A Combination of Curcumin with either Gramicidin or Ouabain Selectively Kills Cells that Express
the Multidrug Resistance-linked ABCG2 Transporter

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Running title: Drug combinations evoke collateral sensitivity against ABCG2

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Keywords: ABC transporter, drug resistance, drug transport, ATPase, Na+ K+ ATPase, ABCG2
transporter, collateral sensitivity, curcumin, gramicidin A, ouabain

Background: The ABCG2 transporter is an ATP-dependent efflux pump that contributes to
multidrug resistance.

Results: Curcumin in combination with gramicidin or ouabain reduces intracellular ATP
levels in ABCG2-expressing cells and selectively kills these cells over parental cells.

Conclusion: ABCG2-expressing cells display collateral sensitivity toward these combinations of
compounds.

Significance: Understanding ABCG2-mediated collateral sensitivity is helpful in finding ways to
combat multidrug resistance.

ABSTRACT

This paper introduces a strategy to kill selectively multidrug resistant cells that express
the ABCG2 transporter (also called breast cancer resistance protein, BCRP). The
approach is based on specific stimulation of ATP hydrolysis by ABCG2 transporters with
sub-toxic doses of curcumin combined with stimulation of ATP hydrolysis by the Na+ K+
ATPase with sub-toxic doses of gramicidin A or ouabain. After 72 h of incubation with the drug
combinations, the resulting overconsumption of ATP by both pathways inhibits the efflux
activity of ABCG2 transporters, leads to depletion of intracellular ATP levels below the
viability threshold, and kills resistant cells selectively over cells that lack ABCG2
transporters. This strategy, which was also tested on a clinically relevant human breast
adenocarcinoma cell line (MCF-7/FLV1), exploits the overexpression of ABCG2
transporters and induces caspase-dependent apoptotic cell death selectively in resistant cells.
This work thus introduces a novel strategy to exploit collateral sensitivity (CS) with a
combination of two clinically used compounds that, individually, do not exert CS. Collectively,
this work expands current knowledge on ABCG2-mediated CS and provides a potential strategy for discovery of
CS drugs against drug-resistant cancer cells.

This paper reports a collateral sensitivity (CS) strategy (1) to kill selectively resistant cells that
overexpress the ABCG2 transporter. This transporter is an ATP-binding cassette (ABC)
protein (2) that is located in the plasma membrane of cells in the blood brain barrier, intestines, and
placenta (2–6). Expression of ABCG2 transporters is a marker for stem cells (7) and is being
discussed as a functional marker of cancer stem cells (CSCs) (8). ABCG2 transporters, together
with multidrug resistance protein 1
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(MDR1/ABCB1, also called P-glycoprotein, P-gp) and multidrug resistance-associated protein 1 (MRP1/ABCC1), prevent accumulation of xenobiotics and steroids in the human body (3,6). This efflux activity is also believed to render stem cells resistant to drugs and oxidative stress as well as maintaining stem cells in an undifferentiated state (7,8). Overexpression of efflux pumps in malignant cells can, however, lead to a multidrug resistance (MDR) phenotype that results in the failure of cancer chemotherapy (4,9). This efflux-induced drug resistance has motivated attempts to circumvent ABC transporter-mediated MDR in cancer cells. Inhibitors of MDR transporters, however, have had limited success in clinical trials due to excessive systemic side effects when administered in combination with chemotherapeutic drugs (10-12).

One alternative strategy to address MDR is the so-called ATP depletion strategy that takes advantage of increased metabolic needs of cancer cells (13,14). This approach kills tumor cells by inhibiting ATP synthesis, which leads to apoptosis and necrosis (15) in fast growing cells (16). All previously explored approaches have in common that they achieved ATP depletion by inhibiting ATP synthesis. Despite the potential of this strategy for cancer therapy, it appears difficult to inhibit the energy metabolism of tumor cells selectively as host cells are dependent on the same ATP-generating pathways (13).

An emerging strategy to address MDR is to exploit mechanisms of drug resistance in order to target these resistant cells (17). Several research groups reported that resistant cells were more sensitive to certain compounds than their parental cells (12,18-20). This little known and mechanistically underexplored effect is called collateral sensitivity (CS) or hypersensitivity (recently reviewed by Pluchino et al. (18) and Szakacs et al. (1)). Collateral sensitivity has been reported in cells expressing ABCB1 (P-gp) (12) and ABCC1 (MRP1) (21) transporters. For instance, Gatenby and coworkers recently presented a compelling potential approach to cancer therapy by combined administration of low doses of verapamil and low doses of 2-deoxyglucose to suppress resistant P-gp expressing cells by adaptive administration of chemotherapy with the goal of keeping tumor burden constant in disseminated cancers that are typically fatal when treated with conventional chemotherapy regimen (12,20). With regard to cells expressing ABCG2 (BCRP) transporters, we found only two previous reports on CS, in both cases a single compound induced CS (22,23).

Here, we introduce an approach that exploits the MDR phenotype to achieve targeted ATP depletion by variation of a strategy described by Karwatsky et al. (24) and Silva et al. (12) in cells overexpressing P-gp: Rather than inhibiting ATP synthesis, we selectively stimulate ATP hydrolysis by ABCG2 transporters and thereby induce a lethal reduction of ATP levels in MDR cells but not in parental cells.

We accomplished this selective stimulation of ATP hydrolysis in ABCG2 expressing cells by treatment with a combination of sub-toxic concentration of curcumin with either gramicidin A (gA) or ouabain. Curcumin, the bioactive compound in the South Asian spice turmeric, is an effective chemosensitizer that modulates the function of ABCB1, ABCC1, and ABCG2 transporters without being transported by these efflux pumps (6,25). Instead, curcumin inhibits drug efflux and increases the efficacy of many anticancer agents in multidrug resistant cancers (25,26). Curcumin also stimulates ATP hydrolysis by these transporters and we exploited this activity to increase consumption of ATP in ABCG2 expressing cells. In order to kill ABCG2 cells selectively over parental cells, we amplified the ATP depletion effect of curcumin with a second ATP-depleting process, the activation of the Na+ K+ ATPase. To this end, we treated cells with a combination of sub-toxic (micromolar) concentrations of curcumin with either gramicidin A (gA) or ouabain. Curcumin, the bioactive compound in the South Asian spice turmeric, is an effective chemosensitizer that modulates the function of ABCB1, ABCC1, and ABCG2 transporters without being transported by these efflux pumps (6,25).

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**MATERIALS AND METHODS**

**Chemicals**—We purchased Eagle’s minimal essential medium (EMEM), Dulbecco’s Modified Eagle’s Medium (DMEM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) from Sigma-Aldrich (St. Louis, MO). We used dimethyl sulfoxide (DMSO) as a solvent, and oncogenic transformation occurred in high concentrations (29), while it stimulated the Na+ K+ ATPase activity at nanomolar concentrations (28). Stimulating these two ATP-depleting processes together lowered the intracellular ATP levels in ABCG2-expressing cells sufficiently to kill them selectively over parental cells.

