Xanthines downregulate the drug transporter ABCG2
and reverse multidrug resistance

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Nonstandard abbreviations used: multidrug resistance (MDR), breast cancer resistance protein (BCRP), ATP binding cassette (ABC), mitoxantrone (MX), cancer stem cells (CSCs), Bodipy-prazosin (BP), Fumitremorgin (FTC)
Abstract

ABCG2 is an ATP-binding-cassette (ABC) transporter that confers multidrug resistance (MDR) to tumor cells by extruding a broad variety of chemotherapeutic agents, ultimately leading to failure of cancer therapy. Thus, the downregulation of ABCG2 expression and/or function has been proposed as part of a regimen to improve cancer therapeutic efficacy. In this study, we found that a group of xanthines including caffeine, theophylline and dyphylline can dramatically decrease ABCG2 protein in cells that have either moderate (BeWo, a placental choriocarcinoma cell line) or high (MCF7/MX100 cells, a breast cancer drug resistant cell subline) levels of ABCG2 expression. This downregulation is time-dependent, dose-dependent, and reversible. Using lysosomal inhibitors, we found that xanthines decreased ABCG2 by inducing its rapid internalization and lysosome-mediated degradation. As a consequence, caffeine treatment significantly increased the retention of an established ABCG2 substrate in MCF-7/MX100 cells but not in parental MCF-7 cells, and sensitized the MDR cells to the chemotherapeutic agent mitoxantrone (MX); combination treatment with MX and caffeine decreased the IC₅₀ of MX ~10-fold and induced a greater degree of apoptotic cell death compared to MX treatment alone. Taken together, our results describe a novel function for this large class of therapeutically-relevant compounds and suggest that a subset of xanthines could be developed as combination therapy to improve the efficacy of anticancer drugs that are ABCG2 substrates.
Introduction

The ABCG2 (BCRP) protein was first identified due to its overexpression in breast cancer cells exhibiting a MDR phenotype in the absence of other “classical” drug transporters (Doyle et al., 1998). Although a member of the same ABC family of membrane transporters as P-glycoprotein (Pgp) and Multidrug Resistance Proteins (MRPs), ABCG2 belongs to a unique subclass whose genes encode a half transporter with one classic nucleotide-binding domain and six transmembrane segments. Initially believed to exist as a homodimer, recent evidence suggests that ABCG2 may function as an oligomer consisting of 8–12 identical subunits (McDevitt et al., 2006) and transports a broad spectrum of natural and synthetic substrates including some cancer chemotherapeutics.

Given the role of ABCG2 in conferring MDR, there has been considerable focus on the expression of this transporter in human tumors, and its correlation with clinical outcome. Elevated expression of ABCG2 was first reported in patients with acute myeloid leukemia (AML) (Ross et al., 2000), and subsequently confirmed in both AML and acute lymphocytic leukemia patients (Plasschaert et al., 2003; van den Heuvel-Eibrink et al., 2002; Wilson et al., 2006). ABCG2 conferred resistance to flavopiridol in AML patients (Nakanishi et al., 2003) and its overexpression was higher following relapse of AML; hence, ABCG2 was suggested to act as a prognostic indicator for this disease (Benderra et al., 2004; Uggla et al., 2005). The role of ABCG2 in the resistant phenotype of solid tumors is less clear, although ABCG2 overexpression has been described in several tumor types (Diestra et al., 2002; Turner et al., 2006). Interestingly,
ABCG2 expression was rapidly increased in hepatoblastoma in response to certain chemotherapeutics (Vander Borght et al., 2008), reminiscent of the induction of Pgp observed following treatment of metastatic sarcoma with doxorubicin (Abolhoda et al., 1999). ABCG2 is also expressed in adult normal tissues, including the intestine, colon, placenta, and blood-brain barrier (BBB), where it plays a role in restricting the oral bioavailability and pharmacokinetics of its substrates and likely plays a critical role in the homeostasis of endogenous compounds such as heme, porphyrins, riboflavin, and estrogens (Grube et al., 2007; Krishnamurthy et al., 2007; Ni et al., 2010; van Herwaarden et al., 2007).

