Histone deacetylase inhibitors influence chemotherapy transport by modulating expression and trafficking of a common polymorphic variant of the ABCG2 efflux transporter

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Running title: HDI impact on the expression and trafficking of Q141K ABCG2
Abstract

Histone deacetylase inhibitors (HDIs) have exhibited some efficacy in clinical trials but it is clear that their most effective applications have yet to be fully determined. In this study, we show that HDIs influence the expression of a common polymorphic variant of the chemotherapy drug efflux transporter ABCG2, which contributes to normal tissue protection. As one of the most frequent variants in human ABCG2, the polymorphism Q141K impairs expression, localization and function, thereby reducing drug clearance and increasing chemotherapy toxicity. Mechanistic investigations revealed that the ABCG2 Q141K variant was fully processed but retained in the aggresome, a perinuclear structure where misfolded proteins aggregate. In screening for compounds that could correct its expression, localization and function, we found that the microtubule disrupting agent colchicine could induce re-localization of the variant from the aggresome to the cell surface. More strikingly, we found that HDIs could produce a similar effect but also restore protein expression to wild-type levels, yielding a restoration of ABCG2-mediated specific drug efflux activity. Notably, HDIs did not modify aggresome structures but instead rescued newly synthesized protein and prevented aggresome targeting, suggesting that HDIs disturbed trafficking along microtubules by eliciting changes in motor protein expression. Together, these results showed how HDIs are able to restore wild-type functions of the common Q141K polymorphic isoform of ABCG2. More broadly, our findings expand the potential uses of HDIs in the clinic.
Introduction

ATP-binding cassette transporters (ABC) are energy-dependent transporters involved in absorption, distribution, and excretion of drugs, and in cancer drug resistance (1). ABCG2 is a member of the G subfamily of human ABC transporters, and is a half-transporter that must dimerize for function (2). ABCG2 transports numerous anticancer agents including mitoxantrone, etoposide, topotecan, and tyrosine kinase inhibitors (3). Additionally, ABCG2 substrates include antivirals, antibiotics, and toxins, highlighting the apparent role of ABCG2 transporters in normal tissue protection, including the blood-brain and maternal-fetal barriers. It has also been observed that ABCG2 may play a protective role with respect to exposure to smoke or dietary carcinogens (4, 5).

The most studied ABCG2 polymorphism is the non-synonymous SNP C421A, which results in a glutamic acid to lysine substitution at amino acid 141 (Q141K), localized in the ATP binding domain. C421A allele frequency is very common in the Japanese and Chinese population (26-35%) while it is 10-12% for Caucasians and <1% in the African population (3). ABCG2 harboring Q141K has impaired protein expression, incomplete trafficking to the plasma membrane, and decreased function. Clinically, the Q141K polymorphism has been linked to reduced clearance of some ABCG2 substrate drugs including diflomotecan, irinotecan, topotecan, gefitinib, rosuvastatin, atorvastatin, fluvastatin, simvastatin and sulfasalazine (6). In addition to its impact on pharmacokinetics, the Q141K SNP has recently been linked to at least 10% of all gout cases, since the decreased ABCG2 function reduces urate elimination (7).

Due to the clinical significance of the Q141K polymorphism in anticancer drug pharmacokinetics and potential involvement in carcinogenesis, we studied processing and trafficking of the intracellular trapped variant protein, and ways of rescuing Q141K ABCG2 to restore it to its wild-type phenotype. Previous studies have demonstrated that the defective folding and trafficking of some ABC transporter variants of P-glycoprotein (Pgp) or the cystic fibrosis transmembrane conductance regulator (CFTR) could be partially rescued by ligands that act as pharmacological chaperones to correct the folding defect (8-11). These compounds function by promoting interactions between protein domains during folding. In the same way, previous studies in our laboratory showed that mitoxantrone, a substrate of ABCG2, had the ability to rescue ABCG2 mutant protein (12). Here, we screened various compounds:
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mitoxantrone, the microtubule disruptor colchicine, and four different histone deacetylase (HDAC) inhibitors. HDAC proteins were first discovered for their involvement in gene regulation via histone deacetylation. It appears, however, that HDACs have broader activities than expected and have been shown, for example, to deacetylate various transcription factors or proteins involved in cell growth, death or cellular transport. In recent years, clinical development of HDAC inhibitors has emerged in cancer therapy, and several classes of HDAC inhibitors have been explored in preclinical models and reached the clinic (13, 14).

We found that HDAC inhibitors (HDIs) induced ABCG2 variant rescue and we attempted to elucidate the mechanism of action of HDIs on ABCG2 processing.

