure 5), we calculated apparent K_m and V_{max} for wild type PfMDR1 of 2.14 mM and 62.9 μmol Pi released/mg PfMDR1/min, respectively, at pH 7.50 and 10 mM Mg^{++} (Table 3). Other eukaryotic 12 helix ABC transporters have been reported to exhibit K_m values for basal ATPase activity that range from 0.5 to 2.5 mM and V_{max} of 0.5−5 μmol/mg/min. However, relatively few eukaryotic ABC V_{max} values have previously been quantified using integral native membrane preparations and are instead more often calculated using purified detergent extracted enzyme that could conceivably have lost some level of activity. Also, unlike PfMDR1 (see below), other ABC proteins exhibit up to 20-fold stimulation in the presence of various drugs (see Discussion). Thus, basal ATPase activity of heterologously expressed PfMDR1 is commensurate with the higher end of previously measured, optimized (e.g., drug stimulated) activity for various ABC transporters but to our knowledge represents the highest level of basal activity yet recorded for a 12 helix ABCB protein.

A hallmark feature of most ABC transporters involved in drug resistance phenomena is that drugs to which they confer resistance either stimulate or inhibit their ATPase activity. As summarized elsewhere (*19*), reported effects are usually stimulatory, mild (2−5-fold), or quite significant (10−20-fold), and are often, but not always, further modulated by VPL and other chemo-reversal agents. However, the range

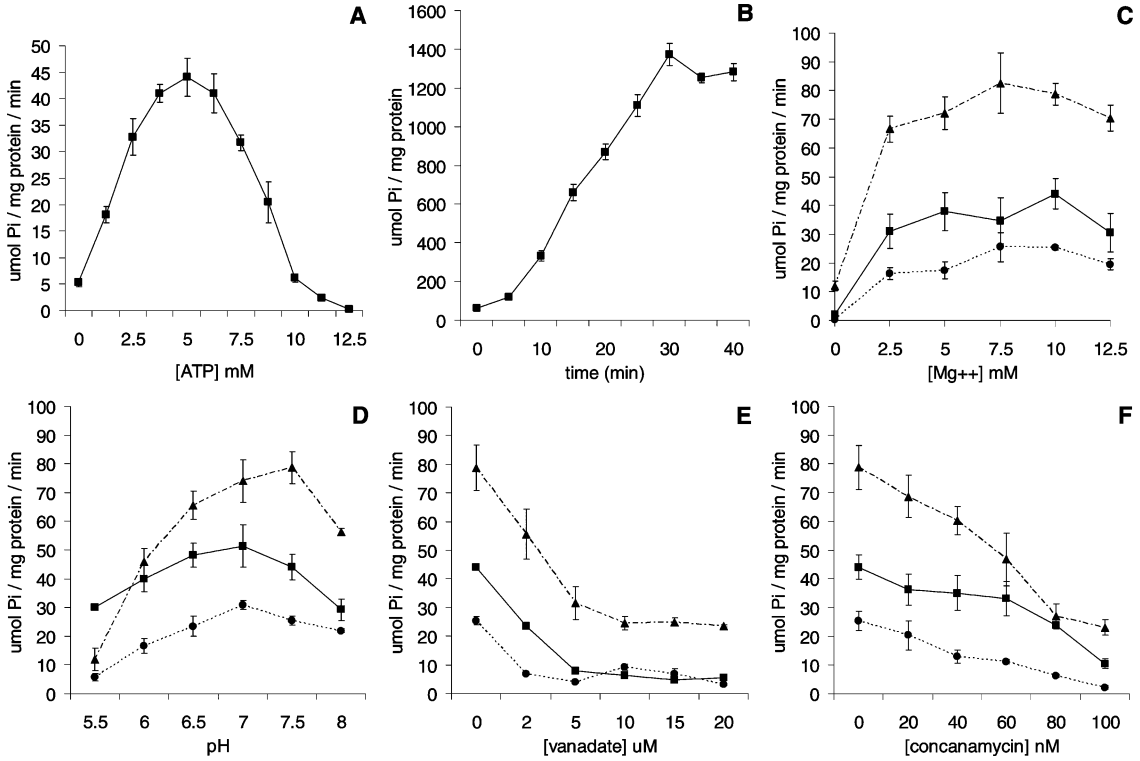

FIGURE 4: ATPase activity of recombinant PfMDR1 isoforms. PfMDR1 ATPase plate assay vs [ATP] (A) and time (B) for WT (3D7) PfMDR1. The results for Dd2 and 7G8 isoforms (not shown) are similar in shape but have different amplitude as described in the following Figures. Also summarized is Mg^{++} dependence (C), pH dependence (D), vanadate sensitivity (E), and concanamycin sensitivity (F) for 3D7 (■, solid lines, all panels), Dd2 (▲, dashed lines) and 7G8 (●, dotted lines) PfMDR1 isoforms.