Of interest is the observation that ABCG2 appears to play a critical role in both normal and cancer stem cells (CSCs); indeed, the observation that ABCG2 is often enriched in normal stem cells and CSCs relative to their more mature progeny has led to the proposed use of ABCG2 as a universal stem cell marker (Bunting, 2002; Zhou et al., 2001). While the function of ABCG2 in CSCs is not well understood, some studies suggest that it is required for maintaining the “stemness” of the population, perhaps by enhancing proliferation and/or decreasing differentiation potential. While this requires further investigation, there is ample evidence supporting a role for ABCG2 in protecting both normal and cancer stem cells from various stressors, including cancer chemotherapeutics (Dean et al., 2005; Krishnamurthy and Schuetz, 2006).

Given the pleiotropic roles of ABCG2 in drug disposition, drug resistance and CSC survival, inhibition or downregulation of ABCG2 may be a valid approach to reverse ABCG2-mediated drug resistance and to increase oral absorption of certain
chemotherapeutic drugs. Moreover, the putative role of ABCG2 in maintenance of CSCs suggests that ABCG2 inhibitors, unlike other drug transporter inhibitors, may function to reduce or eliminate the CSC population.

In the current study, we have investigated the use of xanthine derivatives as ABCG2 inhibitors. Xanthines are common in the human diet, where they are consumed in the form of nutrients, stimulants and drugs. Caffeine (1,3,7-trimethylxanthine), the best known, best-studied and most widely consumed xanthine, is a purine alkaloid, with a variety of pharmacological effects. It is used in the treatment of migraines, and postprandial hypotension and obesity and as a respiratory stimulant in neonates (SAWYNOK, 1995). Caffeine’s effect on cell cycle transition results in the sensitization of some human tumors to ionizing radiation and alkylating agents such as nitrogen mustard and cisplatin (Sarkaria et al., 1999). Analogous to caffeine, many other xanthine-related heterocyclins with enhanced potency and selectivity towards specific biological targets have provided powerful tools for research, and potential therapeutic agents for intervention in a variety of diseases (Daly, 2007). For example, theophylline, a caffeine metabolite found in tea, is used for the treatment of asthma and has anti-inflammatory properties. Dyphylline (7-(2,3-dihydroxypropyl)-theophylline) is used as a bronchodilator in the treatment of asthma, chronic bronchitis, and emphysema.

Additional xanthine derivatives are currently in preclinical and clinical studies (McCarty et al., 2002).
We now show that caffeine and several related xanthines can dramatically decrease ABCG2 protein in multiple cell lines by targeting this cell surface transporter for internalization and lysosomal degradation. This downregulation has functional consequences. Exposure to caffeine increased intracellular accumulation of the ABCG2 substrate mitoxantrone (MX), resulting in a 10-fold increased sensitivity of tumor cells to the cytotoxic effects of this agent. These findings suggest that xanthines can be developed as combination therapy to improve the efficacy of drugs that are ABCG2 substrates.
Materials and methods

Cell culture and Chemicals

BeWo cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in F-12K medium (ATCC, #30-2004) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, GA) at 37 °C in 5% (v/v) CO2. MCF-7 parental cells and the MX-resistant subline MCF-7/MX100 were kindly provided by Dr. Susan Bates, National Institutes of Health, MD. Both cell lines were grown in improved minimum essential medium (IMEM, Invitrogen) containing 2g/l glucose, 2mM L-glutamine, 1mM sodium pyruvate and 10% fetal bovine serum at 37° in 5% (v/v) CO2. MCF-7/MX100 cells were maintained in the presence of 100nM MX (Honjo et al., 2001).

Caffeine and caffeine analogs (theophylline, pentoxifylline, dyphylline, paraxanthine, theobromine, 7-(β-hydroxyethyl) theophylline, 7-methlxanthine), ammonium chloride and leupeptin were purchased from Sigma-Aldrich (St. Louis, MO).

Protein analysis

For western blot analysis, cells were washed twice with cold phosphate-buffered saline and lysed in RIPA buffer (50 mMTris-HCl, pH 7.4, 150 mMNaCl, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% deoxycholate, Na salt) plus protease inhibitor cocktail (Roche Diagnostics), and 100 mg/ml phenylmethylsulfonyl fluoride. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce). Equal amounts of total protein (5 to 15μg) were analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting using mouse monoclonal antibody (clone BXP-21) against ABCG2 (1:1,000; Kamiya), or
rabbit monoclonal antibody against GAPDH (1:1000; Cell Signaling). The secondary antibody was either horseradish peroxidase-conjugated goat-anti-mouse IgG (1:2,500; Amersham) or horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology). Immunoreactive bands were visualized using an enhanced chemiluminescent system (Pierce) according to the manufacturer's recommendations.

