ABCG2 membrane transporter in mature human erythrocytes is exclusively homodimer

Mara L. Leimanis, Elias Georges *

Institute of Parasitology, McGill University, Ste. Anne de Bellevue (Montreal), Que., Canada H9X-3V9

Abstract

The human ABCG2 protein, a member of ABC transporter family, was shown to transport anti-cancer drugs and normal cell metabolites. Earlier studies have demonstrated the expression of ABCG2 in hematopoietic stem cells and erythroid cells; however little is known about the expression and activity of ABCG2 in mature erythrocytes. In this report, we show that ABCG2 in mature human erythrocytes migrates with an apparent molecular mass of 140 kDa, under reducing conditions, on Fairbanks SDS gel system. In contrast, tumor cells expressing higher levels of ABCG2 show no detectable homodimers, when resolved under identical reducing conditions. Analysis of the same membrane extracts from tumor cells and human erythrocytes on Laemmli SDS gel system, where samples are boiled in the presence of increasing concentrations of disulfide reducing conditions and then analyzed, migrate with an apparent molecular mass of 70 kDa or a monomer. Drug transport studies using Pheophorbide A, a substrate of ABCG2, show the protein to be active in erythrocytes. Furthermore, Fumitremorgin C, a specific inhibitor of ABCG2 increases the accumulation of Pheophorbide A in erythrocytes and drug-resistant cells but not in the parental drug-sensitive cells. Given the ability of ABCG2 to transport protoprophyrin IX or heme, these findings may have implications on the normal function of erythrocytes.
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The breast cancer-resistant protein (or ABCG2, also known as the mitoxantrone-resistant protein, and ABC placenta) is a member of the ATP-dependent binding cassette (ABC) family of transporters. Similar to other well-characterized ABC transporters, namely P-glycoprotein (P-gp1 or ABCB1) and multi-drug resistance protein 1 (MRP1 or ABCC1), ABCG2 was initially discovered in a multidrug-resistant cell line, MCF7/AdrVp [1]. ABCG2 has been shown to confer drug resistance in tumor cells, and to mediate the transport of anti-cancer drugs, including mitoxantrone, methotrexate, camptothecans (SN-38, topotecan), and flavopiridol (for recent reviews see [2,3]). In normal tissue, ABCG2 is found in the canalicular membrane of the liver, in the epithelia of the small intestine, colon, kidney, placenta, and sweat glands. More recently,

ABCG2 expression has been found in hematopoietic stem cells. This latter "side population" of progenitor cells is representative of pluripotent stem cells. It has been suggested that the expression of ABCG2 protects this "side population" from cytotoxic substrates [4,5]. Furthermore, increased expression of ABCG2 has been demonstrated in erythroid maturation and was shown to decrease intracellular protoporphyrin IX, a natural substrate of ABCG2 [6]. Consistent with these findings, erythrocytes from ABCG2 knocked-out mice showed significant increase in intracellular protoprophyrin IX and a decrease in survival further confirming the protective role of ABCG2 in normal tissue [7]. Recently, ABCG2 has been implicated in the transport of heme, and was shown to enhance hypoxic cell survival through interactions with heme [8].

The human ABCG2, with a molecular mass of 72 kDa, encodes one transmembrane domain (TMD, with six transmembrane α-helices) and one nucleotide binding domain

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(NBD) all within a 655 amino acids primary sequence. This is in contrast with other ABC transporters (e.g., ABCB1 and ABCC1), which has led some to label ABCG2 "a half-transporter". Consistent with the latter, two monomers are necessary to form a fully active ABCG2 transporter. Several reports have described ABCG2 as a functional homodimer, possibly as a homotetramer [9–14]. These latter studies have suggested that homodimerization through inter-disulfide bonds between two ABCG2 monomers. Mutational analysis of all 12 cysteine residues in human ABCG2, including the three extracellular cysteines, Cys592, 603, and 608, suggested that inter-disulfide bridge at Cys-603 in the 3rd extracellular domain between two monomers is important, but not essential, for oligomerization of ABCG2 [15,16].