Materials and methods

Cell Culture

Flp-In-293 cells were obtained from Invitrogen. WT and Q141K ABCG2 sublines were created by transfection of the pcDNA5/FRT/V5-His-TOPO vector (Invitrogen) expressing WT or Q141K ABCG2 into Flp-In-293 cells, a HEK293 subline bearing a single integrated Flp Recombination Target site (Invitrogen), according to the manufacturer’s instructions. Cells were authenticated by STR-DNA technology (Radil) after establishment of different ABCG2 sublines, then aliquoted and frozen. After being thawed, cells were cultured in MEM supplemented with 10% FBS, 100µg/ml hygromycin B (Invitrogen), 2mM glutamine and 100units/L penicillin/streptomycin (BioFluids).

Chemicals

Mitoxantrone and cycloheximide were purchased from Sigma; panobinostat, vorinostat and 17AAG from ChemieTek; valproic acid from EMD4Biosciences; and tubastatin from Biovision. Romidepsin was obtained from the Developmental Therapeutics Program of NCI/NIH.

Immunofluorescence

Immunofluorescence was performed as previously described (12), and the following primary antibodies were used: anti-ABCG2 BXP-21 (Kamiya Biomedical), anti-giantin, anti-TGN46,
anti-HDAC6, anti-γ-tubulin or anti-vimentin (Abcam). Nuclei were stained with DAPI and cells were visualized on a Zeiss LSM510 META laser scanning microscope (Zeiss), equipped with an Apo63x1.4oil DIC II objective.

**RNA extraction and quantitative PCR**

Isolation of total RNA was performed with High Pure RNA isolation kit (Roche). cDNA synthesis was performed from 1µg RNA in a reverse transcriptase reaction using MMLV enzyme and random hexamers (Invitrogen). cDNA ABCG2 was amplified in a Light Cycler thermocycler using LightCycler TaqMan master mix (Roche Diagnostics) and probes from Roche Universal ProbeLibrary. The expression levels of ABCG2 were normalised with rRNA.

**Western blot**

Blots were probed with the following primary antibodies: anti-ABCG2, anti-GAPDH (American Research Products), anti-BiP (BD Biosciences), anti-Hsp90, anti-Hsc70 (StressGen), anti-Hsp70 (Cell Signaling), anti-acetyl-histone H3 (Santa Cruz), and anti-dynamitin (Abcam). Secondary antibody (LI-COR) were used to visualize quantifiable results with the Odyssey System (LI-COR).

**Endo H and PNGaseF assay**

Cell lysates were incubated with N-Glycosidase F or Endoglycosidase H (ProZyme) at 37 °C for 3 h, and immunoblotted.

**Processing experiments**

Cells were incubated 24h at 37 °C with 3µM MG132 (EMD4Biosciences), 10nM bafilomycin or 2mM 3-methyladenine (Sigma). Proteins were then extracted and immunoblotted.

**Flow Cytometry**

Flow cytometry examining ABCG2 with anti-ABCG2 antibody 5D3 (eBioscience) was performed as previously described (12). Cells were analysed with a FACSort flow cytometer with CellQuest software (BD Biosciences). Transport studies were performed as previously described (12) with 200nM BODIPY-prazosin (Invitrogen) with or without 10µM fumitremorgin.
C (FTC) (Developmental Therapeutics Program NCI/NIH), an ABCG2 inhibitor. The rate of cell death was measured via Annexin V / PI assay (BD Pharmingen), according to the manufacturer’s protocol.

**siRNA**

Cells were transfected with untargeted siRNA or siRNA against dynamitin (SMARTpool, Pharmacon), according to the manufacturer’s protocol. After 48h, immunoblotting was performed to determine the efficacy of the knockdown.

**Statistical analysis**

A student’s T test was performed for each experiment represented with a bar graph and asterisks indicate a significant difference (P<0.05) compared with control.

**Results**

**Determination of Q141K ABCG2 trafficking**

Flip-In-293 cells were selected for this study, as one copy of the vector is known to insert at a unique specific site, resulting in comparable RNA levels. To evaluate ABCG2 wild-type (WT) and Q141K variant localization in cells, immunofluorescence experiments were performed. As previously reported (15-17), we saw that Q141K ABCG2 proteins had incomplete trafficking and cytoplasmic retention, whereas WT proteins were mainly localized in the cell membrane (Figure 1A). Also noted was staining of large aggregates of proteins suggesting that the Q141K ABCG2 variant was accumulated in a localized cellular area.

Although Q141K and WT ABCG2 mRNA levels were equal, variant protein levels were 4-fold lower, suggesting a post-translational mechanism regulates expression (Figure 1B). Accordingly, the Q141K variant showed 5-fold decreased surface expression compared to WT.