For immunohistochemistry, cells grown on glass coverslips were washed three times with PBS, fixed in 4% paraformaldehyde solution and permeabilized in 0.2% Triton-X-100 solution for 10 min. Cells were washed with PBS three times at each interval. Coverslips were incubated with 2% BSA in 0.1% Triton X-100 PBS buffer at room temperature for 1 h, followed by incubation with monoclonal ABCG2 antibody BXP-21 (1:250); in 0.1% Triton X-100 in a humid chamber, washed three times with PBS, then incubated with Alexa Fluor® 488-conjugated goat anti-mouse IgG at 37 °C for 1 h. Coverslips were mounted, sealed on glass slides with DAPI mounting medium and observed using a Nikon Eclipse TE2000-U confocal microscope (NY, USA).

**Efflux assay**

Efflux assays were performed as previously described (Robey et al., 2001) with minor modifications. Briefly, cells were collected and suspended in complete medium alone or complete medium containing 500 nM Bodipy-prazosin (an ABCG2 substrate; Invitrogen, Molecular Probes, Eugene, OR) with or without 10 µM Fumitremorgin C (FTC, a known ABCG2 inhibitor (Rabindran et al., 2000)) and incubated at 37°C in 5% CO₂ for 30 min.
The incubations were stopped with the addition of 4ml cold PBS, and cells were washed three times with ice-cold PBS and incubated for 1 h at 37°C in 5% CO₂ in complete media with or without 10 µM FTC. Cells incubated in blank medium were used as the control for cell autofluorescence. Dead cells and debris were gated out for each treatment by propidium iodide (PI) staining. Fluorescence was visualized using a Coulter Cytomics FC500 Flow Cytometer with a 488-nm argon laser and 530-nm band pass filter.

**Cytotoxicity and cell death assays**

MX cytotoxicity was assayed as follows. BeWo or MCF-7/MX100 cells were plated in 96-well plates at a density of 5,000 cells/well. Twenty-four hours after plating, cells were treated with or without caffeine for another 24 hours. After caffeine pretreatment, the medium was replaced with medium containing increasing concentrations of MX and cells were incubated for 72 h at 37°C. Cell death was assayed using CellTiter 96 AQueous One solution according to the manufacturer’s instructions (Promega, Madison, WI).

Cytotoxicity was assessed by monitoring the absorbance at 490 nm using a Multiskan Spectrum microplate reader (Thermo scientific). The IC₅₀ value was defined as the drug concentration resulting in 50% cell death. Both the fitted sigmoidal dose response curve and IC₅₀ were calculated using Prism 4.

The Guava EasyCyte flow cytometry analysis system (Guava Technologies, Hayward, CA) was utilized to determine percent of apoptotic cells. Assays were conducted
according to the manufacturer’s instructions. Briefly, total cells were collected and washed with cold PBS, then 5 µL of annexin V-phycoerythrin, a marker for early apoptosis, and 5 µL of 7-amino-actinomycin (7-AAD), a cell-impermeant dye indicating late apoptosis or dead cells (Guava PCA-96 Nexin Kit), were added to cell suspensions. After 20 mins incubation and thorough mixing, samples were analyzed on a Guava PC.
Results