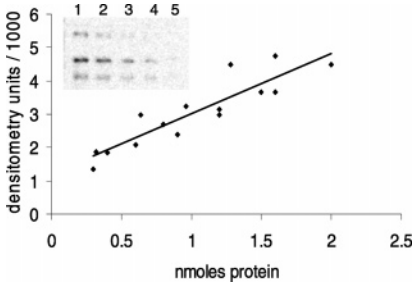

FIGURE 5: Densitometry standardization of PfMDR1 membrane content. Hexa His molecular mass standards (Bio Rad) were serially diluted (inset, lanes 1−5) to yield varible nanograms of protein per lane (150, 120, 90, 60, 30; 120, 96, 72, 48, 24; and 100, 80, 60, 40, 20 for 100 kDa (inset, top band); 75 kDa (middle); and 50 kDa (bottom) standards, respectively). After staining and quantitative densitometry using procedures identical to those for PfMDR1-containing membranes and conversion to moles, we plotted density vs nmol hexa His tagged protein (♦). We used a linear fit to these data ($y = 1802.8x + 1201.7$, $R^2 = 0.81$) and the predicted molecular mass of 171.81 kDa to determine nmol PfMDR1 per mg PM. Dividing this by the amount of PM protein loaded on various gels and averaging across several gels, we find that at 24 h induction PfMDR1 isoforms 3D7, Dd2, and 7G8 constitute 1.19%, 1.46%, and 3.07% of PM protein, respectively (see also Figure 2). These data also allow us to quantitatively compare turnover results across multiple PM samples and multiple PfMDR1 isoforms (or half transporters).

Table 3: Kinetic Parameters and Inhibitor Sensitivities[a]

	3D7	Dd2	7G8
pH optimum	7.0	7.5	7.0
V_{max} (μmol Pi/mg/min)	62.9	109.9	42.7
K_m (mM)	2.14	2.00	3.42
vanadate IC_{50} (μM)	2.25	4.00	1.25
concanamycin IC_{50} (nM)	82.6	67.4	42.9

[a] pH optima, V_{max}, K_m, and sensitivity to vanadate and concanamycin for the different PfMDR1 isoforms analyzed in this study. IC_{50} values were calculated using a sigmoidal fit to the data shown in Figure 4.

of drug concentrations used in such assays varies widely and is often nonphysiologic. In the case of PfMDR1 (which resides within the digestive vacuolar (DV) membrane), the definition of the physiologically relevant drug concentration that should be examined is somewhat ambiguous. For example, *in vitro* IC_{50} data suggest that 10−20 nM CQ is physiologically relevant for CQS strains (e.g., strain 3D7), whereas 100−200 nM is relevant for CQR (e.g., those expressing Dd2 and 7G8 PfMDR1 alleles or overexpressing the 3D7 allele (see Discussion)). Parasite cytosolic concentrations for CQ and other quinolines are predicted to be near growth medium concentrations (*20*); therefore, if drugs interact with PfMDR1 via the cytosolically disposed face (the face harboring ABCB NBDs), then concentrations near IC_{50} would be physiologically relevant with respect to possible PfMDR1 interactions. However, the DV membrane maintains a high ΔpH, which acts to strongly concentrate weak base antimalarial drugs within the DV. If the drug is effectively diprotic (e.g., CQ), then this effect goes as the square of the net pH gradient (the pH scale is logarithmic), whereas if the drug is effectively monoprotic (e.g., MQ and QN), then it is linearly related to the pH scale (*20*).

Therefore, we analyzed PfMDR1 ATPase activity with or without MQ, QN, and CQ in either the absence (Figure 6) or the presence (Figure 7) of VPL and over two concentration ranges that reflect reasonable anticipated equilibrium cytosolic (lower range, left-hand side of each panel in Figures 6 and 7) and DV (higher range, right-hand side of each panel in Figures 6 and 7) concentrations. (Data for both ranges are consolidated in Figures 6 and 7.) CQ, MQ, and QN were