We hypothesized that Q141K variant underexpression was due to protein degradation. To explore whether Q141K ABCG2 was degraded by the ubiquitin-proteasome, the endosome-lysosome, or the autophagic pathways, we treated the cells with the proteasome inhibitor MG132; bafilomycin A1, which inhibits lysosomal degradation; and 3-methyladenine (3-MA),
which inhibits autophagosome formation. Bafilomycin and MG132 treatment increased total protein expression (between 150 and 180%) for WT and Q141K protein (Figure 1C). These data suggest that in normal processing, a small portion of WT and Q141K ABCG2 are degraded via the proteasomal pathway while the fully processed proteins that reach the surface are degraded by the lysosome, as observed for other transmembrane proteins (18). Interestingly, the inhibition of the autophagic pathway induced a 3-fold increase in Q141K ABCG2 levels but barely affected WT protein expression. This indicates that autophagy is the main post-translational mechanism leading to a loss of Q141K variant expression. To support this conclusion, we induced autophagy by starvation or rapamycin, and observed a further decreased in variant levels after 16h and 24h treatments (supplementary Figure S1). We also observed that a higher proportion of Q141K variant is ubiquitinated compared to the WT protein (supplementary Figure S2). Moreover, whereas ABCG2 is ubiquitinated when it is targeted to the proteasome, it is not when it is targeted to the autophagosome.

To examine the processing of Q141K ABCG2, whole cell lysates from Flp-In-293 cells were deglycosylated with PNGase F and Endo H. ABCG2 contains a polysaccharide chain on asparagine 596 (19). PNGase F removes these glycans at any stage of processing while Endo H cleaves immature oligosaccharides. No change in molecular mass was observed after Endo H digestion (Figure 1D), suggesting that both WT and Q141K ABCG2 were completely processed, and not retained in the endoplasmic reticulum.

We next evaluated the localization of the retained ABCG2 variant in Flp-In-293 cells by immunofluorescence colocalization experiments. Two Golgi markers were used: giantin, a cis/medial Golgi marker, and TGN46, a trans-Golgi network marker (20). Results demonstrated that Q141K was not retained in Golgi (Figure 1E). We then asked whether Q141K ABCG2 proteins were retained in aggresomes. These structures, localized around the centrosome, are inclusion bodies where misfolded proteins aggregate (21). In figure 1F, we observed that the intracellular retained ABCG2 variant was mainly localized around gamma-tubulin, a centrosome marker, a finding consistent with localization in the aggresome (22). Thus the major fraction of Q141K variant accumulated in the aggresomes. While WT ABCG2 was mainly on the membrane, it was also discernable in the aggresome.

Together, these results indicated that wild-type ABCG2 was normally processed and mainly localized in the cytoplasmic membrane, while a small fraction undergoes proteasome
degradation or entrapment in the aggresome. The Q141K ABCG2 variant reached its mature form but was mainly sequestered in the aggresome and degraded via autophagy. A small fraction of variant also reached the surface or underwent proteasomal degradation.

**Impact of mitoxantrone on ABCG2 protein expression and localization.**

Studies in CFTR or Pgp have identified compounds that act as pharmacological chaperones to promote folding and trafficking of mature proteins to the cell surface (8-11). We previously demonstrated that mitoxantrone (MX) increased the processing of an ABCG2 mutant (12). The effects of MX were thus assayed in Q141K ABCG2 variant trafficking. We first evaluated by qPCR, immunoblot and flow cytometry analysis whether MX exposure induced ABCG2 expression in Flp-In-293 cells (Figure 2A). Treatment with 5µM MX for 24h caused a small increase in WT and Q141K total ABCG2 expression (around 125% for mRNA and 120% for protein compared to untreated cells). Surface expression showed a 160% induction in WT cells and a 250% induction in Q141K cells (Figure 2A). The subcellular localization of ABCG2 following MX was evaluated by immunofluorescence confocal microscopy. Q141K variant showed a marked homogeneous staining pattern, with loss of aggresome localization, while Flp-In-293 WT cells demonstrated no change (Figure 2B). Lastly, ABCG2 net efflux was determined by FACS in Flp-In-293 WT and Q141K cells after MX treatment (Figure 2C). Pretreatment with MX for 24h caused a weak increase of substrate net efflux (113% and 128% for WT and Q141K ABCG2, respectively).

Taken together, these results suggested that mitoxantrone stopped variant aggresome targeting, but recruitment to the cell surface was still incomplete.