Xanthines downregulated ABCG2 expression in cancer cells

Xanthines, particularly methylxanthines, are found in over 60 plant species and are among the most widely consumed substances in our diet. To evaluate the effect of xanthines on the expression of the drug transporter ABCG2 in tumor cells, we utilized the mitoxantrone (MX)-resistant human breast cancer cell line MCF-7/MX100, which is resistant to MX relative to its MCF-7 parent cells by virtue of its overexpression of ABCG2 (Fig 1a, c), and the human placental choriocarcinoma cell line BeWo (Fig 1b, d), which has intrinsic expression of functional ABCG2. Exposure to the methylxanthine caffeine reduced ABCG2 protein levels in a dose- (Fig 1a, b) and time- (Fig 1c, d) dependent manner. Effects could be seen as early as six hours post-treatment with maximum decrease (~ 60-75%) achieved by 24 hrs (IC50 = 0.4mM (BeWo) and 2.5 mM (MCF-7/MX100)). This effect was reversible; when caffeine was removed and replaced with fresh medium 24 hrs post-treatment, ABCG2 levels gradually recovered, reverting to normal levels within the next 24 hours (Fig 1 e and f). Notably, while some caffeine-induced cell cycle arrest was observed in MCF-7/MX100 cells, this arrest was not observed in BeWo cells (data not shown), yet the degree of downregulation of ABCG2 by caffeine in both cell lines was similar. Therefore, it does not appear that caffeine-induced cell cycle inhibition plays a role in the regulation of ABCG2 levels by xanthines. Moreover, caffeine did not have a general effect on membrane protein stability, since there was minimal impact on the expression of EGFR, and no apparent effect on Na+/K+-ATPase (data not shown).
A number of naturally occurring xanthines are structurally similar to caffeine and share some of its pharmacological effects. To determine the generality of caffeine’s effect on ABCG2 expression, we analyzed ABCG2 protein levels following exposure to several of these caffeine analogs. As shown in Figure 2a, among the analogs we tested theophylline, a direct metabolite of caffeine, was the most potent compound in terms of downregulating ABCG2 protein levels (Fig 2b); 0.2 mM of theophylline was sufficient to reduce ABCG2 protein levels by 50%; maximum decrease (90%) was achieved by 1.75 mM. Other compounds, such as paraxanthine and 7-β-hydroethyl-theophylline, did not significantly affect ABCG2 expression levels. The structure-activity relationship of a large group of xanthines is currently under investigation.

**Caffeine induces internalization and lysosomal degradation of ABCG2**

To determine the molecular basis for the downregulation of ABCG2, we first considered the possibility that caffeine affected the synthesis or processing of ABCG2 mRNA. Indeed, we have previously shown that caffeine can induce the alternative splicing of a subset of genes (Shi et al., 2008). However, quantitative PCR analysis of ABCG2 RNA from untreated and caffeine-treated cells revealed no impact on either ABCG2 RNA levels or splicing in either MCF-7/MX100 or BeWo cells (data not shown), suggesting that the effect of caffeine occurred at the level of protein synthesis or degradation.
To examine the fate of ABCG2 protein in the presence of caffeine, cells were subjected to immunofluorescence staining following caffeine treatment. As shown in Figure 3a, both MCF-7/MX100 and BeWo cells exhibited intense membrane staining and some cytoplasmic staining in the presence of an ABCG2-specific antibody. Following treatment with high levels of caffeine, there was a significant decrease in the total fluorescence intensity in both cell lines, and membrane-localized ABCG2 was largely attenuated. At lower concentrations (0.8 mM), more intracellular staining was observed as compared to the untreated cells, suggesting internalization of ABCG2 protein in the presence of caffeine. Again, this effect was time-dependent (Figure 3b), with internalization apparent following 4 hours of caffeine treatment and peaking between 10 and 24 hrs following caffeine exposure. By 24 hrs, little ABCG2-related staining was observed in the cell, suggesting that caffeine had induced the internalization and subsequent degradation of ABCG2 protein.

It has been previously reported that wild type ABCG2 is primarily degraded through the lysosomal-mediated pathway, although misfolded ABCG2 is degraded by ubiquitin-mediated proteasomal machinery [Nakagawa, et al., 2009]. To determine whether caffeine or its analog, theophylline, enhanced ABCG2 lysosomal degradation, we examined the effect of lysosome inhibitors on caffeine-mediated ABCG2 downregulation. Figure 4 shows a western blot analysis of ABCG2 expression following methylxanthine exposure in the presence or absence of the lysosomal inhibitors ammonium chloride (NH₄Cl) (Fig 4a) or leupeptin (Fig 4b). Ammonium chloride, which inactivates lysosomal enzymes by neutralizing the luminal pH of the lysosome,
prevented caffeine and theophylline from decreasing ABCG2 protein levels (Fig 4a). This effect could be seen as early as 4 hrs post-treatment (data not shown). A similar result was observed when the lysosomal inhibitor leupeptin was used (Fig 4b).

To confirm this result, immunofluorescence was performed on cells treated with caffeine in the absence or presence of either NH₄Cl or leupeptin (Fig 4c and d). As previously shown, caffeine caused an overall decrease in the ABCG2 fluorescence signal, and the ratio between the membrane and cytosol signals remained similar to the untreated samples. In the presence of NH₄Cl a decrease in ABCG2 signal intensity was still observed following caffeine treatment, but there was a marked increase in cytosol staining, particularly around the perinuclear region. This intracellular accumulation of ABCG2 was even more prominent following treatment with the specific lysosomal protease inhibitor leupeptin. These results suggest that caffeine induced the internalization of ABCG2 protein, probably through endocytosis, and directed it to the lysosomal degradation pathway, which was blocked by NH₄Cl or leupeptin treatment.