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bromide (MTT) cell cytotoxicity assay kits from ATCC (Manassas, VA). We obtained fetal bovine serum (FBS), OPTIMEM reduced serum medium, improved minimum essential medium (IMEM), Dulbecco’s phosphate buffered saline (PBS), 0.05% w/v trypsin-EDTA, penicillin, streptomycin, BODIPY-FL-prazosin, Annexin V-FITC and ethidium homodimer I from Invitrogen (Grand Island, NY). We purchased Aprotinin from Roche Diagnostics (Indianapolis, IN). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell lines and culture conditions**—HEK-293 cells transfected with the empty pcDNA3.1 vector (HEK-293 parental cells) or pcDNA3.1 vector containing the ABCG2 gene (HEK-293 ABCG2 cells) were maintained in EMEM supplemented with 10% v/v FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and G418 at a concentration of 2 mg/mL (32). MCF-7 human breast cancer cell line was exposed to increamentally increasing concentrations of flavopiridol. The resulting resistant subline, MCF-7/FLV1 overexpressed ABCG2 while neither P-gp nor MRP overexpression was detected (33). Control MCF7 and MCF7/FLV1 (482R) cells overexpressing ABCG2 were cultured in RPMI with 10% v/v FBS in the absence or presence of 1 μg/mL flavopiridol, respectively (33).

**Cytotoxicity assay**—We seeded 5,000 cells per well in 96-well plates and cultured overnight. After adding various concentrations of compounds and incubating for an additional 72 h, we replaced the medium with OPTIMEM and determined the cell viability using an MTT cell cytotoxicity assay kit, according to the manufacturer’s instruction. We calculated the cell viability using the following formula: Cell survival in % = (mean absorbance in test well)/(mean absorbance in untreated well) × 100. We calculated IC_{50} values with their errors from best curve fits of mean values of viability as a function of the drug concentration to the following equation y = A2 + (A1-A2)/(1 + (x/x0)^p) using Origin software version 7.5.

Fluorescence-based life verses death staining of HEK-293 cells and HEK-ABCG2 cells after 72 h of incubation with the specified compound/s was performed with calcein (green, representing live cells) and ethidium bromide (red, representing dead cells).

**Flow Cytometry analysis of efflux activity by ABCG2**—We trypsinized cells in phenol-red free IMEM for 30 min at 37° C in the absence or presence of 250 nM BODIPY-prazosin combined with 10 μM Fumitremorgin C (FTC), a ABCG2 inhibitor, and that in the presence of 250 nM BODIPY-prazosin without FTC. This difference therefore quantified FTC-inhibitable efflux of BODIPY-prazosin. We calculated the BODIPY-prazosin efflux (in %) for each condition using the following formula: (FTC-inhibitable BODIPY-prazosin efflux in the presence of compound/s)/(FTC-inhibitable prazosin efflux in the absence of compound/s) × 100 %.

**Isolation of crude membranes from ABCG2-expressing HEK-293 cells**—We prepared crude membranes from HEK-ABCG2 cells as described previously (34) with some modifications. Briefly, we scraped cells from their culture dishes into ice cold PBS containing 1% w/v aprotinin and centrifuged at 500 × g for 10 min to collect the pellet. We resuspended these cells in a lysis buffer containing 10 mM Tris-HCl (pH = 7.5), 10 mM NaCl, 1 mM MgCl2, and 1% w/v aprotinin and incubated on ice for 45 min. We disrupted cells using a Dounce homogenizer (30 strokes with pestle A and B). We removed undisrupted cells and nuclear debris by centrifugation at 500 × g for 10 min. We diluted the supernatant 2-fold in resuspension buffer containing 10 mM Tris-HCl (pH = 7.5), 50 mM NaCl, 250 mM sucrose, and 1% w/v aprotinin. Finally, we collected the membrane vesicles by centrifugation at 100,000 × g for 60 min and resuspended in resuspension buffer. We stored the membranes in small aliquots at -70 °C. Using the Amido Black protein method described by Schaffner and Weissmann (35) with bovine serum albumin (BSA) as a standard, we determined the protein content of each preparation.

**ATPase assay**—We used crude membranes from ABCG2 expressing HEK-293 cells (100 μg protein/mL) and incubated them with varying concentrations of compounds in the presence and
absence of 300 μM vanadate in ATPase assay buffer for 10 min at 37 °C. This ATPase assay buffer contained 50 μM KCl, 5 mM NaN3, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 2 mM ouabain, and 50 mM Tris-HCl (pH = 7.5). We started the reaction by adding 5 mM ATP and incubated for 20 min at 37 °C. We terminated the reaction with the addition of SDS solution (0.1 mL of 5 % w/v SDS) and quantified the amount of inorganic phosphate released by a sensitive colorimetric reaction as described previously (34). We recorded the specific activity of the transporter as vanadate-sensitive ATPase activity (34).

We determined the Na⁺ K⁺ ATPase activity in crude membrane vesicles from ABCG2 expressing cells treated with the ABCG2 inhibitor FTC at a concentration of 10 μM to completely inhibit the ATPase activity of the ABCG2 transporter. We determined total ATP hydrolysis in crude membrane vesicles from ABCG2 cells in the absence of the Na⁺ K⁺ ATPase inhibitor ouabain and in the absence of FTC.

**Determination of cellular ATP levels**—We determined cellular ATP levels with a luciferase assay (36) using a bioluminescence assay kit from Sigma Aldrich. We seeded 5 × 10⁵ cells per well in 96-well plates and cultured overnight. We incubated the cells for 1 h at 37 °C with or without drug treatments. We lysed the cells with lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% w/v Triton X-100, 0.1% w/v SDS, and 1% w/v deoxycholate) and mixed 100 μL (2.5 × 10⁵ cells) of cell suspension with an equal volume of luciferase solution in ATP assay dilution buffer as indicated by the manufacturer. We mixed the plate for 3–5 s and immediately placed the plate in the luminometer (Fluoroskan Ascent FL). We compared the observed light intensity from the cell samples to the one from several ATP standards to determine the ATP concentration of each sample.

**Determination of Membrane Potentials by Whole-Cell Current Clamp Recordings**—We plated cells on poly-L-lysine coated 35 mm glass bottom Petri dishes (MatTek, Ashland, MA) before performing whole cell recordings in current clamp mode. The standard pipette solution consisted of 130 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM ATP, 0.2 mM GTP and 0.5 mM EGTA (pH = 7.2). The bath solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 5 mM glucose (pH = 7.4). We fabricated the patch electrodes with resistances of 2.0–4.0 MΩ from borosilicate glass (Sutter Instruments, Novato, CA) using a P-87 puller (Sutter Instruments, Novato, CA). We recorded the membrane potentials using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and digitized using a Digidata 1322A (Molecular Devices, Sunnyvale, CA). Data acquisition was done using the pClamp9 software (Molecular Devices, Sunnyvale, CA).

**Apoptosis/Necrosis Assay**—We trypsinized and harvested cells that were incubated in the absence or presence of 35 nM gA, a combination of 35 nM gA and 2 μM curcumin, 7.5 nM ouabain, and a combination of 7.5 nM ouabain and 2 μM curcumin for 24, 48, and 72 hours. We washed cells with PBS and binding buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES, and 2.5 mM CaCl₂ with pH 7.4) before incubation with Annexin V-FITC and ethidium homodimer I (EthD I) for 15 min at room temperature in the dark. Then we determined the percentage of early apoptotic and late apoptotic or necrotic cells by flow cytometry. To inhibit the activity of caspase-3/7, we incubated cells in the presence of 50 μM Z-VAD-FMK, a well-known caspase inhibitor, for 2 hours prior to the addition of other compounds.

**Caspase-3/7 Activity Assay**—We trypsinized and harvested cells that were incubated for 72 hours in the absence or presence of 35 nM gA, a combination of 35 nM gA and 2 μM curcumin, 7.5 nM ouabain, and a combination of 7.5 nM ouabain and 2 μM curcumin. We washed cells with 0.1 % w/v BSA-PBS and stained cells with CellEvent caspase-3/7 green detection reagent (Invitrogen, Grand Island, NY) for 30 min at 37 °C in the dark. Then, we determined the population of caspase-3/7 activated cells by flow cytometry. To distinguish early apoptotic cells from late apoptotic or necrotic cells, we labeled cells with both CellEvent caspase-3/7 green detection reagent and SYTOX AADvanced dead cell stain (Invitrogen, Grand Island, NY) as instructed by the manufacturer. We subsequently determined the percentage of early apoptotic and late apoptotic or necrotic cells by flow cytometry.