**Impact of HDIs on ABCG2 protein expression, localization and function.**

We previously reported the ability of HDIs to increase ABCG2 mRNA levels (23, 24). Four HDIs were evaluated for their impact on ABCG2 expression and processing. Romidepsin affects primarily class I HDACs while panobinostat, vorinostat and valproic acid are more broad spectrum HDIs (13). We first determined the effect of HDIs on ABCG2 mRNA and protein expression in the ABCG2 Flp-In-293 sublines treated for 24h with 46nM romidepsin, 100nM panobinostat, 10µM vorinostat or 1mM valproic acid (Figure 3A). Following treatment with romidepsin, panobinostat and vorinostat, ABCG2 mRNA levels showed a 250-350% increase,
presumably due to transcriptional regulation following altered histone acetylation (23). Total and surface protein expressions were similarly increased (210-310% for WT ABCG2 and 260-375% for Q141K ABCG2). Valproic acid induced the weakest effect on mRNA and protein ABCG2 expression (150-200% increase). The same experiments performed in mock Flp-In-293 cells showed that endogenous ABCG2 was not expressed, even after HDI treatment (supplementary Figure S3), assuring that we only studied the transfected ABCG2.

Localization of ABCG2 was examined by confocal microscopy after HDI treatment. Again, basal plasma membrane localization of WT far exceeded that in Flp-In-293 Q141K cells, where ABCG2 was localized in aggresomes (Figure 3B). After exposure to HDIs, WT ABCG2 showed minimal change in localization, while a dramatic change was seen for Q141K ABCG2. Panobinostat, vorinostat, and especially romidepsin induced a significant decrease in ABCG2 inside the cell and increased staining along the cell surface. In figure 3C, we observed in detail that, in romidepsin-treated Flp-In-293 Q141K cells, the aggresome-localized ABCG2 has almost disappeared in favor of a strong surface localization. ABCG2 specific efflux was then determined by FACS after HDI treatment in Flp-In-293 WT and Q141K cells. In Flp-In-293 WT cells, 24h HDI pretreatment led to a weak increase of substrate net efflux compared to untreated cells (110-120%). However, the change in transport ability following exposure to the HDIs in Flp-In-293 Q141K cells was notably higher: 200% for romidepsin, 163% for panobinostat and 210% for vorinostat, compared to untreated Flp-In-293 Q141K cells (Figure 3D). Increased Q141K ABCG2 function was confirmed by a cell death assay using the ABCG2 substrate, pheophorbide A (Figure 3E). Indeed, Flip-In-293 Q141K cells pretreated for 24h with romidepsin showed a decrease in cell death after pheophorbide A treatment compared to cells not pretreated. Notably, it appears that WT is also rendered more efficient after HDI in the cell death assay, although the efflux assay was not sufficiently sensitive to detect this increasing WT ABCG2 efflux (saturating levels).

Altogether, these results indicate that romidepsin, vorinostat and panobinostat induced an increase in WT and Q141K ABCG2 expression that was associated with an improved trafficking to the cell surface in the Q141K variant. The increase also came with gain of Q141K ABCG2 function, indicating that the surface variant was functional.
Transcriptional effects of HDIs on Q141K ABCG2 rescue

Our goal was to determine the mechanism by which HDI treatment leads to improvement of ABCG2 mutant trafficking. We first determined whether the increased ABCG2 expression on the cell surface required new protein synthesis. We treated cells simultaneously with HDIs and cycloheximide (CHX), an inhibitor of protein biosynthesis. We verified that CHX does not affect the histone and α-tubulin acetylation induced by HDIs (supplementary figure S4). We then observed, in both Flp-In-293 WT and Q141K cells, that the MX and HDI-induced increases in surface expression were abolished by inhibition of protein synthesis (Figure 4A), and the same results were obtained with the transcription inhibitor actinomycin D (supplementary figure S5). These experiments indicated that HDI- and MX-induced rescue require transcription events. Whereas MX did not induce increased protein expression, the protein synthesis inhibitor CHX abrogated the MX-induced variant surface increase. This suggests that mitoxantrone only modified the trafficking of the neosynthesized ABCG2, and would not act on Q141K ABCG2 already trapped in the aggresome.

We also monitored the effect of romidepsin on mRNA and surface expression of ABCG2 over time. We observed that the drug-induced increase in mRNA expression of the two proteins were only detectable after 12h, and that the increased ABCG2 surface expression appeared subsequently, after 16h of treatment (Figure 4B). These results support the conclusion that the transporter cell surface rescue was more likely due to a gene transcription-related event, than to a direct acetylation-induced cytoplasmic event.