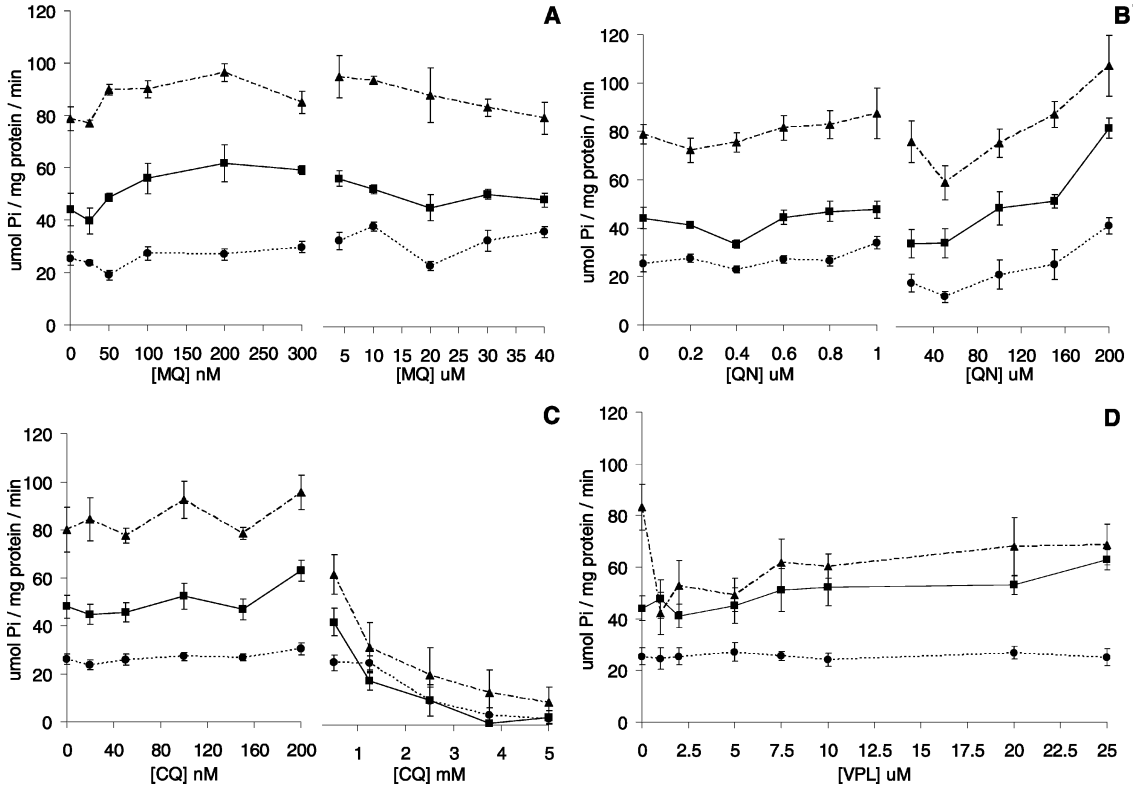

FIGURE 6: PfMDR1 ATPase activity under standard conditions ([ATP] = 5 mM; [Mg^{++}] = 10 mM; time = 15 min) vs MQ (A), QN (B), CQ (C), and VPL (D) for 3D7 (■, solid lines) Dd2 (▲, dashed lines), and 7G8 (●, dotted lines) isoforms. In this Figure and Figure 7, we consolidate data over two physiologically relevant concentration ranges that reflect expected drug concentrations either in the parasite cytosol (left-hand side; 0.2 × IC_{50} to 5 × IC_{50}) or the digestive vacuole (right-hand side; the cytosolic range × pH gradient fold concentration expected for monoprotic (MQ and QN) or diprotic (CQ) quinolines (*20*)).

chosen for this initial analysis because on the basis of data with field isolates (*5*) and *P. falciparum* transfectants (*8*, *9*), the hypotheses that have been offered to date include that mutation and/or overexpression of PfMDR1 confers the highest degree of resistance to MQ and QN but no appreciable resistance to the related drug CQ. (However, in the Discussion section, we note some disagreement in the literature with respect to this last point.) Whether or not the mutation of PfMDR1 to Dd2 or 7G8 isoforms confers higher levels of MQ or QN resistance compared to 3D7 is controversial but has been a popular hypothesis.

Drugs to which ABC transporters confer resistance are expected to stimulate ATPase activity; thus, surprisingly, QN did not stimulate PfMDR1 over the range of concentrations that corresponds to expected cytosolic (0−400 nM Figure 6A and B). Over a wide range of MQ concentrations that spans both expected cytosolic and digestive vacuolar (0.05−5.0 μM), a mild (30−40%) but reproducible stimulatory effect was measured for 3D7 and Dd2 PfMDR1 isoforms (Figure 6A, squares and triangles, respectively), in multiple independent purified PM preparations (Materials and Methods). The effect was proportionally greatest for 3D7 PfMDR1. Interestingly, the 7G8 isoform (Figure 6A, circles) was not appreciably stimulated at similar [MQ].

A significant QN stimulation was found for 3D7 and Dd2 isoforms (Figure 6B, squares and triangles, respectively) but only at very high dosages that correspond to the upper limit of what is expected within the DV- for QN-resistant *P. falciparum*. Again the effect was proportionally greatest for 3D7 and significantly reduced for the 7G8 isoform. At 200 μM QN, the stimulatory effect was nearly 60% (1.6-fold) and 100% (2-fold) for the Dd2 (triangles, Figure 6B) and 3D7 (squares) isoforms, respectively, and, similar to the case for MQ, noticeably less for the 7G8 isoform (circles).

Interestingly, the largest effects on ATPase activity were seen for CQ (Figure 4C), to which PfMDR1 is not believed to confer resistance (*1*, *6*−*9*). A trend in mild stimulation of ATPase activity was seen for the 3D7 and Dd2 isoforms at the higher range of anticipated cytosolic concentrations (Figure 6C, left-hand side top two traces) that was of marginal significance. However, CQ then very strongly inhibited all isoforms at anticipated DV concentrations (Figure 6C, right-hand side). Net stimulation was largest for the 3D7 isoform and again was not apparent for the 7G8 isoform; however, in relative terms, the 7G8 isoform was inhibited to a similar extent at high [CQ].