**Statistical analysis**—Unless indicated otherwise, we repeated experiments at least three times and performed each experiment in triplicate. In bar graphs, each bar represents mean values ± standard error of the mean from at least two (typically three or more) separate experiments. We determined the statistical significance of
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RESULTS AND DISCUSSION

A combination of curcumin with either gA or ouabain selectively kills HEK-ABCG2 cells—Figure 1 shows that sub-toxic concentrations (defined here as concentrations that killed ≤ 10 % of cells, LD10) of curcumin (2 μM) in combination with sub-toxic concentrations of gA (35 nM) or ouabain (7.5 nM) were able to kill most of the cells with the MDR phenotype (HEK-293 ABCG2), while almost all parental cells (HEK-293 control) remained viable. We observed this selective cell death only in the presence of the combination of compounds (Fig. 1 and Table 1). Curcumin, gA, or ouabain by themselves, were slightly less toxic to HEK-ABCG2 cells than to the parental cells (Fig. 1 and Table 1). These results suggest that the MDR phenotype turned from an advantage that incurred a slight resistance towards these compounds individually to a disadvantage that incurred sensitivity towards combined exposure to curcumin with either gA or ouabain. These results are therefore on the one hand similar to those reported as CS (reviewed by Pluchino et al. (18) and Szakacs et al. (1)), in that resistant cells are more sensitive to certain chemicals than the parental cells. On the other hand, these results are different from previous cases of CS, in that the resistant cells are actually less sensitive against each compound individually, while the combination of curcumin with either gA or ouabain evokes CS.

Table 1 shows that in the presence of curcumin, resistant cells died at four times lower gA and two times lower ouabain concentrations than parental cells. Based on these results, we tested whether curcumin, gA, or ouabain would have a similar sensitization effect in ABCG2-expressing cells when administered at sub-toxic concentrations together with mitoxantrone, a well-known substrate of ABCG2 transporters (5). Table 1 shows that in the presence of curcumin, HEK-ABCG2 cells were still more resistant towards mitoxantrone than parental cells. Although curcumin reduced (i.e. reversed) the resistance of these cells towards mitoxantrone significantly, it could not cause CS as we observed in Figure 1 with the combination of curcumin with either gA or ouabain. The presence of sub-toxic concentrations of gA or ouabain had almost no effect on the resistance of ABCG2 cells towards mitoxantrone. Therefore, the specific toxicity of a combination of the ABCG2 modulator curcumin with gA or ouabain towards HEK-ABCG2 cells could not be replicated with a combination of the well-known ABCG2 transporter substrate mitoxantrone.

Inhibiting the function of ABCG2 transporters reverses cytotoxicity of curcumin in combination with gA or Ouabain towards HEK-ABCG2 Cells—To determine whether the selective cell death of HEK-ABCG2 cells by treatment with curcumin and gA or curcumin with ouabain was dependent on the activity of the ABCG2 transporter, we incubated cells with sub-toxic doses of curcumin in combination with gA in the presence or absence of 10 μM FTC, a specific inhibitor of ATP hydrolysis and thus of active transport by the ABCG2 transporter (37). We found that in the presence of FTC, the selective cytotoxicity of curcumin and gA or curcumin and ouabain towards HEK-ABCG2 cells was lost and the viability was restored to that of parental cells (Fig. 2). This result indicates that active ABCG2 transporters were indeed responsible for selective killing of HEK-ABCG2 cells in the presence of curcumin with either gA or ouabain.

A combination of curcumin with either gA or ouabain inhibits efflux by ABCG2—To determine if sub-toxic concentrations of curcumin in combination with either gA or ouabain would affect the efflux activity of ABCG2 transporters, we used flow cytometry to quantify the efflux of the fluorescent substrate BODIPY-prazosin (Bp) in HEK-ABCG2 and parental cells in the presence and absence of curcumin in combination with gA or ouabain (Fig. 3) (8). Table 2 summarizes the mean fluorescence intensity (MFI) and % efflux in HEK-ABCG2 cells under various conditions. Curcumin, gA, or ouabain individually had either no effect or a small effect on efflux of BODIPY-prazosin by ABCG2. In contrast, the combination of curcumin with gA inhibited efflux by ABCG2 completely and was as effective as the inhibition by FTC. Similarly, the combination of curcumin with ouabain reduced efflux by ~ 60 %. For comparison, the accumulation of BODIPY-prazosin remained unaffected under all conditions.
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in parental cells (Fig. 3). These results demonstrate a strong effect on the efflux activity of ABCG2 transporters by the combined treatments.

A combination of curcumin with either gA or ouabain increases ATP turnover in HEK-ABCG2 Cells—Since simultaneous treatment with subtoxic concentrations of curcumin with gA or ouabain inhibited the transport of BODIPY-prazosin by ABCG2 transporters, we hypothesized that selective killing of ABCG2 cells after exposure to the drug combinations was not caused by transport activity (Table 2) but rather by increased ATP hydrolysis by the ABCG2 transporter and Na⁺ K⁺ ATPase. This hypothesis was based on previous observations that curcumin stimulates ATP hydrolysis by ABCG2 transporters presumably without being a substrate (26) and that nanomolar concentrations of gA and ouabain can increase Na⁺ K⁺ ATPase activity (27,30).

To quantify ATP hydrolysis by the ABCG2 transporter, we performed experiments with crude membranes from HEK-ABCG2 cells in the presence of 2 mM ouabain (to inhibit ATP hydrolysis by the Na⁺ K⁺ ATPase) (26). Figure 4 shows that curcumin-induced stimulation of ATP hydrolysis by ABCG2 transporters was 3.7 times higher than the basal rate of ATP hydrolysis. Gramicidin A or ouabain, in contrast, had no effect on the ATP hydrolysis rate by ABCG2 transporters. For comparison, the maximal stimulation of ATP hydrolysis by 20 μM mitoxantrone was double the basal rate. These results demonstrate that curcumin increases ATP hydrolysis by ABCG2 transporters approximately twice as strongly as mitoxantrone, while gA or ouabain exerts its cytotoxic effect without directly modulating ATP hydrolysis by ABCG2 transporters.

To quantify ATP hydrolysis by the Na⁺ K⁺ ATPase, we performed experiments with crude membranes from HEK-ABCG2 cells in the presence of 10 μM FTC (to inhibit all ATP hydrolysis by the ABCG2 transporter) (26). Figure 4 shows that gA increased the rate of ATP hydrolysis by the Na⁺ K⁺ ATPase by a factor of 2.6. Since ouabain is known to have both stimulatory and inhibitory effects on the Na⁺ K⁺ ATPase (28,29,38,39), we determined the effect of various concentrations of ouabain on the Na⁺ K⁺ ATPase (Fig. 5A). We found that ouabain concentrations of 1 μM and above inhibited the Na⁺ K⁺ ATPase in crude membranes from HEK-ABCG2 cells, while ouabain concentrations between 1 pM and 10 nM activated the Na⁺ K⁺ ATPase relative to its basal activity. A similar activation/inactivation response was reported for the ATPase activity of P-gp as a function of various verapamil concentrations (24). Maximal activation of the Na⁺ K⁺ ATPase in membrane vesicles occurred at 10 pM ouabain, leading to a 2.3 ± 0.1 fold increase in the rate of ATP hydrolysis compared to the absence of ouabain (Fig. 4 and 5A). We found that 2 μM curcumin also increased ATP hydrolysis by the Na⁺ K⁺ ATPase by a factor of 1.8 compared to basal activity (27,30).