Comparing the data from MX and HDI effect on Q141K ABCG2 localization and function, we noted that MX induced a relocalization of the variant in a homogeneous cytoplasmic pattern and weakly on the plasma membrane, linked to a weak increase in ABCG2 function. HDIs induced mainly Q141K ABCG2 trafficking to membrane, accompanied by a greater increase in protein function. From these results, we hypothesized that HDIs not only induced ABCG2 expression, but also genes encoding proteins that enhanced ABCG2 folding and/or trafficking.

In order to find an ABCG2 folding partner, we focused on chaperone protein expression after 24h HDI treatment. We did not observe changes in Bip, Hsc70, Hsp70 or Hsp90 expression (supplementary figure S6), suggesting that HDI-mediated correction of trafficking cannot be ascribed to induction of these chaperones.
Non-transcriptional mechanisms of HDIs inducing Q141K ABCG2 rescue.

Considering ABCG2 acetylation as a possible mechanism of rescue, we immunoprecipitated ABCG2 and immunoblotted for acetylated lysine, but saw no effect of HDIs on the transporter acetylation state (data not shown).

We observed that Q141K ABCG2 was retained in aggresomes. The formation of aggresomes begins with the coalition of small, unfolded protein aggregates, which are dispersed in the periphery of the cytoplasm, and travel on microtubules towards the microtubule organizing center region. There, they remain as stable particulate structures entangled with intermediate filaments like vimentin, until degradation by autophagy or by the proteasome (21). In several models, HDAC6 has also been involved in retrograde transport by binding both misfolded proteins and the motor protein dynein, which then transports the misfolded protein-HDAC6 complexes towards aggresomes by travelling along microtubules (21, 25, 26).

We asked whether HDIs disturbed aggresome maintenance. In figure 5A, we observed that the microtubule-organizing center (centrosome) and vimentin were not disrupted by exposure to romidepsin, indicating maintenance of the aggresome structure.

We next examined the role of microtubules, known to be involved in retrograde transport, in ABCG2 localization. A previous study has shown that romidepsin did not alter the microtubule structure (27), but it is possible that HDIs disturb the trafficking along microtubules. To study the involvement of microtubule/retrograde transport in ABCG2 rescue, we used colchicine, a microtubule polymerization inhibitor. Surprisingly, immunofluorescence experiments showed that colchicine induced a dispersion of the ABCG2 variant aggregates and a relocalization of ABCG2 to the surface (figure 5B), much as observed with HDI treatment. Total ABCG2 expression was unchanged after colchicine treatment (Figure 5C). Based on the colchicine results, we concluded that HDI-induced ABCG2 rescue could also involve inhibition of retrograde transport along microtubules.

We then asked how HDIs could disturb retrograde transport. To evaluate HDAC6 involvement in Q141K ABCG2 rescue, we treated Flip-In-293 Q141K cells with the HDAC6 inhibitor tubastatin (28). In figure 5D, we observed that the HDAC6 inhibition observed after 24h treatment with 2.5µM tubastatin did not induce ABCG2 variant rescue. Moreover, cotreatment of tubastatin with romidepsin (class I HDAC inhibitor) did not induce an additional effect. No change was expected with the other HDIs, knowing that they already inhibit HDAC6.
We concluded that HDAC6 inhibition was not involved in variant rescue. We then evaluated the involvement of the motor protein complex in ABCG2 rescue. It has been shown that overexpression of dynamitin induces the disruption of dynein from microtubules, leading to abrogation of aggresome formation (29). We studied dynamitin expression after HDI treatment and observed that romidepsin, panobinostat, and vorinostat, but not valproic acid, induced dynamitin overexpression (Figure 5E). Knowing that valproic acid is the only HDI that does not induce ABCG2 rescue, these results suggest that HDIs might inhibit ABCG2 retrograde transport via dissociation of dynein from microtubules, due to dynamitin overexpression. To confirm these data, we performed a siRNA assay to inhibit the expression of dynamitin, and we assessed the effect of HDIs on ABCG2 Q141K rescue after dynamitin knockdown. As shown in figure 5F, dynamitin knockdown blunted the effect of HDIs on Q141K ABCG2 rescue, with a significant effect for romidepsin.

**Discussion**

We saw that the Q141K variant was retained in aggresomes, and then degraded by the autophagic pathway. The aggresome was discovered by Johnson et al (21) as a cellular area where misfolded undegraded CFTR molecules accumulate. They proposed that aggresome formation was a general response of cells, occurring when proteasome capacity is exceeded by misfolded protein production. Aggresome formation facilitates the capture of aggregated proteins by the autophagic pathway (30-33), which we observed to be the case for Q141K ABCG2. A previous study had demonstrated in the same Flp-In-293 cell model that Q141K ABCG2 was subjected to ubiquitin-mediated proteasomal and lysosomal degradation, with a 2-fold increase in expression after treatment with both inhibitors (17). We also found that a fraction of ABCG2 variant was degraded via the proteasome and lysosome, with a 1.8-1.9-fold increase after inhibition. The authors concluded that it undergoes lysosomal and proteasomal degradations, but did not explore the possibility of an autophagic pathway.