The expression of ABCG2 in hematopoietic stem cells and erythroid cells can protect normal tissue from toxic agents, similar to other ABC transporters. Moreover, the enhanced expression of ABCG2 in hematopoietic stem cells has led some to speculate a role for ABCG2 in preventing the accumulation of a differentiating factor in stem cells [4]. While others have observed the expression of ABCG2 in Ter119+ erythroid precursors and natural killer lymphocytes [4]; however little is known about the expression and activity of ABCG2 in mature erythrocytes. In this report we demonstrate that ABCG2 is expressed in mature human erythrocytes isolated from at least eight different adults. In these cells, ABCG2 migrated almost exclusively as 140-kDa protein on Fairbanks SDS gel system under reducing conditions. By contrast, ABCG2 from mitoxantrone-resistant tumor cells (MCF7/Mitox) migrated exclusively as a 72 kDa under identical Fairbanks gel system. Interesting, analysis of ABCG2 from mature erythrocytes and MCF7/Mitox tumor cells using the Laemmli gel system, in the presence of reducing agent, migrates as a monomer (or 72-kDa protein). The significance of these finding in the normal transport functions of ABCG2 in erythrocytes is discussed.

Materials and methods

Materials. ABCG2-specific antibody BXP-21 was purchased from Kamiya Biomedical Co. (Seattle, WA). Na^+/K^+-ATPase-specific monoclonal antibody was purchased from Sigma. All other chemicals were of the highest grade available.

Cell culture and plasma membrane preparation. Human breast carcinoma cells MCF7 and their drug-resistant clone, MCF7/Mitox, were grown in α-MEM containing 10% fetal calf serum (Bio Media). Plasma membranes were prepared as described previously [17]. Protein concentrations were determined by the Lowry method [18]. Erythrocytes ghosts were prepared as described previously [19]. Briefly, freshly drawn erythrocytes in the presence of sodium-citrate were first washed with PBS three times, and then run on a Ficoll gradient to remove leukocytes. Erythrocytes were washed three more times with ice-cold PBS prior to lysis in hypotonic buffer 5P8 (5 mM sodium phosphate, pH 8.0). Hemolysis was initiated by rapid mixing in the presence of protease inhibitors (2 mM PMSF). Lysed erythrocytes were centrifuged at 4 °C for 10 min at 14,000 rpm (16,000g) and the supernatant removed by aspiration. The latter wash was repeated four more times and the final pellet suspended in PBS with protease inhibitors.

Western blots. Plasma membranes from tumor cells (MCF7 and MCF7/Mitox cells) or erythrocytes (10–100 μg) were resolved by SDS–PAGE using the Fairbanks gel system [20] and transferred to nitrocellulose membranes using wet electroblotting as outlined by Towbin et al. [21]. The nitrocellulose membrane was blocked in 5% fetal bovine serum, 5% skim milk, and 7.5 mM NaN_3 in PBS, and incubated with various ABCG2-specific antibodies at varying dilutions (1:1000–1:3000 v/v) overnight at 4 °C. Membranes were washed, and incubated with varying dilutions (1:3000–1:6000 v/v) of goat anti-rabbit or mouse antibody conjugated to horseradish peroxidase. Immuno-reactive proteins were visualized by chemiluminescence using Pico or Femto SuperSignal Substrate (Pierce).

Flow cytometry analysis. ABCG2 activity in MCF7 mammalian tumor cells was assessed by FACS analysis as previously described [22] with some modifications. Briefly, cells were washed twice with incubation buffer (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, $CaCl_2 \times 2H_2O$ 1.8 mM, $MgCl_2 \times 6H_2O$ 1 mM, glucose 10 mM, pH 7.4). Freshly drawn human erythrocytes were collected by finger prick in EDTA-filled 0.5 mL Eppendorf tubes and re-suspended in PBS and run on an equal volume Ficoll gradient (to remove leukocytes), and subsequently washed twice with incubation buffer. Cells MCF7 (0.5×10^6–1.0×10^6 cells/ml) and whole erythrocytes (0.5% hematocrit) were incubated with 2 μl PhA with or without ABCG2-specific or non-specific inhibitors: 1–10 μM FTC, 50 μM verapamil, 50 μM *cis*-platin, and incubated in 37 °C hot water bath for 30 min. Cells were then washed once with ice-cold incubation buffer and then incubated for 1 h at 37 °C in PhA-free medium with ABCG2-specific or non-specific inhibitors. Following the efflux phase cells are washed once again with ice-cold incubation buffer, resuspended in 1 mL of incubation buffer and kept on ice in the dark and analyzed immediately (within 30 min), using the FACS Aria flow cytometer (Becton–Dickinson, CA). PhA fluorescence was measured with a 488-nm argon laser and a 670-nm filter. At least 10,000 events were collected for all of the flow cytometry studies, and by gating forward versus side scatter from a dot blot we were able to determine cellular debris and dead cells from our target population. Results are representative of at least two separate experiments done in triplicate, and calculated as % increase of mean channel fluorescence (MCF).