In some cases, VPL alone and/or non lethal doses of VPL along with drugs to which the ABC transporter confers resistance further stimulates ATPase activity of the transporter. Physiologic (nontoxic) dosages of VPL (1−2 μM) had a significant effect on Dd2 PfMDR1 ATPase activity but not on 3D7 and 7G8, which appeared insensitive over this range (Figure 6D). The Dd2 isoform was inhibited by approximately 2-fold at 1 μM VPL, and 40−50% stimulation from this value was then seen at progressively higher (toxic) concentrations (Figure 6D, triangles). Again, the 7G8 isoform was much less influenced by the drug; in this case, virtually no effect was measured for this isoform (Figure 6D, circles) across a wide range of [VPL].

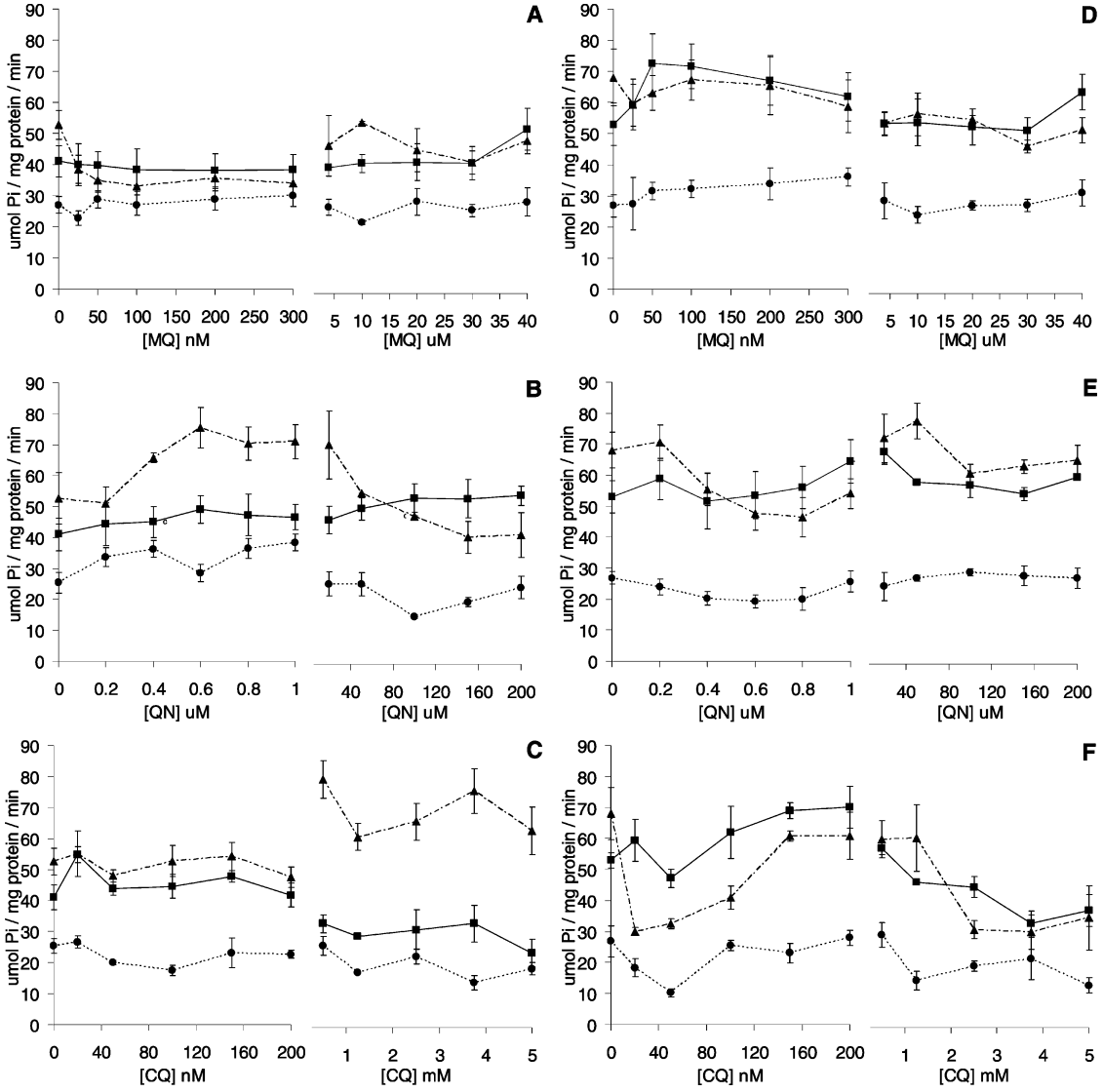

FIGURE 7: PfMDR1 ATPase activity vs MQ (A and D), QN (B and E), and CQ (C and F) for 3D7 (■, solid lines), Dd2 (▲, dashed lines), and 7G8 (●, dotted lines) isoforms in the presence of either 2 μM (A, B, and C) or 20 μM (D, E, and F) VPL. Note that we consolidate data over two physiologically relevant quinoline concentration ranges that reflect either expected parasite cytosol (left-hand side) or digestive vacuole (right-hand side) concentrations.