It is interesting to note that the expressed WT and Q141K ABCG2 variants are found in a fully mature form, contrary to what has been observed with other ABC transporters. For example, CFTR with the ΔF508 deletion results in a defectively folded protein that fails to mature and is rapidly degraded (34, 35). In the same way, only wild-type Pgp is processed in
mature form while some mutants are intracellularly retained in an immature form (36).

We sought to rescue Q141K ABCG2 trafficking using HDIs. The HDIs induced ABCG2 mRNA, total, and surface protein expression with a conserved ratio, for WT as for Q141K variant. More importantly, HDIs caused a strong relocalization of the Q141K variant from aggresome to cell surface, and increased Q141K-mediated efflux. We sought to understand why these HDI-induced Q141K proteins, instead of accumulating in the aggresomes, were able to traffic to the cell surface. The rescue by the HDIs required protein neosynthesis, and an approximate 16-hour delay. As suggested for mitoxantrone, these results imply that HDIs improve neosynthesized transporter folding rather than affecting the already aggresome-trapped variant. They also implicate an increase in trafficking mechanisms via transcription regulation rather than by a more immediate signaling or acetylation event. We observed that dynamitin was overexpressed, probably leading to the inhibition of retrograde transport via the disruption of dynein from microtubules. The partial inhibition of the HDI effect on ABCG2 rescue after dynamitin knockdown suggested that inhibition of retrograde transport is a part of HDI mechanism of action. Other proteins involved in protein folding or trafficking might also be regulated. For example, Hutt et al (37) have shown that SAHA-induced rescue of CFTR variant was also mainly due to gene transcription regulation. Interestingly, they demonstrated by siRNA screen of all class I and II HDACs that inhibition of HDAC7 restored CFTR trafficking and activity on cell surface. HDAC7 regulated a set of genes involved in CFTR folding, maturation, trafficking and channel activation. HDAC1 inhibition also restored CFTR variant trafficking, but not its function.

We observed that colchicine was also able to rescue Q141K ABCG2 trafficking. This drug, which inhibits microtubule polymerization, is already known to disrupt aggresome formation and retrograde transport (38). We deduced that HDIs similarly inhibit Q141K retrograde transport towards the aggresome, likely via dynamitin overexpression. HDIs might also directly inhibit HDACs involved in retrograde transport. For example, Kim et al (39) recently demonstrated that, during an inflammatory stimulus, HDAC1 competed with the adaptor proteins for binding to motor proteins that travel along the microtubules, impairing axonal transport in neurones. This suggests a new role for HDAC1 in protein trafficking. Nawrocki et al. (40) also demonstrated that inhibition of HDAC6 led to disruption of the aggresomes, but our experiments suggested that this deacetylase was not involved in the Q141K ABCG2 rescue.
Based on our results, we propose a multi-pathway mechanism for HDI-induced Q141K ABCG2 rescue to surface (Figure 6). HDIs induced Q141K ABCG2 surface localisation by an increase in ABCG2 transcription coupled to dynamitin overexpression, which reduced aggresome targeting by inhibition of dynein/microtubule retrograde transport. Other protein partners might also improve the neosynthesized variant folding and trafficking, as observed with the CFTR variant (37).

HDI impact on the expression and trafficking of Q141K ABCG2

HDI are promising anti-cancer agents. They were initially thought to simply reverse aberrant epigenetic changes associated with cancer, but it was soon observed that the inhibition of HDAC activity led to pleiotropic activities via various cellular and molecular pathways. These broad effects make it difficult to identify the key molecular events that determine the biological response to HDIs. Here, we highlighted a new molecular mechanism: HDIs acting as modulators of protein trafficking.

The use of molecules acting as “correctors” of variant protein processing and “potentiators” of variant protein activity, largely developed in vitro, has just shown its first clinical success. Ramsey et al (November 2011, (41)) observed in a phase III clinical trial that the CFTR potentiator Ivacaftor, used to treat CFTR variant carriers, was associated with a sustained 17% relative improvement in lung function. These successful results highlight the potential of this emerging treatment strategy in the clinic and open doors to a new use of HDIs as ABCG2 defective variant corrector to treat gout and imply that a chemoprevention strategy could be identified if ABCG2 were found to be important in carcinogenesis. Regardless of therapeutic relevance, this study offers insights into the processing of a protein of increasing importance in pharmacology, blood brain barrier, and normal tissue protection.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, NCI, CCR. We acknowledge Drs. To and Polgar for the Flp-In-293 cell line establishment.
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Figure Legends