Results and discussion

Several reports have now shown that ABCG2 mediates the transport of normal cell metabolites, including protoprophyrin IX and heme [6,7]. Given these latter findings, it was of interest to examine the expression of ABCG2 in normal tissue that contains high levels of heme, such as mature human erythrocytes. Fig. 1A shows a Western blot of plasma membrane extracts from drug-sensitive (MCF7) or -resistant (MCF7/Mitox) tumor cells and human erythrocytes probed with ABCG2-specific monoclonal antibody, the BXP-21. The results in Fig. 1A show a BXP-21 reactive polypeptide with an apparent molecular mass of 72 kDa in drug-resistant tumor cells (MCF7/Mitox, lane 2) using a Fairbanks gel system. The parental drug-sensitive cells (MCF7, lane 1) showed a much weaker signal for this 72-kDa polypeptide; while membrane extracts from several human erythrocytes (lanes 3 and 4) showed a significant expression of this 72-kDa protein. These observations are consistent with the expression of ABCG2 protein in breast tumor cells that have been selected for mitoxantrone resistance [23] from the drug-sensitive breast tumor cell lines MCF7. In addition, lanes 3 and 4 revealed a 140-kDa BXP-21 reactive protein that was not observed in membrane extracts from tumor cells (lanes 1 and 2). The 140-
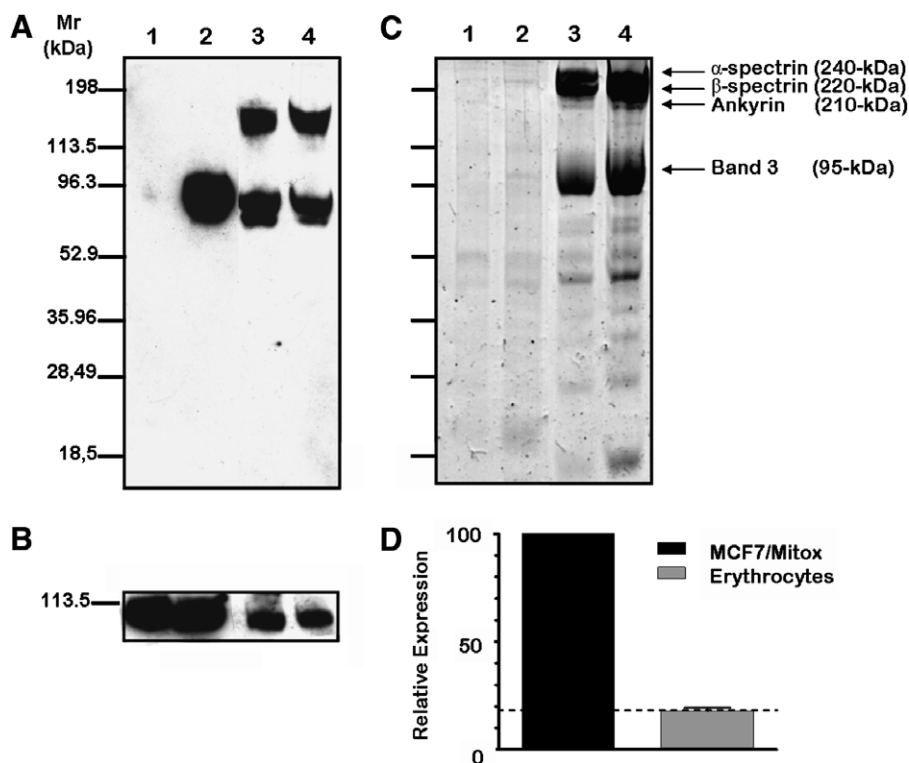
Fig. 1. Detection of ABCG2 homodimer in mature erythrocytes. Plasma membranes from MCF7 parental and MCF7/Mitox drug resistant or erythrocyte ghosts (10–50 μg) were loaded onto Fairbanks gel system, and transferred onto nitrocellulose membranes. (A) Western blot probed with an ABCG2-specific monoclonal antibody, BXP-21. A 72 kDa reactive polypeptide is detected in MCF7/Mitox and to a much less extent in MCF7 parental cells (lanes 2 and 1, respectively). A 72 kDa reactive polypeptide is also detected in lanes 3 and 4 of (A) which contain resolved membrane proteins from human erythrocyte (lane 3: South Asian female, B+ blood type or lane 4: Caucasian male, A+ blood type). (B) The same Western blot as in (A) probed with a monoclonal antibody to Na^+/K^+-ATPase, hence confirming equal protein loading between lanes 1 and 2 or 3 and 4. (C) Coomassie blue stained gel of replicate samples as described in (A). Densitometric quantification of ABCG2 expression in mature erythrocyte membranes relative to MCF7/Mitox is shown in (D) is represented by a histogram as mean ± SD of seven independent Western blot analyses.