With regard to the total ATP hydrolysis rate by ABCG2 transporters and the Na⁺ K⁺ ATPase together, Figure 4 shows that the experimentally measured total ATP hydrolysis rate induced by gA, ouabain, or curcumin by themselves was approximately the sum of the ATP hydrolysis rate by ABCG2 transporters and the Na⁺ K⁺ ATPase, as expected. In contrast, the combination of curcumin with gA or ouabain caused a synergistic effect on the rate of ATP hydrolysis, leading to a 5-fold and 4-fold increase compared to the basal hydrolysis rate, respectively. Therefore, we hypothesized that the resulting ATP turnover of ~140-170 nmol Pi/(min × mg protein) may be sufficiently fast to deplete intracellular ATP levels in HEK-ABCG2 cells below the viability threshold.

Gramicidin A and ouabain alter the resting membrane potential of Cells—Gramicidin A affects energy metabolism by forming pores in cellular membranes and thereby altering their resting membrane potential (30,31). This membrane depolarization stimulates the Na⁺ K⁺ ATPase, leading to an increased rate of ATP hydrolysis and a decrease in intracellular ATP content (30,31). In the case of ouabain, the mechanism of activation of the Na⁺ K⁺ ATPase is not well understood. Ouabain binds to the Na⁺ K⁺ ATPase but Oselkin et al. proposed that its stimulatory effect at pico- and nanomolar concentrations may be exerted indirectly by a cascade of intracellular signaling events (40). In order to explore the effect of gA and ouabain on the resting membrane potential (RMP) of HEK-
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ABCG2 and parental cells, we performed whole-cell current clamp experiments. Figure 5B shows that even in the absence of curcumin, gA, or ouabain, the RMP of HEK-ABCG2 cells was approximately half that of HEK-293 parental cells. Hoffman et al. have previously linked membrane depolarization to an ABC-transporter mediated phenomenon in MDR cells (41). Figure 5B also shows that gA, curcumin, ouabain, or a combination of curcumin with either gA or ouabain induced further depolarization of the RMP in both cell types.

Since gA is a pore-forming peptide (27,42), we expected a strong effect on the RMP as shown in Figure 5B. Surprisingly, however, ouabain at concentrations in the low nanomolar range depolarized both the HEK-ABCG2 and parental cells to a similar extent as gA. In light of the result that ouabain stimulated ATP hydrolysis by the Na\(^+\) K\(^+\) ATPase (Fig. 5A), this depolarization suggests one plausible mechanism for gA- and ouabain-induced stimulation of the Na\(^+\) K\(^+\) ATPase as part of a cellular regulation response to restore the RMP. Since ouabain does not form pores in membranes (43), the mechanism for ouabain-induced depolarization remains unclear.

A combination of curcumin with either gA or ouabain selectively depletes intracellular ATP levels in HEK-ABCG2 Cells—To determine whether the increased ATP turnover may reduce the intracellular ATP level, we measured intracellular ATP concentrations in HEK-ABCG2 cells and parental cells. Figure 6A shows that the basal intracellular ATP levels in HEK-ABCG2 and parental cells were similar. Treatments with curcumin, gA or ouabain individually decreased the intracellular ATP levels up to ~20%. In contrast, exposure to a combination of curcumin with gA or ouabain depleted intracellular ATP levels by 40 to 50% in HEK-ABCG2 cells while these levels remained almost unaffected in the parental cells. These results hence demonstrate the synergistic effects by the drug combinations on intracellular ATP depletion in HEK-ABCG2 cells.

To determine whether the selective ATP depletion in resistant cells in the presence of the drug combinations was dependent on active ABCG2 transporters, we repeated the experiments in the presence of 10 \(\mu\)M FTC. In this case, the intracellular ATP concentration in HEK-ABCG2 cells was the same as in untreated cells for all tested conditions (Fig. 6B). These results show that curcumin-stimulated ATPase activity of ABCG2 transporters combined with the gA- or ouabain-stimulated ATPase activity of the Na\(^+\) K\(^+\) ATPase were responsible for depleting the intracellular ATP levels in cells with ABCG2 transporters below the viability threshold (15), while leaving ATP levels in the parental cells unaffected.

Inhibitors of oxidative phosphorylation and glycolysis in combination with curcumin do not preferentially kill HEK-ABCG2 cells—Gramicidin A is an uncoupler of oxidative phosphorylation (44) and inhibits glycolysis in prokaryotes (45); both of these activities inhibit ATP synthesis (44). In order to dissect whether inhibition of oxidative phosphorylation and glycolysis might be relevant for the results reported here, we tested the effect of carbonyl cyanide meta-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation (46), and 2-deoxy glucose (2-DG), an inhibitor of glycolysis (47), in combination with curcumin. We selected these two compounds because, like gA, they were not substrates of the ABCG2 transporter in the sense that the IC\(_{50}\) values from cytotoxicity assays with HEK-293 parental cells and HEK-ABCG2 cells were not significantly different for these two compounds (Fig. 7 and Table 3). We found that the combination of curcumin with CCCP or 2-DG killed HEK-ABCG2 cells and parental cells alike, indicating that inhibiting oxidative phosphorylation or glycolysis was not sufficient to selectively kill resistant cells. Therefore, these results provide further evidence that gA and ouabain enhance the cytotoxicity of curcumin in HEK-ABCG2 cells by stimulating ATP turnover by the Na\(^+\) K\(^+\) ATPase rather than by inhibiting oxidative phosphorylation or glycolysis.

Inhibitors of the electron transport chain do not affect CS by curcumin with gA or ouabain—In order to further investigate the mechanism by which stimulated ATP hydrolysis and the resulting low intracellular ATP levels might kill HEK-ABCG2 cells, we investigated a previously proposed mechanism of CS. Laberge et al. showed that cells expressing P-gp exhibited CS towards the P-gp substrate verapamil (48,49). In this case, verapamil by itself killed the resistant cells preferentially over the parental cells. Along with others before (49,50), the authors showed that low concentrations of verapamil (~10 \(\mu\)M)
increased the ATPase activity of P-gp and that this effect could reduce intracellular ATP levels by 50%. Laberge et al. proposed that hypersensitivity of P-gp expressing cells toward verapamil was caused by this increased demand for ATP, which caused an increased rate of oxidative phosphorylation and concomitant increased production of reactive oxygen species (ROS) (51). They hypothesized that accumulation of ROS ultimately initiated apoptosis of the resistant cells. The authors showed that two inhibitors of the electron transport chain, rotenone and antimycin A (52), amplified the collateral sensitivity to verapamil in the P-gp expressing cell line, while the same concentration of these compounds had no effect on the parental cell line. Based on findings by Li et al. (52), Laberge et al. proposed that rotenone and antimycin A exerted their effect through ROS-mediated toxicity and subsequent apoptosis.