**Caffeine potentiates cytotoxicity of the ABCG2 substrate mitoxantrone**

To determine the functional impact of the downregulation of ABCG2 by methylxanthines, we determined whether caffeine could impact the accumulation of an ABCG2 substrate, using a flow cytometry assay (Robey et al., 2001). MCF-7/MX100 (a high ABCG2 expresser) and its parental cell line MCF-7 (a low ABCG2 expresser) were treated with caffeine for 24 hours, then cells were collected and incubated with the ABCG2- specific fluorescence substrate Bodipy-prazosin (BP). Following incubation,
cells were grown in fresh media without substrate, and the intracellular concentration of BP was determined as a measure of ABCG2 expression/function. The highly potent but toxic ABCG2 inhibitor, Fumitremorgin C (FTC), was used as a positive control. As shown in Figure 5a, accumulation of BP in the highly expressing MCF-7/MX100 cells was markedly increased in the presence of caffeine, suggesting that caffeine inhibited transport of this substrate by ABCG2. In contrast, there was little effect of caffeine on BP accumulation in the low-expressing MCF-7 parental cell line (Figure 5b), confirming that the effect of caffeine on BP uptake was specific for ABCG2.

MX is used in the treatment of certain cancers, including metastatic breast cancer and acute myeloid leukemia, where it has been shown to induce programmed cell death or apoptosis (Kluza et al., 2004). Therefore, in order to further confirm the functional effect of caffeine on ABCG2 and investigate the potential of using methylxanthines as chemosensitizing agents to treat ABCG2-mediated multidrug resistance, we analyzed the effect of caffeine on MX-induced apoptosis in MCF7/MX100. Cells were pretreated with caffeine for 24 hours to achieve maximum downregulation of ABCG2 protein, then treated with 10 or 100uM MX for an additional 24 hours. The apoptotic status of the cells was determined using annexin staining and flow cytometry. As shown in Figure 5c, when compared to untreated cells, treatment with caffeine alone (7mM) induced relatively little apoptosis (1.6%), while MX alone caused a dose-dependent apoptotic effect. However, pretreatment of cells with caffeine prior to MX exposure (10uM) increased MX-induced apoptosis ~3-fold, and shifted the IC_{50} by ~10-fold (IC_{50} = 946.5 \pm 1.71 \mu M for MX alone; 81.4 \pm 1.31 \mu M for caffeine and MX (Fig 5d)). Parental MCF-7...
cells were intrinsically more sensitive to MX (IC_{50}=3.233 \pm 1.84 \mu M) due to their relatively low levels of ABCG2; as expected, caffeine had little effect on MX cytotoxicity in MCF-7 cells (IC_{50}=2.174 \pm 1.44 \mu M for caffeine treated cells), again supporting the hypothesis that caffeine is sensitizing cells to MX by downregulating ABCG2 expression.
Discussion

In the present study, we show that xanthines, including the widely-consumed methylxanthine caffeine and the respiratory therapeutic theophylline, can downregulate the MDR membrane protein ABCG2 by inducing its translocation and subsequent lysosomal degradation. As a consequence of this downregulation, xanthines inhibited efflux of an ABCG2 substrate and sensitized drug resistance breast cancer cells to mitoxantrone, an antineoplastic agent commonly used to treat acute myeloid leukemia, metastatic breast cancer and non-Hodgkin’s lymphoma.

Previous studies have suggested that caffeine can modulate cancer drug sensitivity. The plethora of cellular affects attributed to caffeine have implicated a number of pathways/targets in this sensitization. The primary mechanism that has been suggested is through caffeine-mediated inhibition of ATM/ATR kinases, resulting in cell cycle arrest and sensitization of certain tumor cells to DNA damaging agents and irradiation (Lu et al., 2008; Sarkaria et al., 1999). We can achieve ABCG2 downregulation in the absence of a cell cycle block in the BeWo cell line, and induction of cell cycle arrest with different agents did not lead to the downregulation of ABCG2 (data not shown), suggesting that this is not the mechanism by which caffeine downregulates ABCG2. Another mechanism by which caffeine has been proposed to potentiate tumor cell death is through direct DNA binding to prevent repair of drug-induced DNA damage (Tornaletti et al., 1989), although there is little information to support this hypothesis. Notably, early studies suggested that methylxanthines could promote the antitumor activities of chemotherapeutic agents such as Adriamycin and doxorubicin by increasing the

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intracellular accumulation of these drugs both \textit{in vitro} and \textit{in vivo} (Sadzuka et al., 1995; Sadzuka et al., 1999), although the mechanism by which this interference with drug transport was accomplished had not been investigated. Our finding that methylxanthines alleviated multidrug resistance by downregulating ABCG2 expression reveals a novel molecular action of this class of compounds.