Figure 1. Determination of Q141K ABCG2 localization, processing and degradation pathways.
A. ABCG2 was detected by immunofluorescence in Flp-In-293 cells. B. mRNA and protein were detected by qPCR and immunoblot, and ABCG2 surface expression was determined by flow cytometry. C. Flp-In-293 cells were incubated 24h with bafilomycin (10nM), MG132 (3µM), or 3-MA (2mM), and ABCG2 expression was determined by immunoblot. D. Enzymatic deglycosylation was performed with PNGase F and Endo H, then ABCG2 was detected by immunoblot. E. and F. Flp-In-293 cells showing nuclei (blue), ABCG2 (red) and Golgi marker (giantin, E. above), transgolgi marker (TGN46, E. below) or γ-tubulin (F.) in green. White arrows in some panels point to the site of aggresome.

Figure 2. Impact of mitoxantrone on ABCG2 protein expression and localization.
A. Flp-In-293 cells were submitted to 24h exposure to 5µM MX then ABCG2 mRNA, protein and surface expression were detected by qPCR, immunoblot, and flow cytometry,
respectively. Q141K mRNA, total protein and surface protein expression was 93%, 28% and 21%, respectively, of WT ABCG2. B. ABCG2 was demonstrated by confocal microscopy in Flp-In-293 WT and Q141K cells after 24h treatment with MX. C. ABCG2 relative efflux was determined by FACS after 24h treatment with MX. Q141K relative efflux was 39.5% of WT ABCG2.

**Figure 3. Impact of HDIs on WT and Q141K ABCG2 protein expression, localization and function.**

A. ABCG2 mRNA, total and surface protein were respectively quantified by qPCR, immunoblot, and FACS analysis in Flp-In-293 WT and Q141K cells following 24h exposure to 46nM romidepsin (RD), 100nM panobinostat (PN), 10µM vorinostat (VR) or 1mM valproic acid (VA). B. ABCG2 repartition was visualized in cells by immunofluorescence after 24h HDI exposure. C. ABCG2 and γ-tubulin were observed in Q141K cells after 24h RD treatment (magnified example). D. ABCG2 net efflux was determined by FACS after 24h treatment with HDIs. E. Cells were pretreated or not with RD dilutions for 24h, then treated for 24h with pheophorbide A (1µM for WT and 0.5µM for Q141K cells), and cell death was measured by flow cytometry.

**Figure 4. Transcriptional effects of HDIs on ABCG2 Q141K rescue**

A. Quantification of ABCG2 surface expression by FACS in Flp-In-293 WT and Q141K cells after 24h exposure to MX and HDIs, in presence or not of cycloheximide (CHX). B. Time course of ABCG2 mRNA (top panel) and surface expression (low panel) in Flp-In-293 cells during RD treatment (46 nM) was realized by qPCR and FACS using the 5D3 antibody, respectively.

**Figure 5. Non-transcriptional effects of HDIs on ABCG2 Q141K rescue.**

A. Vimentin and γ-tubulin localization were observed by confocal microscopy in Flp-in-293 Q141K cells after a 24h treatment with 46 nM romidepsin. B. The effect of colchicine (1µM, 24h) on ABCG2 localization was determined by immunofluorescence in Flp-In-293 Q141K ABCG2 cells. C. ABCG2 total expression was quantified by immunoblot in Flp-In-293 WT and Q141K cells after 24h exposure to 1µM colchicine. D. Quantification of ABCG2 surface
expression by FACS in Flp-In-293 Q141K cells after 24h exposure to HDIs in presence or not of 2.5µM tubastatin. E. Quantitation of dynamitin expression by immunoblot after 24h treatment with HDIs. F. The knockdown of dynamitin was observed after 48h transfection in Q141K cells by immunoblot (left panel). The effect of dynamitin knockdown was then assessed on ABCG2 Q141K protein expression (surface and total) after a subsequent 24h HDI treatment.