In the case of the MQ and QN stimulatory effects noted above, 1−2 μM VPL did not further stimulate but in fact negated or shifted the concentration dependence for MQ- or QN-induced stimulation, respectively (Figure 7A and B). Similar to the effect of VPL alone, the effects were noticeably larger for the Dd2 isoform, wherein 2 μM VPL in combination with anticipated DV concentrations of MQ (e.g., 200 nM) actually inhibited ATPase activity more than VPL alone (compare Figure 6A to Figure 7A triangles). VPL abrogated stimulation at 200 μM QN (Figure 7B vs Figure 6B) but interestingly shifted QN stimulation for Dd2 to lower [QN] (Figure 7B, triangles). At 2 μM concentration, VPL did not fully negate the ATPase inhibition seen at high doses of CQ for the 3D7 isoform (Figure 6C, squares) but did so for the Dd2 isoform (Figure 7C, triangles). In sum, in these examples, chemosensitizing doses of VPL along with quinoline appear to have primarily opposing effects on ATPase activity, relative to the effects seen in the presence of the same dose of quinoline antimalarial alone.

Finally, we also examined ATPase activity of the 3D7 half transporter constructs (Materials and Methods and ref *15*) expressed either alone (N term and C term; see *x*-axis) or together (coex) and compared this to the behavior of the full length 3D7 isoform (FL) (Figures 8 and 9). As expected (*15*), ATPase activity of N term and C term was lower than that of FL (Figure 8A and B), even after normalization to number of NBDs (Materials and Methods), yet they exhibited similar [ATP] dependency (Figure 8A, open circles and open triangles vs squares, respectively). The two half transporters expressed together (coex; Figure 8A, solid diamonds) did not show maximum ATPase activity until higher [ATP], relative to FL 3D7 (squares). Interestingly, the pH dependency for ATP hydrolysis was lost for the half transporters yet was steeper for coex (Figure 8B). Unlike the case for Pgp (*15*), wherein N term activity was found to be noticeably higher than C term activity, and the basal activity of coex Pgp (both Pgp N term and C term polypeptides expressed together) was approximately 70% that of the FL Pgp,

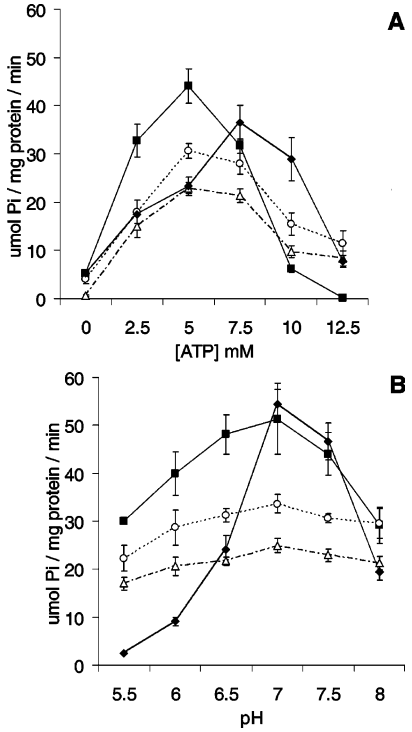

FIGURE 8: ATPase of individual WT half transporters vs WT coex vs WT full length. (A) Dependency on [ATP]. (B) pH dependency. Full length, (■, solid lines); N-terminal, (△, dashed line); C-terminal, (○, dotted line); coexpressed halves, (♦, thatched line).

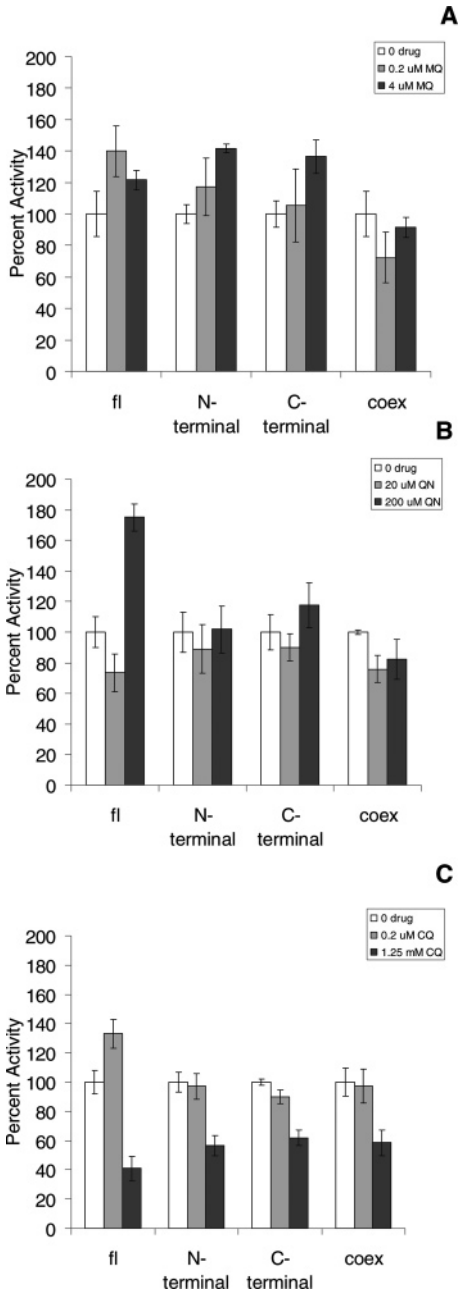

FIGURE 9: ATPase of individual WT half transporters (N-terminal and C-terminal) vs WT coex (both half transporters expressed together in the same cell) vs WT full length (fl) in the presence of no drug (open bars) vs cytosolic (gray bars) or DV (black bars) concentrations of MQ (A), QN (B), and CQ (C).