Xanthines join a growing list of potential ABCG2 inhibitors; to date, none of these inhibitors are in clinical use. Some ABCG2 inhibitors bind directly to the transporter, hindering its efflux activity; certain tyrosine kinase inhibitors fall into this class. The observation that xanthines reversed ABCG2-mediated multidrug resistance by inducing its degradation places it in a second class of compounds, those that affect ABCG2 expression. Recently, a novel inhibitor was identified that falls into both classes; the binding of this agent inhibits ABCG2 function and expression by targeting the transporter for lysosomal degradation (Peng et al., 2010). Our studies now show that xanthines, including those already in clinical use, also induce the lysosomal degradation of ABCG2. Indeed, early studies tested xanthines as part of a combinational therapeutic regimen to achieve better efficacy of anticancer drugs; caffeine was shown to potentiate etoposide and doxorubicin and prolong the overall survival of patients with high-grade soft tissue sarcoma, osteosarcoma, lymphoma of bone and metastatic carcinoma (Hayashi et al., 2005; Kimura et al., 2009; Takeuchi et al., 2007). Though the mechanism of this potentiation by caffeine was not determined in these studies, and it may involve caffeine’s role in regulation of the DNA repair process, it is notable that both etoposide and doxorubicin are substrates of ABCG2, and interesting to speculate
that downregulation of ABCG2 by caffeine may have mediated the increased tumor sensitivity to these drugs. That said, given the high concentrations of caffeine required to down-regulate ABCG2 in vitro, coupled with the narrow therapeutic window and multiple mechanisms of action of this agent, it is unlikely that caffeine itself will be clinically useful for modulation of ABCG2. Instead, we are currently using caffeine and caffeine analogs as 1) tools to further dissect the mechanism underlying regulation of degradation of ABCG2 protein, and 2) as lead compounds for the identification/development of more potent/less toxic agents. Apropos of this latter point, we have recently investigated the effect of a synthetic xanthine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), on ABCG2 levels. DPCPX is in clinical trials for the treatment of cystic fibrosis; single doses up to 1000 mg were tested with no apparent toxicity (McCarty et al., 2002). Importantly, DPCPX was able to downregulate ABCG2 levels to the same degree as caffeine, but at concentrations (5-50 uM, data not shown), that are more likely to be achievable in vivo. Animal studies are planned to evaluate the antitumor effect of this agent in combination with chemotherapeutics that are ABCG2 substrates.

One complication of most of the chemotherapeutic regimens currently in use is that their targets are also expressed in normal cells. This is also the case with ABCG2, which is found in normal stem cells, endothelial barriers and excretory cells of the liver, kidney and intestines (Robey et al., 2009). This presents both advantages and disadvantages. On the one hand, downregulation of ABCG2 in secretory tissues may enhance drug bioavailability; in contrast, decreased ABCG2 levels may also lead to greater toxicity of
chemotherapeutic agents in some cell types. Thus, as is the case with most cancer-directed agents, unwanted effects of candidate xanthines on normal cells/tissues will need to be considered as in vivo studies progress. It is also important to note that ABCG2 is expressed in cancer stem cells, where it may play a role in stemness maintenance and proliferation. Thus, downregulation of ABCG2 may prove useful for the elimination of this oft-times drug-resistant subpopulation. Studies addressing these questions are underway.