**Figure 6: Proposed mechanism of multiple target HDIs-mediated Q141K ABCG2 rescue.**

HDIs act on Q141K ABCG2 trafficking by inducing ABCG2 overexpression (1), and likely a trafficking/folding partner overexpression (3), as well as by inhibition of retrograde transport from cytoplasm to aggresome (2).
Figure 2

A. ABCG2 expression

<table>
<thead>
<tr>
<th>mRNA</th>
<th>protein</th>
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</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Q141K</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% compared to untreated cells</td>
<td>% compared to WT</td>
<td>% compared to WT</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. ABCG2 relative efflux

<table>
<thead>
<tr>
<th>Flp-In-293 WT</th>
<th>Flp-In-293 Q141K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Q141K</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. ABCG2 relative efflux

<table>
<thead>
<tr>
<th>WT</th>
<th>Q141K</th>
</tr>
</thead>
<tbody>
<tr>
<td>% compared to untreated cells</td>
<td>% compared to WT</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>


**Figure 3**

A. mRNA, protein, surface

```
<table>
<thead>
<tr>
<th></th>
<th>mRNA</th>
<th>protein</th>
<th>surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image" alt="WT mRNA" /></td>
<td><img src="image" alt="WT protein" /></td>
<td><img src="image" alt="WT surface" /></td>
</tr>
<tr>
<td>Q141K</td>
<td><img src="image" alt="Q141K mRNA" /></td>
<td><img src="image" alt="Q141K protein" /></td>
<td><img src="image" alt="Q141K surface" /></td>
</tr>
</tbody>
</table>
```

B. Flp-In-293

```
<table>
<thead>
<tr>
<th></th>
<th>ctl</th>
<th>RD</th>
<th>PN</th>
<th>VR</th>
<th>VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image" alt="WT ctl" /></td>
<td><img src="image" alt="WT RD" /></td>
<td><img src="image" alt="WT PN" /></td>
<td><img src="image" alt="WT VR" /></td>
<td><img src="image" alt="WT VA" /></td>
</tr>
<tr>
<td>Q141K</td>
<td><img src="image" alt="Q141K ctl" /></td>
<td><img src="image" alt="Q141K RD" /></td>
<td><img src="image" alt="Q141K PN" /></td>
<td><img src="image" alt="Q141K VR" /></td>
<td><img src="image" alt="Q141K VA" /></td>
</tr>
</tbody>
</table>
```

C. ABCG2 / γ-tub / nucleus in RD-treated Q141K cells

```
<table>
<thead>
<tr>
<th></th>
<th>ABCG2</th>
<th>γ-tub</th>
<th>nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image" alt="WT ABCG2" /></td>
<td><img src="image" alt="WT γ-tub" /></td>
<td><img src="image" alt="WT nucleus" /></td>
</tr>
<tr>
<td>Q141K</td>
<td><img src="image" alt="Q141K ABCG2" /></td>
<td><img src="image" alt="Q141K γ-tub" /></td>
<td><img src="image" alt="Q141K nucleus" /></td>
</tr>
</tbody>
</table>
```

D. ABCG2 relative efflux

```
<table>
<thead>
<tr>
<th></th>
<th>abcg2 efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image" alt="WT abcg2 efflux" /></td>
</tr>
<tr>
<td>Q141K</td>
<td><img src="image" alt="Q141K abcg2 efflux" /></td>
</tr>
</tbody>
</table>
```

E. Pheo A-induced cell death

```
<table>
<thead>
<tr>
<th></th>
<th>Pheo A-induced cell death</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image" alt="WT Pheo A-induced cell death" /></td>
</tr>
<tr>
<td>Q141K</td>
<td><img src="image" alt="Q141K Pheo A-induced cell death" /></td>
</tr>
</tbody>
</table>
```

*Significant difference compared to untreated cells.*
**Figure 4**

### A. ABCG2 surface expression

Bar graph showing ABCG2 surface expression in WT and Q141K cells with and without 10 μg/ml CHX treatment. Graphs for untreated cells and cells treated with MX, RD, PN, VR, and VA are included.

### B. ABCG2 expression

Line graphs showing mRNA and surface expression of ABCG2 over time in WT and Q141K cells with treatments.
Figure 5

A. Vimentin / γ-tubulin / nucleus in Flip-In-293 Q141K ABCG2 cells
ctl | RD

B. ABCG2 labelling in Flip-In-293 Q141K ABCG2 cells
ctl | colch

C. ABCG2 total expression

D. Surface ABCG2 expression + tuba
ctl | RD | PN | VR | VA

E. DNMT expression
ctl | RD | PN | VR | VA

F. siRNA dynamitin in Flip-In-293 Q141K cells
dynamitin expression

ABC2 surface / total expression
Figure 6

Diagram illustrating the retrograde transport inhibition process involving microtubules, dynein/dynactin, aggresome, vimentin, centrosome, ABCG2, and chaperones. The process includes steps for misfolded ABCG2 being corrected, aggregated, and transported back to the cell surface.
Histone deacetylase inhibitors influence chemotherapy transport by modulating expression and trafficking of a common polymorphic variant of the ABCG2 efflux transporter

Agnes Basseville, Akina Tamaki, Caterina Ieranò, et al.

Cancer Res  Published OnlineFirst April 3, 2012.