PfMDR1 N term was found to have similar activity relative to C term, and at optimum pH, coex PfMDR activity was 100% that of the FL PfMDR1. On the basis of these data, it is likely that communication between the two halves (which is also believed to be important with regard to drug stimulation effects) is quite different for PfMDR1 relative to the well studied Pgp.

Similar to the drug effects noted for Pgp half transporters (*15*), QN and CQ drug stimulatory effects observed for the FL PfMDR1 were lost when the protein was expressed as N term or C term forms (Figure 9B and C). However, mild MQ stimulation, but at higher dose (corresponding to predicted DV concentrations), was preserved for the N- and C-terminal half transporters (Figure 9A, gray vs black bars) as were inhibitory effects seen at high dose CQ (Figure 9C, solid bars).

DISCUSSION

Reproducible high level overexpression of large polytopic integral plasma or vacuolar membrane proteins is frequently difficult and for *P. falciparum* proteins has only been accomplished after *de novo* synthesis of optimized genes (*14*). Yet, molecular analysis of several of these (PfCRT, PfNHE1, and PfMDR1) is crucial for further understanding the molecular basis of antimalarial drug resistance. In this study, we have used methods similar to those reported previously for the much smaller PfCRT protein (*14*) to create synthetic yeast optimized versions of *pfmdr1* genes and now report reproducible, high level overexpression of WT (strain 3D7) PfMDR1 and two common isoforms (Dd2 and 7G8) that are expressed in common CQR laboratory strains of *P. falciparum*. The proteins are well expressed in *P. pastoris*, well localized to the yeast PM in either half transporter or full length forms, and are efficiently biotinylated when an *E. coli* transcarboxylase biotin acceptor domain is fused to the C-terminus. After optimizing a plate-based assay, we used purified yeast PM fractions to perform an initial characterization of PfMDR1 isoform ATPase activities versus key antimalarial drugs.

The definition of the molecular mechanism behind any ABCB transporter-mediated drug resistance phenomenon remains elusive. In the particular case of PfMDR1 versus antimalarial drug resistance, there is some disagreement and controversy in the relevant literature. First, importantly, any antimalarial resistance mediated *in vivo* by mutated or

alternately expressed PfMDR1 appears to be dependent upon the simultaneous expression of CQR-conferring mutant PfCRT (*1*). That is, mutation and/or increased expression of PfMDR1 likely does not promote appreciable drug resistance in and of itself, as shown recently for strain GC03 parasites that express wild type PfCRT (*8*). The nature of this PfCRT/PfMDR1 interaction, whether it is direct or indirect and so forth, is not known but obviously merits additional study using heterologous expression and purification methods. Second, the relative importance of PfMDR1 overexpression versus mutation (as well as overexpression of WT vs overexpression of mutant PfMDR1 isoforms) in contributing to antimalarial drug resistance is currently a topic of debate. Notwithstanding these two complexities, the data in this article do help to further define an emerging model for the role of PfMDR1 in antimalarial drug resistance.

From prokaryotes to humans, there are multiple examples wherein either overexpression or mutation of either 6 helix or 12 helix ABC proteins contributes to pleiotropic resistance phenomena (resistance to antibiotics, heavy metals, antiparasitic drugs, anticancer drugs, etc.). In reviewing all of this, it is difficult and perhaps dangerous to attempt to draw too many general conclusions. However, there are important comparisons that are relevant for interpreting these data for PfMDR1. PfMDR1 is a 12 helix 2 NBD member of the ABCB subfamily that is believed to confer resistance to hydrophobic weakly basic compounds. These drugs are in some respects structurally reminiscent of cancer chemotherapeutics, to which overexpression of the human ABCB protein MDR1 (P-glycoprotein; Pgp) confers resistance. A similar parallel could be drawn using the yeast ABCB protein PDR5, which is known to be similar to Pgp in many respects (*21*).

Reports of how Pgp or PDR5 ATPase activities are altered by the drugs to which these proteins confer resistance vary (*15*, *19*, *21*−*27*), but with a few exceptions, they are stimulatory and typically at least 2−3-fold and occasionally even as high as 10−20-fold. Notably, PfMDR1 differs from these related resistance conferring ABCB proteins in that the drug to which it is currently believed to confer the highest level of resistance (MQ) stimulates ATPase activity by at most only 30−40%. Also, again in contrast to Pgp and PDR5, the MQ chemoreversal agent VPL does not further stimulate within the relevant concentration range. This suggests, but does not prove, that relative to Pgp and PDR5 versus drugs to which they confer resistance, MQ interacts more weakly with PfMDR1, and VPL may interact in a different fashion altogether. This would be consistent with PfMDR1 playing only a small role in conferring resistance to MQ and other quinoline-based antimalarials, as has been recently suggested by several laboratories based on QTL or transfection results (*4*, *8*, *9*). ATPase stimulation is somewhat greater at very high levels of QN, but even in this case, it is below what has typically been measured for Pgp or PDR5. Overall then, drug stimulation of PfMDR1 ATPase activity is unlike that for any previously characterized drug resistance associated ABCB protein and is instead curiously much more consistent with recent results for Cdr1p, a member of the architecturally distinct ABCG subfamily that is involved in antifungal drug resistance (*28*). We note that PfMDR1 may confer mildly altered sensitivity to other antimalarial compounds outside the quinoline class (e.g., reactive endoperoxides (*9*)), but this is currently controversial, and if it is indeed correct, the effect is very mild.