It has been previously reported that ABCG2 can be degraded through both proteasome- and lysosome-mediated pathways. Mutated or misfolded newly synthesized ABCG2 protein is rapidly eliminated via ubiquitin-mediated proteasome degradation prior to reaching the membrane (Nakagawa et al., 2009). In contrast, membrane-associated wild-type ABCG2 is normally degraded through the lysosome (Wakabayashi et al., 2007). How xanthines induce the translocation and lysosomal degradation of ABCG2 is not yet clear. One possibility is that xanthines directly interact with ABCG2 and negatively regulate its stability; indeed it has been reported that some nucleosides and nucleoside analogues can bind to the drug pocket of ABCG2 (de Wolf et al., 2008), although there is no evidence that nucleobases can do the same. Notably, we have observed that some of the xanthines tested did not mimic caffeine’s effect on ABCG2. For example, theophylline, dyphylline and theobromine actively reduced the protein level of ABCG2, whereas 7-(β-Hydroxyethyl) theophylline, paraxanthine and 7-methylxanthine did not significantly impact its expression. The fact that the size of the side chain modification on the xanthine ring did not correlate with activity, and that
caffeine is closely related to a nucleobase rather than a nucleoside, reduces the likelihood that xanthines act by direct binding to ABCG2. While we have not yet ruled this out, our current hypothesis favors a model whereby active xanthines impact/induce a signaling event that triggers the translocation and degradation of ABCG2, perhaps via a regulated process such as ubiquitin-mediated endocytosis. Recent studies have demonstrated the importance of monoubiquitination as a critical signal for selective degradation (d'Azzo et al., 2005). For example, the ion transporter ENaC can be recognized by the E3 ligase Nedd4-2 for ubiquitination in response to metabolic stress, triggering its subsequent endocytosis and lysosomal degradation (Bhalla et al., 2006). While we have yet to determine whether/which caffeine-induced signaling pathway regulates ABCG2 degradation, a previous study demonstrating that inhibition of PI3K activity led to the intracellular translocation of ABCG2 and subsequent decrease in the efflux of ABCG2 substrates (Bleau et al., 2009) supports this hypothesis, and suggests an avenue of further investigation. Clarifying the mechanisms by which xanthines induce lysosomal degradation of ABCG2 will inform the development of more specific ABCG2 modulators.

Taken together, our data demonstrate that a class of xanthine derivatives, including many already in clinical use, can modulate the expression of ABCG2 and sensitize cells to chemotherapeutic agents that are substrates for this transporter. This defines a new function for this large family of compounds, and identifies a new class of agents that may be useful in sensitizing tumors to the cytotoxic effects of chemotherapeutic drugs. As we further dissect the mechanism/pathway through which xanthines induce the
degradation of ABCG2, it is likely that more specific and efficacious inhibitors will be identified.

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**Authorship contributions**

*Participated in research design:* Ding, Shi, Scotto.

*Conducted experiments:* Ding, Pabon.

*Performed data analysis:* Ding, Shi, Scotto.

*Wrote or contributed to the writing of the manuscript:* Ding, Scotto.
References


Footnotes

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Figure legends

Figure 1. Caffeine downregulated ABCG2 expression in cancer cell lines. (a) ABCG2 protein levels were decreased by caffeine in a dose-dependent manner in ABCG2-overexpressing MCF-7/MX100 cells. Cells were treated with increasing concentrations of caffeine for 24 hours and western blot analysis was performed using antibody BXP-21. (b) Similar results were obtained in placental choriocarcinoma BeWo cells. (c, d) Caffeine (14mM) reduced ABCG2 protein levels in a time-dependent manner, with maximum reduction achieved by 24hrs, in MCF-7/MX100 (c) and BeWo cells (d). (e, f) Caffeine-mediated downregulation of ABCG2 protein was reversible. MCF-7/MX100 (e) and BeWo (f) cells were treated with caffeine for 24 h and then washed with drug-free medium and incubated for the times indicated prior to lysate preparation and western blot analysis. Graphs display quantifications of the western blot using Quantity One Software (BioRad, Inc.). In all cases, levels of ABCG2 protein were normalized to a GAPDH loading control and to the untreated sample.

Figure 2. Methylxanthines and caffeine analogs downregulated ABCG2 expression. (a) BeWo cells were treated with caffeine (14mM), theophylline (14mM), dyphylline (14mM), theobromine (2.5mM), paraxanthine (1.5mM), 7-(β-Hydroxyethyl) theophylline (14mM), and 7-methylxanthine (1mM) for 24 hours and ABCG2 protein levels were analyzed by western blot. GAPDH was used as a loading control. (b) Theophylline decreased ABCG2 protein levels potently and dose-dependently. BeWo cells were treated with theophylline at concentrations for 24 hours. GAPDH was used
as a loading control. (c) Quantification of the effect of theophylline on ABCG2. ABCG2 protein levels were normalized to the GAPDH loading control and the percent protein determined relative to the levels in untreated cells.