kDa polypeptide has also been previously described as a homodimer of ABCG2 [13]. Hence, the assumption that the 72-kDa polypeptide is ABCG2 is consistent with: (i) tumor cell lines selected for resistance to mitoxantrone express mostly ABCG2 [24]; (ii) ABCG2 encodes 655 amino acid polypeptide migrates with a molecular mass of 72 kDa on SDS–PAGE [13,15]; (iii) BXP-21 is a well-characterized monoclonal antibody raised against 126 amino acids polypeptide (271–396 AA of ABCG2) and binds to an intracellular epitope in ABCG2 [25]. Although equal amounts of membrane proteins were loaded in each of the lanes of Fig. 1A, it was not possible to compare the loading between two membrane preparations derived from breast tumor cells (MCF7 and MCF7/Mitox) and mature erythrocytes. Consequently, a comparison was only possible between MCF7 and MCF7/Mitox or the two erythrocytes samples. Fig. 1B shows the Western blot of the same resolved proteins as in Fig. 1A probed with a monoclonal antibody specific to the α subunit of Na^+/K^+-ATPase (lanes 1 and 2 or 3 and 4, respectively). The results of Fig. 1B show 110-kDa polypeptide in membranes from breast tumor cells and mature erythrocytes, confirming equal loading between the parental and Mitox-resistant tumor cells or the two different erythrocyte samples. Inter-estingly, membrane extracts from breast tumor cells expressed much higher levels of the Na^+/K^+ ATPase α subunit than from mature erythrocytes.