The CQ profile, differences in this profile for 3D7 versus Dd2 isoforms, and the effects of VPL on these profiles are intriguing. The biphasic (mild stimulatory followed by strongly inhibitory) profile observed across the wide range of [CQ] inspected is unusual but is again somewhat reminiscent of Pgp, which has been observed to exhibit biphasic profiles for several compounds to which it mediates resistance (*19*). But because the prevailing hypothesis is that PfMDR1 does not mediate resistance to CQ, these data are surprising. The nearly complete inhibition of PfMDR1 by high DV concentrations of CQ could be quite important for interpreting complex patterns of active versus passive drug transport proposed to be linked to the CQR phenotype (*29*, *30*).

In the case of Pgp, although exceptions can be found, VPL typically further stimulates ATPase activity when combined with weak base chemotherapeutics that stimulate. In the present case, 2 μM VPL reverses the stimulatory effect seen at 200 nm MQ and 200 μM QN as well as the mild CQ-based stimulation and completely reverses the CQ inhibition seen for Dd2 PfMDR1 but not 3D7. These effects could be due in part to the fact that unlike Pgp, PfMDR1 does not appear to be simulated by VPL alone to any appreciable extent. A weak interaction with VPL is consistent with the recent conclusions of Cooper et al. (*31*) that link VPL chemoreversal of quinoline drug resistance in *P. falciparum* to PfCRT mutations and not to the mutation or altered expression of PfMDR1. Conversely, Kirk and colleagues (*32*) have recently suggested that PfMDR1 mutations influence the intrinsic antiplasmodial activity of VPL. (The compound is toxic to *P. falciparum* above 2 μM.) Although the effects are mild, we do note differences in how 7G8 versus Dd2 versus 3D7 isoforms respond to VPL. ATPase activity of the 7G8 isoform is not affected by VPL alone, and data in ref *32* suggest that mutations found in the 7G8 isoform confer increased sensitivity to VPL. Thus, stimulation of PfMDR1 ATPase by toxic levels of VPL (Figure 5D, right side, top traces) may be linked to decreased sensitivity to VPL. This might be consistent with 3D7 and Dd2 PfMDR1 acting to concentrate VPL within the DV, away from targets elsewhere in the cell.

How the other ATPase effects we measure correspond to patterns of drug resistance believed to be partially mediated by PfMDR1 *in vivo* is less clear. As mentioned, for *P. falciparum*, low levels of resistance that may be mediated by PfMDR1 require the presence of mutated PfCRT, and the relative importance of PfMDR1 overexpression versus mutation in contributing to this resistance is currently a topic of debate. Recent field studies (*5*) suggest that for isolates harboring CQR-conferring *Pfcrt* mutations, gene duplication of wild type (3D7) *pfmdr1* is associated with increased resistance to MQ and QN but does not further influence the level of CQR. *In vitro*, some increased resistance to MQ and QN was seen for strains expressing the N86Y (Dd2) mutant isoform (*5*), but no *in vivo* correlation was observed. Relatedly, recent gene disruption studies using a strain exhibiting *pfmdr1* gene duplication presumably due to CQ selection (*33*) have shown the converse, namely, that decreased expression of PfMDR1 confers increased susceptibility to MQ and QN.

In light of these observations, interestingly, the basal ATPase activity we measure is conspicuously higher for Dd2 and 3D7 isoforms versus 7G8, with Dd2 being the highest. Although allele specific overexpression trends have not been examined in any detail to our knowledge, on the basis of this result, we predict that selection for overexpression of 7G8 PfMDR1 would be less common than overexpression of wildtype or Dd2 isoforms in drug resistant *P. falciparum*. Furthermore, our data suggest that if the net level of PfMDR1 ATPase activity is the relevant factor in conferring resistance, overexpression of Dd2 PfMDR1 under CQ selection pressure would be more efficient than overexpression of WT. Meaning, in the presence of CQS IC_{50} levels of CQ (the clinically relevant level of CQ selection pressure), two copies of Dd2 PfMDR1 would provide similar increased PfMDR1 ATPase activity relative to four copies of 3D7 (nearly 10 copies of 7G8 would be required to obtain the same level of PfMDR1 ATPase activity). Interestingly, we note that Dd2 CQR parasites show elevated cytosolic pH (*20* and refs therein) relative to 3D7, which would act to further stimulate PfMDR1 (Dd2 isoform of PfMDR1) in these strains. That is, if overexpression of PfMDR1 is indeed a small but important contribution to antimalarial drug resistance and because ATPase activity is fundamental to how this ABCB protein confers drug resistance (as is the case for all other ABCB), we predict that higher levels of overexpression of WT isoforms (meaning >2 copies) will be found to be more common than that for Dd2. We also predict that overexpression of 7G8 or 7G8-like isoforms yet to be discovered will be less well correlated with drug resistance (relative to 3D7 and Dd2) because reasonable increases in copy number will not impart similar levels of drug stimulated PfMDR1 ATPase activity. We also note that in further predicting the relative effects of these PfMDR1 isoforms, precise quantification of cytosolic [ATP] and pH for the malarial parasite will be crucial. Only a handful of measurements have been reported, but available data suggest (*34*) that due to anaerobic metabolism, ATP concentrations are lower than those for other eukaryotes, perhaps close to 1−2 mM.