Motivated by these results, we investigated the effect of rotenone and antimycin A on HEK-ABCG2 and parental cells in the presence and absence of curcumin and gA or curcumin and ouabain. HEK-ABCG2 cells were slightly more sensitive to rotenone and antimycin A than the parental cells (SR = 1.5 for both compounds and therefore not significant). Interestingly, however, administration of rotenone or antimycin A did not lead to a significant increase in CS of HEK-ABCG2 cells that were also exposed to curcumin and gA or curcumin and ouabain (Table 4 and Fig. 8). These experiments also indicated that rotenone and antimycin A were not substrates of ABCG2 transporters (Table 4). The absence of a significant effect by rotenone or antimycin A indicated that ROS-mediated toxicity was insufficient to amplify CS towards the drug combinations in HEK-ABCG2 cells. Hence, mechanisms other than ROS-induced apoptosis may be involved in the selective cell death of these cells in response to intracellular ATP depletion by a combination of curcumin with gA or ouabain.

**Selective cell death occurs through caspase-dependent apoptosis**—In a landmark paper, Leist et al. showed that the intracellular ATP concentration acts as a switch between apoptosis and necrosis (15). At ATP concentrations significantly above 50% of basal levels, cell death by apoptosis is favored, while ATP concentrations below 50% trigger necrosis. In fact, caspase activation in the apoptotic pathway requires ATP, such that low ATP levels inhibit apoptosis (53). Since combined treatments with curcumin and gA or curcumin and ouabain lowered intracellular ATP in HEK-ABCG2 cells to levels close to the 50% threshold (Fig. 6), it was unclear whether ATP depletion causes selective cell death of HEK-ABCG2 cells over parental cells through apoptosis or necrosis.

To answer this question, we conducted an apoptosis/necrosis assay with both HEK-ABCG2 and parental cells that were exposed to curcumin in combination with either gA or ouabain. After only 24 hours of treatment, a significant fraction (~5%) of resistant cells was already identified as late apoptotic or necrotic. After 72 hours, this fraction reached over 50% in the presence of curcumin and gA and ~15% in the presence of curcumin and ouabain. In comparison, less than 5% of parental cells that were treated identically or ABCG2-expressing cells that were treated with a single compound (i.e. gA or ouabain) were identified as late apoptotic or necrotic after 72 hours (Fig. 9).

To clarify whether the drug combinations induce apoptosis or necrosis, we measured the activation of the effector caspases (i.e. caspase-3 and -7), the hallmark of apoptosis (54), in both HEK-ABCG2 and parental cells. After 72 hours of incubation with curcumin and gA, nearly 50% of resistant cells showed a significant increase in caspase-3/7 activation. This fraction was ~35% when these cells were treated with curcumin and ouabain (Fig. 10A). In contrast, the activity of caspase-3/7 in parental cells remained at the basal level under all conditions (Fig. 10A). In addition, we distinguished early apoptotic cells from late apoptotic or necrotic cells using the caspase-3/7 activity assay similar to the apoptosis/necrosis assay. After 72 hours, ~40% of resistant cells were late apoptotic or necrotic when treated with curcumin and gA; this fraction was ~30% when these cells were treated with curcumin and ouabain (Fig. 10B). These results are consistent with those obtained from the apoptosis/necrosis assay (Fig. 9), suggesting that HEK-ABCG2 cells treated by the drug combinations undergo apoptosis instead of necrosis involving the activation of caspases.

Furthermore, we explored the effect of a caspase-3/7 inhibitor, Z-VAD-FMK, on the selective killing of HEK-ABCG2 cells using the apoptosis/necrosis assay. Interestingly, 50 μM Z-VAD-FMK completely eliminated the selective...
cell death of ABCG2 cells caused by the drug combinations after 72 hours (Fig. 10C). Z-VAD-FMK is not a substrate of ABCG2 transporters since treatment with only the inhibitor induced no significant difference in viability between HEK-293 parental cells (IC\textsubscript{50} = 255 μM) and ABCG2 cells (IC\textsubscript{50} = 226 ± 3.8 μM). Consistently, results from cytotoxicity assays showed that 50 μM Z-VAD-FMK protected HEK-ABCG2 cells against the toxicity of gA in the presence of 2 μM curcumin (IC\textsubscript{50} = 47 ± 5.6 nM in HEK-293 parental and 50 ± 3.8 nM in HEK-ABCG2 cells). Z-VAD-FMK had a similar effect on cells treated with ouabain in the presence of 2 μM curcumin (IC\textsubscript{50} = 29 ± 1.2 nM in HEK-293 parental cells and 33 ± 4.9 nM in HEK-ABCG2 cells). Together, these results indicate that the combination of curcumin with either gA or ouabain selectively kills HEK-ABCG2 cells by a caspase-dependent pathway which ultimately leads to apoptotic cell death.

**Effect of curcumin in combination with gA or ouabain on the human breast adenocarcinoma cell lines expressing ABCG2 transporters (MCF-7/FLV1)—**To test whether this approach would have similar effects on a clinically relevant cell line, we conducted cytotoxicity assays with human breast adenocarcinoma parental cells (MCF-7) and flavopiridol-selected daughter cells that express ABCG2 transporters (MCF-7/FLV1) (33). Surprisingly, the IC\textsubscript{50} value of gA in MCF-7/FLV1 cells was significantly higher (4.5 ± 0.8 fold) than in the MCF-7 parental cell line (Table 5 and Fig. 11A). This result contrasts with Table 1, which shows that the IC\textsubscript{50} values for gA in HEK-293 parental controls and ABCG2 cells were not significantly different (p = 0.14). To explore if this resistance towards gA was either a consequence of ABCG2 efflux activity or of a pleiotropic effect (55,56) as a result of stepwise selection with flavopiridol, we exposed MCF-7/FLV1 cells to gA in the presence of FTC. Despite inhibition of ABCG2 transporters, these cells retained their resistance towards gA (IC\textsubscript{50} = 5.9 μM in the absence of FTC and 5.2 μM in the presence of FTC) providing strong evidence for a pleiotropic effect.

Figure 11A shows that in the presence of 2 μM curcumin, the resistance of MCF-7/FLV1 cells to gA was reversed by 22.5 ± 9 fold and slightly inversed compared to MCF-7 parental cells (Table 5). Therefore, the CS effect on ABCG2-expressing MCF-7/FLV1 cells by curcumin with gA was similar, albeit smaller, than that observed in HEK-ABCG2 cell lines (Fig. 1).

With regard to ouabain, Figure 11B shows that in the presence of curcumin, MCF-7/FLV1 cells showed CS to increasing concentrations of ouabain; and died at 2.8 lower concentrations of ouabain than MCF-7 parental cells. Treatment with curcumin or ouabain individually showed no significant differences in viability between parental cells and the resistant MCF-7/FLV1 cells (Fig. 11B and Table 5).

Similar to results in Figure 2, the presence of FTC restored the viability of MCF-7/FLV1 cells to that of MCF-7 parental cells when we incubated cells with varying concentrations of gA or ouabain in combination with 2 μM curcumin (Table 5 and Fig. 11C, D). Furthermore, we found that gA, ouabain, or the combination of curcumin with gA or with ouabain induced membrane depolarization in MCF-7 and MCF-7/FLV1 similar to that observed in HEK-293 parental cells and ABCG2 cells (Fig. 11E).

These results show that the principle idea of inducing selective cytotoxicity in ABCG2 transfected cells by stimulated depletion of ATP levels could be replicated in the clinically relevant human breast adenocarcinoma cell line MCF-7/FLV1 that expresses ABCG2 transporters. This approach may therefore be generally applicable to resistant cells with significant expression of ABCG2 transporters.

In summary, the CS approach of intracellular ATP-depletion by treatment with agents that stimulate ATP hydrolysis by ABC transporters and ion-motive pumps presented here provides a strategy to target drug resistant cells with minimal effects on non-resistant cells. This method utilizes curcumin in combination with either gA or ouabain, all of which have previously been tested clinically (6,57,58). Therefore, the transporter-induced, synergistic ATP depletion (TISAD) strategy introduced here could inspire a fresh approach for discovery of CS agents against MDR cells.