To rule out the possibility that the BXP-21 reactive polypeptides (e.g., 72 or 140 kDa) is due to non-specific immuno-reactivity of BXP-21 mAb with abundant proteins in erythrocyte ghosts, total cell extracts from tumor cells and erythrocytes were resolved on SDS–PAGE and stained with Coomassie blue. As expected, the results in Fig. 1C show several highly expressed proteins, ankyrin and α-, β-spectrin, which migrates higher than the homodimer bands of ABCG2 (e.g., 210–240 kDa versus 140 kDa) and band 3 which migrates significantly higher than ABCG2 monomer (e.g., 95 kDa versus 72 kDa). It should be mentioned that such cross-tissue comparison is rather difficult, especially where the above 4 proteins (α-, β-spectrin, ankyrin, and band 3) account for the bulk of membrane proteins in erythrocytes. Hence, the expression of ABCG2 in erythrocytes is likely to be underestimated. Quantitative densitometry of ABCG2 immuno-reactive bands as determined by Western blots analyses of seven independent experiments revealed mature erythrocytes to express roughly 20% ABCG2 relative to MCF7/Mitox drug-resistant cells (Fig. 1D).
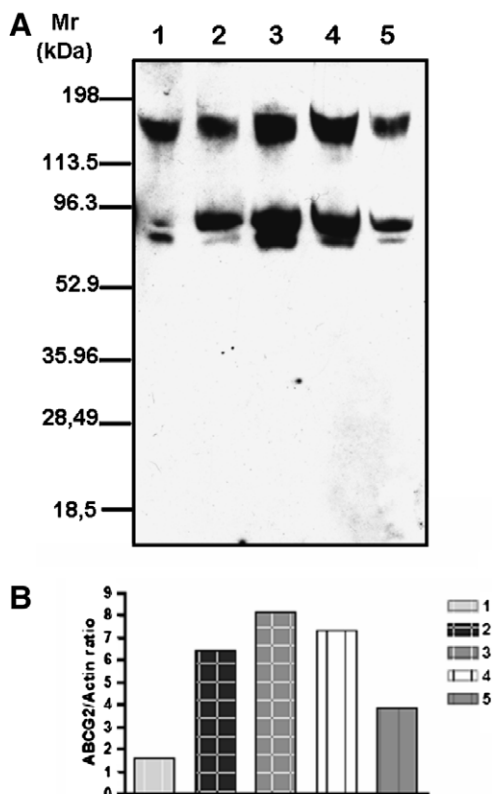
Fig. 2. ABCG2 expression in erythrocytes from different ethnic groups and blood types. Briefly, erythrocyte ghosts (50 µg) were loaded onto Fairbanks gel system, and transferred onto nitrocellulose membranes. Blots were probed with anti-ABCG2 (e.g., BXP-21) mAb. Lanes 1–5 of the Western blot contain resolved proteins from South Asian female, B+ blood type (lane 1); Caucasian male, O+ blood type (lane 2); Caucasian female, B+ blood type (lane 3); Caucasian female, B+ blood type (lane 4); and Caucasian female, O+ (lane 5). Anti-actin polyclonal antibody was used to estimate protein loading. (B) Histogram, showing the relative expression of ABCG2 in different samples (lanes 1–5) relative to actin expression.

rocytes or blood donors, other than the two blood donors shown in Fig. 1A. Fig. 2 shows the results of a Western blot analysis of erythrocyte ghosts from five different subjects resolved on Fairbanks SDS–PAGE and the Western blot probed with BXP-21 mAb (lanes 1–5, South Asian female, B+ blood type; Caucasian male, O+ blood type; Caucasian female, B+ blood type; Caucasian female, B+ blood type; Caucasian female, O+). The results in Fig. 2 (lanes 1–5) demonstrate the presence of ABCG2 homodimer in all five erythrocyte samples. Hence, the presence of ABCG2 homodimer in erythrocytes appears to be independent of blood type or gender (Fig. 2). Earlier studies have demonstrated gender-based differences in the expression of ABC proteins that result in gender-based pharmacokinetics and ultimately contribute to variation seen in drug disposition and therapeutic response and drug toxicity (as reviewed by [26]). Our results in Fig. 2 show no significant differences in ABCG2 expression levels based on the sample set tested (data not shown). In addition we looked at different blood types to determine if any antigenic variability or different ethic backgrounds could influence the expression levels of the ABCG2; again no significant differences were observed between donors of various racial groups (Caucasian: $n = 8$; South-Asian: $n = 3$; African: $n = 1$).

It has been previously shown that two ABCG2 monomers are required for active transport [10–12]. As such, analyses of ABCG2 on SDS–PAGE under non-reducing conditions show 140-kDa homodimer in membranes from drug-resistant tumor cells. Indeed, a higher molecular weight band, in addition to that at 140 kDa of ABCG2 homodimer, has been shown to migrate with an apparent molecular mass of ~210 kDa or trimmer and possibly higher oligomerization [9]. The observation that while both tumor cells and mature erythrocytes expressed ABCG2 monomer, only erythrocyte membranes contained a homodimer, when membrane extracts were examined on Fairbanks SDS–PAGE.