Because ABCB proteins are believed to translocate drug in some fashion from the NBD-disposed side of the membrane (in this case, cytosolic) to the opposite side (in this case, the DV interior) and because the DV is believed to be the primary site of action for MQ, QN, and other quinolines, how PfMDR1 concentrating more drug within the DV leads to resistance has always been a lingering mystery. If the DV is indeed where the primary drug target (ferriprotoporphyrin IX heme) resides, as is generally accepted to be the case (*20*), this would act to encourage drug−target interactions, which is the converse of what is typically promoted by any drug resistance mechanism. As one possibility, we have previously observed that quinoline drugs actually act to prematurely precipitate heme dimers from solution (*35*). These curious nucleation phenomena, which occur at different rates as the drug is varied from vastly substoichiometric to high molar excess, would lower drug target availability. If pH, volume (concentration of heme), and ionic strength were manipulated in certain ways (as indeed seems to be the case for drug resistant malaria (*36*)), synergistic (and quinoline drug specific) heme aggregation would occur.

That is, in all other examples of ABCB-mediated drug resistance of which we are aware, the proteins function to lower the accumulation of drug on the NBD side of the membrane (e.g., the cytosol for a drug resistant tumor cell) because the NBD side is disposed toward the drug target (e.g., cytosolic tubulins in the case of Pgp mediated resistance to vinca alkaloids, colchicines, etc.). Alternatively, resistance-conferring ABC proteins expressed in vacuolar membranes (again with NBD disposed to the cytosol) act to concentrate drug into the vacuole in order to again sequester the drug away from the drug target, which is cytosolically or nuclearly localized. In this case, because PfMDR1 is expressed in the DV membrane, the simple prediction is that PfMDR1 would act to concentrate drugs *at* their site of action (inside the DV where the heme target lies), not *away*. Higher levels of PfMDR1 (as in drug resistant field isolates overexpressing *pfmdr1*) or the drug stimulation of PfMDR1 ATPase shown here would be predicted to concentrate even *more* drug at the site of action (not less). This is again paradoxical, unless this has an unexpected effect on target availability as described in ref *35*. Alternatively, although the concept has not been as extensively explored as has the drug interaction concept, it is also true that drug resistance-conferring members of the ABC transporter family have been observed to mediate movement of ions under various conditions (*17*, *37*). This possibility for PfMDR1 merits additional scrutiny because the pH gradient across the DV membrane is very large and directly or indirectly controls the accumulation of CQ, MQ, QN, and other drugs within the DV as well as the biomineralization of the heme drug target. Even subtle effects on this parameter and others closely linked to it (e.g., DV volume and other ion activities (*36*)), which via this model could then be influenced by drug effects on PfMDR1 ATPase activity, would contribute to resistance in interesting and drug specific ways (*35*).

Along with a better understanding of the nature of quinoline−heme interactions (*35*, *36*), obviously many additional molecular questions remain. The perfected heterologous expression described in this article will be extremely useful in addressing these. For example, it will be interesting in future work to dissect the ATPase effects observed in naturally occurring (e.g., Dd2 and 7G8) versus artificial (*8*) PfMDR1 isoforms versus the wild type. Some of these amino acid substitutions lie within homologous regions that are believed to be involved in drug binding for other ABCB proteins (i.e., residues 1034 and 1042 mutated in 7G8 PfMDR1, which lie within predicted helix 11). Also, how PfCRT and PfMDR1 might interact to further modify either these PfMDR1 properties or PfCRT properties (*14*) will be important to examine.

In conclusion, our data showing higher basal ATPase and shifted pH optimum for the Dd2 PfMDR1 isoform, mild stimulation of PfMDR1 ATPase by physiologically relevant doses of MQ and QN, equal or even slightly higher stimulation for wild type versus other isoforms, and VPL reversal (not simulation) of these effects are in general consistent with the evolving picture presented in refs *5*, *8*, and *9*. Assuming these drug effects on PfMDR1 are relevant to the drug resistance mechanism, overexpression of wild type PfMDR1 will confer many of the same effects, regardless of mutation, to Dd2 or 7G8 isoforms, and in some cases, higher levels of wild type would be predicted to be

more effective than increased expression of some CQR-associated isoforms (i.e., 7G8).

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SUPPORTING INFORMATION AVAILABLE

The full length sequence of the synthetic 3D7 *pfmdr1* gene used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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