In order for this TISAD approach to be applicable to efflux pumps other than ABCG2 transporters, it will be necessary to identify modulators or substrates of these pumps which are able to induce significantly increased ATP hydrolysis rates in MDR cells without inducing significant toxicity in non-resistant cells. In addition, co-administration of a second molecule
may be necessary to induce further ATP depletion by stimulating major ATPases such as the Na⁺-K⁺ ATPase. More generally, any physical or chemical process that induces significant ATP consumption in combination with stimulated ATP hydrolysis by MDR transporters might have the potential to induce CS.

In contrast to the ATP starvation therapy, the TISAD approach introduced here provides targeting of MDR cells by selectively stimulating ATP hydrolysis rather than indiscriminately inhibiting its synthesis. The results presented here suggest that severe ATP-depletion and the resulting induction of cellular apoptosis may be one of the mechanisms by which compounds induce CS in ABC transporter expressing drug-resistant cells. The novel aspect of the CS phenomenon reported here is that two compounds, which are individually neither toxic to the resistant nor to the parental cells, can evoke CS in resistant cells when they act synergistically. The work presented here therefore reveals a mechanism of CS that can be targeted rationally because it uses a known phenotypic difference (i.e. inducible ATP depletion by overexpressed MDR transporters) in order to kill resistant cells.
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FOOTNOTES

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2The abbreviations used are: MDR, multidrug resistant; CSCs, cancer stem cells; CS, collateral sensitivity; EMEM, Eagle’s minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IMEM, improved minimum essential medium; G418, geneticin disulfate; BSA, bovine serum albumin; MFI, mean fluorescence intensity; Bp, BODIPY-prazosin; gA, gramicidin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; 2-DG, 2-deoxy glucose; SDS, sodium dodecyl sulfate; FTC, fumitremorgin C; DTT, dithiothreitol; Tris-Cl, trizma hydrochloride; Z-VAD-FMK, Benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethylketone.
### Table 1. IC₅₀ values, relative resistance, and reversal of resistance of HEK-293 parental cells and HEK-ABCG2 cells towards curcumin, gA, ouabain, and mitoxantrone.

| Compound                        | IC₅₀ HEK-293 control, | IC₅₀ HEK-ABCG2, | p-value of IC₅₀ from HEK-ABCG2 cells relative to IC₅₀ from control cells | Relative Resistance | Sensitization /Reversal of Resistance |
|--------------------------------|------------------------|-----------------|--------------------------------------------------------------------------|--------------------|--------------------------------------|
| Curcumin alone<sup>d</sup>     | 5.0 ± 0.6 µM           | 6.0 ± 0.9 µM    | 0.2                                                                      | 1.2 ± 0.2 fold     |                                      |
| Curcumin + 10 µM FTC           | 5.1 ± 0.6 µM           | 6.3 ± 0.7 µM    | 0.1                                                                      | 1.2 ± 0.2 fold     | 1.0 ± 0.2 fold                      |
| Gramicidin alone               | 100 ± 22 nM            | 138 ± 16 nM     | 0.1                                                                      | 1.4 ± 0.3 fold     |                                      |
| Gramicidin + 2 µM curcumin     | 96 ± 10 nM             | 26 ± 4 nM       | 1E-3                                                                     | 0.3 ± 0.1 fold     | 5.0 ± 1.5 fold                      |
| Gramicidin + 2 µM curcumin + 10 µM FTC | 102 ± 16 nM     | 96 ± 15 nM      | 0.7                                                                      | 1.0 ± 0.2 fold     | 1.4 ± 0.5 fold                      |
| Ouabain alone                  | 14 ± 2 nM              | 14 ± 3 nM       | 1.0                                                                      | 1.0 ± 0.3 fold     |                                      |
| Ouabain + 2 µM curcumin        | 14 ± 2 nM              | 6 ± 1 nM        | 1E-2                                                                     | 0.4 ± 0.1 fold     | 2.5 ± 0.9 fold                      |
| Ouabain + 2 µM curcumin + 10 µM FTC | 15 ± 3 nM           | 16 ± 3 nM       | 0.7                                                                      | 1.1 ± 0.3 fold     | 1.0 ± 0.2 fold                      |
| Mitoxantrone alone             | 5.6 ± 0.8 nM           | 206 ± 12 nM     | 1E-4                                                                     | 37 ± 5.6 fold      |                                      |
| Mitoxantrone + 2 µM curcumin   | 5.1 ± 0.9 nM           | 32 ± 3 nM       | 4E-3                                                                     | 6.2 ± 1.2 fold     | 6.0 ± 2 fold                        |
| Mitoxantrone + 35 nM gramicidin| 5.2 ± 0.6 nM           | 170 ± 4 nM      | 1E-4                                                                     | 33 ± 4.6 fold      | 1.1 ± 0.2 fold                      |
| Mitoxantrone + 7.5 nM ouabain  | 5.3 ± 0.8 nM           | 187 ± 7 nM      | 1E-4                                                                     | 35 ± 5.5 fold      | 1.0 ± 0.2 fold                      |

<sup>a</sup> To determine p-values, we used a two-tailed Student’s t-test based on log(IC₅₀) values. We considered p-values ≤ 0.05 as statistically significant.

<sup>b</sup> Expressed as the ratio between the IC₅₀ value of ABCG2 expressing cells and the IC₅₀ value of control cells. This ratio is the inverse of the selectivity ratio (SR), which is defined as SR = IC₅₀(parental cells)/IC₅₀(MDR cells)<sup>(18)</sup>.

<sup>c</sup> Expressed as the ratio between the relative resistance value of ABCG2 expressing cells in the absence of the second compound and the relative resistance in its presence. In the case of experiments with gA or ouabain, ABCG2 expressing cells are more sensitive to the combined treatment with curcumin than the parental cells (i.e. sensitization). In the case of experiments with mitoxantrone, the ABCG2 expressing cells are less resistant to combined treatment with curcumin or gA (i.e. reversal of resistance).

<sup>d</sup> Chearwae et al., reported previously that curcumin is presumably not a substrate of ABCG2 transporters<sup>(26)</sup>. Based on the slight curcumin resistance of ABCG2-expressing HEK cells compared to the parental HEK cells, it appears, however, also possible that curcumin’s high permeability through membranes makes it difficult to detect whether it is transported by ABCG2 transporters or not. In that sense, curcumin may actually be a substrate of ABCG2 transporters but due to its fast passive back-diffusion through membranes it may appear not to be a substrate. We note, that in general net transport observed in transport assays is the sum of passive and active transport processes.
Table 2. Mean fluorescence intensities (MFI) and % efflux of Bodipy-FL-prazosin (Bp) in HEK-ABCG2 cells treated with the specified compound/s

| Distribution | Treatment                  | MFI a | % efflux b |
|--------------|----------------------------|-------|-----------|
| 1            | 250 nM Bp (substrate)      | 105 ± 66 | 100       |
| 2            | Bp + 10 μM FTC (inhibitor) | 546 ± 4 | 0         |
| 3            | Bp + 2 μM curcumin         | 139 ± 60 | 92 ± 19   |
| 4            | Bp + 35 nM gA              | 108 ± 71 | 99 ± 21   |
| 5            | Bp + 35 nM gA + 2 μM cur   | 540 ± 2 | 1.3 ± 1    |
| 6            | Bp + 7.5 nM ouabain        | 106 ± 42 | 100 ± 19  |
| 7            | Bp + 7.5 nM oua + 2 μM cur | 376 ± 32 | 38 ± 12   |

a Mean fluorescence intensity. Mean value of the mode (i.e. the most frequently measured fluorescence intensity) of each distribution ± standard deviation of the mean from two independent experiments.