ABCG2 contains 12 cysteine residues, including three extracellular cysteines, Cys592, 603, and 608. Earlier reports have suggested that inter-disulfide bridge at

The presence of the 140-kDa ABCG2 homodimer in erythrocytes, but not in tumor cells, under the Fairbanks denaturing conditions prompted us to examine other eryth-
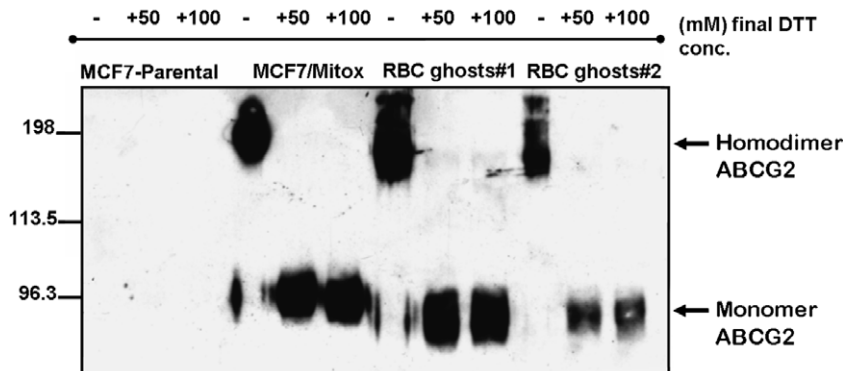

Fig. 3. Effects of increasing concentrations of DTT on homodimer/monomer pools of ABCG2 in MCF7/Mitox and erythrocyte membrane ghosts. Membrane extracts from MCF7/Mitox tumor cells and erythrocyte ghosts were incubated in the absence or presence of DTT (50 and 100 mM final). Membrane proteins (10–50 µg) were resolved on 7.5% Laemmli gel and transferred to nitrocellulose membrane. Membrane was probed with anti-ABCG2 monoclonal antibody BXP-21. (RBC ghosts #1: Caucasian female, B+ blood type; RBC ghosts #2: South Asian female, B+ blood type). The arrows to the left of the figure show the mobility of the homodimer and monomer forms of ABCG2, migrating with apparent molecular masses of 140 and 72 kDa, respectively.

Cys-603 in the 3rd extracellular domain between two monomers is important for oligomerization of ABCG2 [15]. However, given the role of disulfide bridges in ABCG2 oligomerization and functions, it was of interest to determine the effect of increasing concentrations of reducing agents on oligomerization of ABCG2. To this effect, we examined the effects of 50 and 100 mM of DTT on the mobility of ABCG2 on SDS–PAGE, as a measure of ABCG2 oligomerization. The results of Fig. 3 show that increasing concentrations of DTT causes the complete conversion of the dimer ABCG2 (e.g., 140-kDa polypeptide) into the monomer form (or a 72-kDa polypeptide). Taken together these results imply that the homodimer in erythrocytes is reduced under excessively high reducing condition of 50 mM DTT or higher. The variation seen between MCF7/Mitox and erythrocytes has yet to be further explored; however, we speculate that the high oxidative conditions under which erythrocytes operate are likely to play a role in ABCG2 oligomerization, relative to ABCG2 in tumor cells. Indeed, the results in Fig. 3 show that in the absence of exogenously added reducing agent, both tumor and erythrocyte ABCG2 migrated mainly as homodimer. Consequently one is left to speculate that in erythrocytes, ABCG2 homodimerization involves additional disulfides than those formed in ABCG2 extracted from tumor cells. The former ABCG2 (or erythrocyte ABCG2) homodimers are stable to low concentrations of DTT in Fairbanks gel system.