**Figure 3. Caffeine decreased membrane-localized ABCG2 and altered its cellular localization.** (a) Both MCF-7/MX100 and BeWo cells were treated with increasing concentrations of caffeine as indicated, and subjected to immunohistochemistry using BXP-21 antibody. Caffeine decreased plasma membrane-bound ABCG2 in a dose-dependent manner. (b) Caffeine induced alterations of the cellular localization of ABCG2. MCF-7/MX100 and BeWo cells were treated with 7mM caffeine for different time periods as indicated.

**Figure 4. Caffeine and theophylline-induced lysosomal-mediated degradation of ABCG2** (a) The lysosomal inhibitor NH₄Cl prevented caffeine and theophylline from reducing ABCG2 protein levels. BeWo cells were treated with caffeine (7mM), theophylline (7mM), and/or NH₄Cl for 24 hours and ABCG2 protein levels determined. Graph shows the quantification of the western blot image. (b) The lysosomal protease inhibitor leupeptin also blocked caffeine’s effect on ABCG2. Similar treatment combinations were used as for NH₄Cl Quantification is shown below figure (c) Immunofluorescence analysis demonstrates that caffeine promotes the internalization of membrane-localized ABCG2 (green). (d) Single cell view of the caffeine - and NH₄Cl - treated cells.
Figure 5. Caffeine inhibited efflux of an ABCG2 substrate and sensitized cells to the chemotherapeutic agent mitoxantrone. (a) MCF-7/MX100 cells were treated with either 14mM caffeine or 10μM FTC 24 hours prior to assaying cells for their ability to efflux the ABCG2-specific substrate BP. Both caffeine (red dashed line) and FTC treatments (black dotted line) increased the mean fluorescence intensity compared to the untreated cells (black solid line). (b) Parental MCF-7 cells have very low levels of ABCG2 expression. As expected, neither caffeine (red dashed line) nor FTC (black dotted line) had significant impact on the uptake of Bodipy-prozasin in these cells. (c) Caffeine potentiated mitoxantrone-mediated apoptosis in MCF-7/MX100 cells. Cells were treated sequentially with 7mM caffeine and MX at either 10μM or 100μM for 24 hours and percent of apoptotic cells were determined. Results are expressed as mean ± S.D., n = 3. (d, e) Caffeine increased the cytotoxicity of mitoxantrone in MCF-7/MX100 cells (d) but not in MCF-7 parental cells (e). Cells were pretreated with 7mM caffeine for 24 hours followed by increasing concentrations of mitoxantrone for 72 hours prior to the MTS assay. Data are expressed as mean ± S.D., n = 8.
Figure 1

(a) MCF-7/MX100

Caffeine (mM): 0, 1.75, 3.5, 7, 14, 28

ABCG2 | GAPDH

Graph showing ABCG2 protein levels over time with caffeine.

(b) BeWo

Caffeine (mM): 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8

ABCG2 | GAPDH

Graph showing ABCG2 protein levels over time with caffeine.

c. Time (hrs): 0, 2, 4, 8, 24, 48

ABCG2 | GAPDH

Graph showing ABCG2 protein levels over time.

d. Time (hrs): 0, 2, 4, 6, 8, 24, 25

ABCG2 | GAPDH

Graph showing ABCG2 protein levels over time.

(e) Post-treatment incubation time: 0 hr, 7 hr, 12 hr, 24 hr

Caffeine: (-) (+) (-) (+) (-) (+) (-) (+)

ABCG2 | GAPDH

Graph showing changes in ABCG2 protein levels with caffeine over time.

(f) Post-treatment incubation time: 0 hr, 7 hr, 12 hr, 24 hr

Caffeine: (-) (+) (-) (+) (-) (+) (-) (+)

ABCG2 | GAPDH

Graph showing changes in ABCG2 protein levels with caffeine over time.
Figure 2

(a) Xanthines: untreated, Caffeine, Theophylline, Dyphylline, 7-(β-Hydroxyethyl)theophylline, Paraxanthine, Theobromine, 7-methylxanthine

(b) Theophylline (mM): (-) 0.2 0.4 0.8 1.75 3.5 7 14

(c) ABCG2 Protein (%) vs Theophylline (mM)

Graph showing the decrease in ABCG2 protein expression with increasing concentrations of theophylline.
Figure 3

(a) BeWo and MCF-7/MX100 cells treated with different concentrations of Caffeine (Caff): (-), 0.8mM, 3.5mM, and 14mM.

(b) BeWo and MCF7/MX100 cells treated for 0, 4, 7, 10, 24, and 48 hours.