b The difference in MFI values in the presence of 250 nM Bp alone to that in the presence of 250 nM Bp with 10 μM FTC was defined as 100 % efflux activity.
**Table 3.** IC₅₀ values, relative resistance, and reversal of resistance of CCCP and 2-DG in HEK-293 parental cells and in HEK- ABCG2 cells.

| Compound              | IC₅₀ HEK-293 control, | IC₅₀ HEK- ABCG2, | p-value of IC₅₀ from HEK- ABCG2 cells relative to IC₅₀ from control cells a | Relative Resistance b | Reversal of Resistance c |
|-----------------------|------------------------|------------------|------------------------------------------------------------------|------------------------|---------------------------|
| CCCP alone            | 2.3 ± 0.4 μM           | 2.4 ± 0.3 μM     | 0.32                                                             | 1.0 ± 0.2 fold         |                           |
| CCCP + 2 μM curcumin  | 1.8 ± 0.2 μM           | 1.7 ± 0.2 μM     | 0.16                                                             | 0.9 ± 0.2 fold         | 1.1 ± 0.3 fold            |
| 2-DG alone            | 4.0 ± 0.6 mM           | 4.2 ± 0.9 mM     | 0.27                                                             | 1.0 ± 0.3 fold         |                           |
| 2-DG + 2 μM curcumin  | 2.1 ± 0.3 mM           | 2.0 ± 0.4 mM     | 0.18                                                             | 1.0 ± 0.2 fold         | 1.0 ± 0.4 fold            |

a To determine p-values, we used a two-tailed Student’s t-test based on log(IC₅₀) values. We considered p-values ≤ 0.05 as statistically significant.
b Expressed as the ratio of the IC₅₀ value of ABCG2 expressing cells to that of the control cells.
c Expressed as the ratio of the relative resistance value of ABCG2 expressing cells in the absence of the second compound to the relative resistance in its presence.
Table 4. Effect of rotenone or antimycin A on the viability, relative resistance, and reversal of resistance of HEK-ABCG2 and parental cells.

| Compound                    | IC$_{50}$  | IC$_{50}$    | Relative Resistance $^a$ | Sensitization /Reversal of Resistance $^b$ |
|-----------------------------|------------|-------------|-------------------------|-------------------------------------------|
|                             | HEK-293    | HEK-ABCG2,  |                          |                                           |
| Rotenone alone              | 6.9 ± 1.8 nM | 4.6 ± 1.6 nM | 0.7 ± 0.3 fold          |                                           |
| Rot + 2 μM cur + 35 nM gA   | 9.3 ± 3.4 nM | 4.4 ± 1.5 nM | 0.5 ± 0.2 fold          | 1.4 ± 0.9 fold                           |
| Rot + 2 μM cur + 7.5 nM ouabain | 6.4 ± 1.9 nM | 4.5 ± 1.4 nM | 0.7 ± 0.3 fold          | 0.9 ± 0.6 fold                           |
| Antimycin A alone           | 6.0 ± 2.1 mM | 4.0 ± 2.0 mM | 0.7 ± 0.3 fold          |                                           |
| Anti A + 2 μM cur + 35 nM gA | 1.6 ± 0.7 mM | 1.3 ± 0.4 mM | 0.8 ± 0.4 fold          | 0.8 ± 0.6 fold                           |
| Anti A + 2 μM cur + 7.5 nM ouabain | 2.0 ± 1.0 mM | 1.8 ± 1.0 mM | 0.9 ± 0.6 fold          | 0.8 ± 0.7 fold                           |

$^a$ Expressed as the ratio of the IC$_{50}$ value of ABCG2 expressing cells to that of the control cells.

$^b$ Expressed as the ratio of the relative resistance value of ABCG2 expressing cells in the absence of the second compound to the relative resistance in its presence.
**Table 5.** Effect of gramicidin or ouabain on relative resistance and reversal of resistance of ABCG2 expressing MCF-7/FLV1 and parental cells.

| Compound                        | IC\textsubscript{50} MCF-7 control, \(\mu\text{M}\) | IC\textsubscript{50} MCF-7/FLV1, \(\mu\text{M}\) | p-value of IC\textsubscript{50} from MCF-7/FLV1 cells relative to IC\textsubscript{50} from MCF-7 cells \(a\) | Relative Resistance \(b\) | Reversal of Resistance \(c\) |
|---------------------------------|-----------------------------------------------------|-----------------------------------------------|-----------------------------------------------------------------|-------------------|---------------------|
| Curcumin alone                  | 7.3 \pm 0.5 | 5.4 \pm 0.5 | 0.12 | 1.4 \pm 0.2 fold |
| Gramicidin alone                | 190 \pm 20 | 850 \pm 115 | 4E-3 | 4.5 \pm 0.8 fold |
| Gramicidin + 2 \(\mu\text{M}\) curcumin | 50 \pm 11 | 11 \pm 4 | 0.03 | 0.2 \pm 0.1 fold | 23 \pm 9 fold |
| Gramicidin + 2 \(\mu\text{M}\) curcumin + 10 \(\mu\text{M}\) FTC | 49 \pm 13 | 51 \pm 12 | 0.85 | 1.0 \pm 0.1 fold | 4.5 \pm 1.7 fold |
| Ouabain alone                   | 14 \pm 2 | 15 \pm 2 | 0.74 | 1.1 \pm 0.2 fold |
| Ouabain + 2 \(\mu\text{M}\) curcumin | 14 \pm 1 | 6 \pm 2 | 0.02 | 0.4 \pm 0.1 fold | 2.8 \pm 1.0 fold |
| Ouabain + 2 \(\mu\text{M}\) curcumin + 10 \(\mu\text{M}\) FTC | 15 \pm 3 | 16 \pm 3 | 0.70 | 1.1 \pm 0.3 fold | 1 \pm 0.3 fold |

\(a\) To determine p-values, we used a two-tailed Student’s t-test based on log(IC\textsubscript{50}) values. We considered p-values \(\leq\) 0.05 as statistically significant.

\(b\) Expressed as the ratio of the IC\textsubscript{50} value of ABCG2 expressing cells to that of the control cells.

\(c\) Expressed as the ratio of the relative resistance value of ABCG2 expressing cells in the absence of the second compound to the relative resistance in its presence.
FIGURE LEGENDS

Figure 1. A combination of curcumin with either gramicidin (gA) or ouabain selectively kills cells with ABCG2-induced MDR phenotype. A) Sensitivity of parental (HEK-293 control) cells (black) and HEK-ABCG2 cells (red) to gA in the absence (solid curves) or presence (dashed curves) of 2 μM curcumin. B) Sensitivity of HEK-293 control (black) and HEK-ABCG2 cells (red) to ouabain in the absence (solid curves) or presence (dashed curves) of 2 μM curcumin. C, D) Fluorescence-based life versus death assay of HEK-293 control (top) and HEK-ABCG2 cells (bottom) after 72 hours incubation with the specified compound/s.

Figure 2. Reversal of selective cytotoxicity by curcumin in combination with either gA or ouabain in HEK-ABCG2 cells in the presence of FTC. Cell viability of HEK-293 control cells (black) and HEK-ABCG2 cells (red) exposed to increasing doses of gA (A) or ouabain (B) in the presence of 2 μM curcumin and in the absence (dashed curves) or presence (solid curves) of 10 μM FTC.