To determine if ABCG2 is active in erythrocytes, ABCG2-mediated transport in MCF7/Mitox-resistant cells and whole fresh erythrocytes was analyzed by flow cytometry. Cells were incubated with PheA, a fluorescent substrate of ABCG2, in the absence and presence of specific inhibitors (FTC). The results in Fig. 4 show that in the presence of FTC (up to 10 μM), PheA retention is significantly increased (~60%) in MCF7/Mitox cells. Similar incubation of intact erythrocytes with PheA in the presence of increasing concentrations of FTC shows a significant increase in PheA accumulation (~15%), but to a lesser extend than in MCF7/Mitox and more than in MCF7 cells (Fig. 4). It is interesting that our estimate of relative levels of ABCG2 expression in MCF7/Mitox and erythrocytes, roughly 1:4 folds, are consistent with the observed estimate of ABCG2 transport as measured by inhibition of ABCG2 transport with FTC and consequent increase in PheA retention (Fig. 4). Taken together, our results demonstrate that ABCG2 in erythrocytes is functionally active and can mediate active transport. Future work will address the kinetics of ABCG2 transport in erythrocytes relative to tumor cells using inside out *in vitro* transport system. It is interesting to speculate in light of these results that although disulfide bridges appear important to the functions of ABCG2, the fine details of their role on ABCG2 functions remain elusive. For one thing, higher disulfide bonds between two homodimers, as predicted from these results in erythrocyte membranes, does not appear to inhibit ABCG2 function, nor does it cause hyper-activation of ABCG2 transport function. It could however affect the
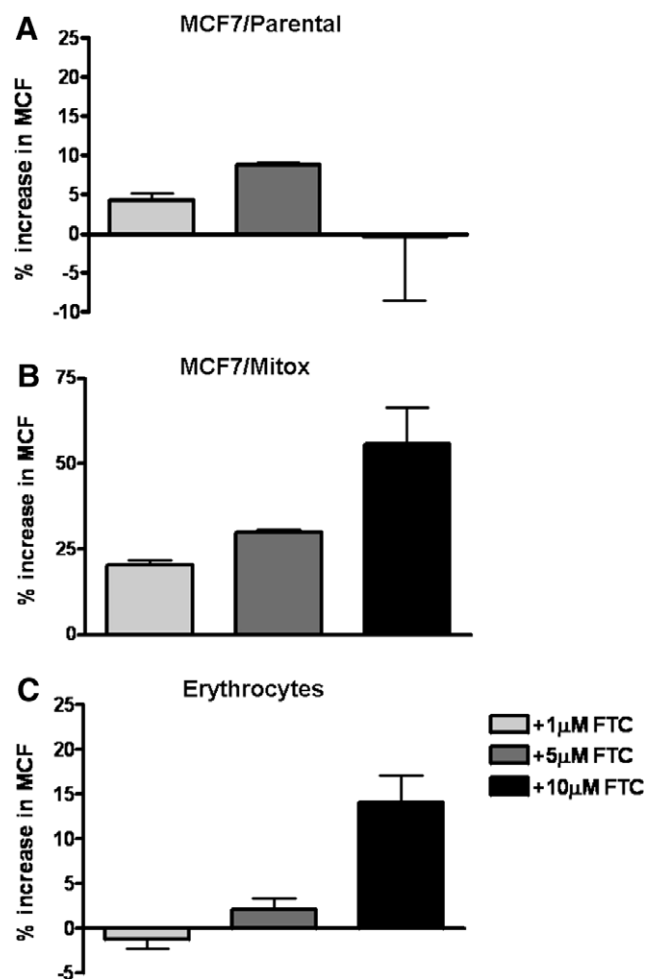

Fig. 4. Modulation of Pheophorbide A (PheA) transport. Drug-sensitive and Mitox-resistant MCF7 tumor cells and erythrocytes are incubated with 2 μM PheA for 30 min at 37 °C in the presence or absence of 1–10 μM fumitremorgin C (FTC). Cells were washed and incubated again for 60 min in PheA-free incubation medium, in the presence or absence of ABCG2-specific inhibitors. Drug accumulation was measured, by FACS analysis, as a function of total remaining fluorescence. (A–C) % change in mean fluorescence in MCF7, MCF7/Mitox, or erythrocytes in the presence of increasing FTC relative to cells incubated with PheA alone. Values are means ± SD of at least one experiment done in triplicate.

turnover of the protein. Therefore, it would be of interest to determine the role of disulfide bridges on the relative stability or turn over of ABCG2 in different membranes from the same or different tissues.

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