Figure 3. Effect of 2 μM curcumin in combination with 35 nM gA or with 7.5 nM ouabain on the accumulation of BODIPY-prazosin (Bp) in HEK-293 control and HEK-ABCG2 cells. HEK-293 control cells (A, B) and HEK-ABCG2 cells (C, D) were incubated in the absence (red curve) or presence of 250 nM Bp alone (1) or in combination with 10 μM FTC (2), 35 nM gA (3), 2 μM curcumin (4), a combination of 35 nM gA and 2 μM curcumin (5), 7.5 nM ouabain (6), and a combination of 7.5 nM ouabain and 2 μM curcumin (7).

Figure 4. Effect of gA, curcumin (cur), ouabain (oua), and a combination of curcumin and gA, or curcumin and ouabain on ATP hydrolysis by the ABCG2 transporter, on ATP hydrolysis by the Na+ K+ ATPase, and on total ATP hydrolysis by both transporters in crude membrane vesicles from HEK-ABCG2 cells. Single, double, and triple asterisks indicate a difference compared to the basal activity with a p-value ≤ 0.05, ≤ 0.01, and ≤ 0.001 respectively.

Figure 5. Effects of gA and ouabain on the ATP hydrolysis rate by the Na+ K+ ATPase in crude membrane fragments from HEK-ABCG2 cells and on the resting membrane potential (RMP) of HEK-293 control and HEK-ABCG2 cells. A) Stimulatory and inhibitory effect of various concentrations of ouabain (red) and gA (blue) on the ATP hydrolysis rate by the Na+ K+ ATPase in crude membrane fragments from HEK-ABCG2 cells. A single asterisk indicates a difference compared to the basal hydrolysis rate (black horizontal line) with a p-value ≤ 0.05; a double asterisk indicates a p-value ≤ 0.01. B) Effect of curcumin (cur), gA, and ouabain (oua) on the RMP of HEK-293 control (black) and HEK-ABCG2 cells (white). Cells were incubated under various conditions as indicated. RMP represents the control without addition of compounds. FTC concentration was used at 10 μM.

Figure 6. Effect of sub-toxic concentrations of curcumin (2 μM), gA (35 nM), or ouabain (7.5 nM) and combinations of curcumin with either gA or ouabain on intracellular ATP levels in HEK-293 control (black bars) and HEK-ABCG2 cells (white bars) in the absence (A) and presence (B) of 10 μM FTC. A double asterisk indicates a difference compared to untreated cells with a p-value ≤ 0.01.

Figure 7. Cell viability of HEK-293 control and HEK-ABCG2 cells as a function of increasing concentrations of carbonyl cyanide meta-chlorophenylhydrazone (CCCP), and 2-deoxy glucose (2-DG). The sensitivity to CCCP (A), and 2-DG (B) in HEK-293 control (black) and HEK-ABCG2 (red) cells was determined in the absence (solid curves) or presence (dashed curves) of 2 μM curcumin. See Table 3 for a statistical analysis of differences in IC_{50} values.

Figure 8. Sensitivity of HEK-293 control and HEK-ABCG2 cells to inhibitors of the electron transport chain. A) Toxicity of increasing concentrations of rotenone on HEK-ABCG2 (red) and parental cells (black) in the absence (solid curve) or presence of 2 μM curcumin and 35 nM gA (dashed curve). B) Toxicity of increasing concentrations of rotenone on HEK-ABCG2 (red) and parental cells (black) in the
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

absence (solid curve) or presence of 2 μM curcumin and 7.5 nM ouabain (dashed curve). C) Toxicity of increasing concentrations of antimycin A on HEK-ABCG2 (red) and parental cells (black) in the absence (solid curve) or presence of 2 μM curcumin and 35 nM gA (dashed curve). D) Toxicity of increasing concentrations of antimycin A on HEK-ABCG2 (red) and parental cells (black) in the absence (solid curve) or presence of 2 μM curcumin and 7.5 nM ouabain (dashed curve). The open symbols in C) and D) indicate that antimycin was not soluble at this concentration. These points were not included in the best curve fits.

Figure 9. A combination of curcumin with either gA or ouabain stimulates selective cell death of HEK-ABCG2 cells over parental cells. Cells were incubated in the absence (A) or presence of various compounds: 35 nM gA (B), a combination of 35 nM gA and 2 μM curcumin (C), 7.5 nM ouabain (D), and a combination of 7.5 nM ouabain and 2 μM curcumin (E) for 24, 48, and 72 hours and were subsequently stained with Annexin V-FITC /ethidium homodimer I (Annexin V-FITC /EthD I) for analysis using a flow cytometer. The dark gray bars indicate the population of early apoptotic cells (Annexin V-FITC + /EthD I –), while the light gray bars indicate the population of late apoptotic or necrotic cells (Annexin V-FITC + /EthD I + ).

Figure 10. Selective cell death of HEK-ABCG2 cells occurs through caspase -3/7 -dependent apoptosis. Cells were incubated in the absence or presence of 35 nM gA, a combination of 35 nM gA and 2 μM curcumin, 7.5 nM ouabain, and a combination of 7.5 nM ouabain and 2 μM curcumin for 72 hours. A) Cells were stained with CellEvent caspase-3/7 green detection reagent and analyzed subsequently by flow cytometry. Bars represent the percentage of caspase-3/7 activated cells of the total population. B) Cells were stained with both CellEvent caspase-3/7 green detection reagent and SYTOX AADvanced™ dead cell stain and analyzed subsequently by flow cytometry. The dark gray bars indicate the population of early apoptotic cells (CellEvent caspase-3/7 green + / SYTOX AADvanced – ), while the light gray bars indicate the population of late apoptotic or necrotic cells (CellEvent caspase-3/7 green + / SYTOX AADvanced + ). C) Cells were incubated with 50 μM Z-VAD-FMK for 2 hours prior to the addition of various compound/s for 72 hours and subsequently stained with Annexin V-FITC/EthD I for flow cytometry analysis. The dark gray bars indicate the population of early apoptotic cells (Annexin V-FITC + /EthD I – ) whereas the light gray bars indicate the population of late apoptotic or necrotic cells (Annexin V-FITC + /EthD I + ). P values indicate no significant difference between HEK-control and ABCG2 cells.

Figure 11. Cell viability of MCF-7 and MCF-7/FLV1 cells as a function of increasing gA or ouabain concentrations. A) Sensitivity of MCF-7 cells (black) and MCF-7/FLV1 cells (red) to gA in the absence (solid curves) or presence (dashed curves) of 2 μM curcumin. B) Sensitivity of MCF-7 cells (black) and MCF-7/FLV1 cells (red) to ouabain in the absence (solid curves) or presence (dashed curves) of 2 μM curcumin. C, D) Cell viability of MCF-7 cells (black) and MCF-7/FLV1 cells (red) exposed to increasing doses of gA (C) or ouabain (D) in the presence of 2 μM curcumin in the absence (dashed curves) or presence (solid curves) of 10 μM FTC. See Table 5 for a statistical analysis of differences in IC50 values.
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

Figure 1
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

Figure 2
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

Figure 3

[Graphs showing fluorescence intensity against the number of cells for HEK-293 and HEK-ABC2 cells under different conditions]
Figure 4
Figure 5
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

Figure 6
Figure 7
Figure 8.
Figure 9
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

Figure 10
Drug Combinations Evoke Collateral Sensitivity Against ABCG2

Figure 11.
A Combination of Curcumin with either Gramicidin or Ouabain Selectively Kills Cells that Express the Multidrug Resistance-linked ABCG2 Transporter

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