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Guggulsterone and bexarotene induce secretion of exosome-associated breast cancer resistance protein and reduce doxorubicin resistance in MDA-MB-231 cells

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Abstract

Many breast cancer cells acquire multidrug resistance (MDR) mediated by ABC transporters such as breast cancer resistance protein (BCRP/ABCG2). Here we show that incubation of human breast cancer MDA-MB-231 cells with farnesoid X receptor antagonist guggulsterone (gug) and retinoid X receptor agonist bexarotene (bex) elevated ceramide, a sphingolipid known to induce exosome secretion. The gug+bex combination reduced cellular levels of BCRP to 20% of control cells by inducing its association and secretion with exosomes. Exogenous C6 ceramide also induced secretion of BCRP-associated exosomes, while siRNA-mediated knockdown or GW4869-mediated inhibition of neutral sphingomyelinase 2 (nSMase2), an enzyme generating ceramide, restored cellular BCRP. Immunocytochemistry showed that ceramide elevation and concurrent loss of cellular BCRP was prominent in Aldefluor-labeled breast cancer stem-like cells. These cells no longer excluded the BCRP substrate Hoechst 33342 and showed caspase activation and apoptosis induction. Consistent with reduced BCRP, ABC transporter assays showed that gug+bex increased doxorubicin retention and that the combination of gug+bex with doxorubicin enhanced cell death by more than 5-fold. Taken together, our results suggest a novel mechanism by which ceramide induces BCRP secretion and reduces MDR, which may be useful as adjuvant drug treatment for sensitizing breast cancer cells and cancer stem cells to chemotherapy.

Keywords

doxorubicin; nuclear receptors; breast cancer resistance protein; bile acid; sphingolipids; cancer stem cells; multidrug resistance; exosomes

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Introduction

Many breast cancer cells acquire multidrug resistance (MDR) by up-regulating the level or activity of a variety of MDR-mediating proteins, most of which are ATP binding cassette (ABC) transporters, increasing the efflux of hydrophobic compounds that are endogenous lipids or cytotoxic agents^{1–2}. Most recently, another form of acquired MDR has been described that relies on the transfer of extracellular lipid vesicles or microparticles from a drug-resistant to a drug-sensitive cancer cell^{3–7}. Extracellular lipid vesicles (EVs) can be shed from the cell membrane (microvesicles, microparticles, or ectosomes) or secreted as multivesicular endosome-derived exosomes^{8–12}. They can carry miRNAs or ABC transporters that confer MDR to the drug-sensitive cell. However, previous studies have not reported that EVs may restore drug sensitivity by depleting ABC transporters from the drug-resistant cell. In particular, cancer stem cells are often resistant to drugs and give rise to metastasis. Therefore, inducing secretion of microvesicle-or exosome-associated ABC transporters may be a strategy for adjuvant cancer treatment by restoring drug sensitivity in cancer and cancer stem cells^{13–17}.

Ceramide is a sphingolipid that induces secretion of exosomes^{9–10, 18}. It is primarily generated by *de novo* biosynthesis in the endoplasmic reticulum (ER) or by hydrolysis of sphingomyelin in the plasma membrane and endolysosomes. We have found that the nuclear bile acid or farnesoid X receptor (FXR) antagonist guggulsterone (gug) activates neutral sphingomyelinase 2 (nSMase2) in murine breast cancer 4T1 cells, which is then followed by induction of apoptosis due to elevation of ceramide¹⁹. Recently, we have shown that activation of nSMase2 induces exosome secretion in astrocytes¹⁰. Therefore, we hypothesized that elevation of ceramide by activation of nSMase2 will also enhance secretion of exosomes and their association with ABC transporters in breast cancer cells.

We observed that the nuclear retinoid X receptor (RXR) agonist bexarotene (bex) elevates ceramide in human breast cancer MDA-MB-231 cells. Therefore, we hypothesized that the combination of gug and bex (gug+bex) will synergistically induce ceramide generation and secretion of exosome-associated ABC transporters. Among the proteins tested, breast cancer resistance protein (BCRP/ABCG2), a doxorubicin efflux transporter that is highly expressed in breast cancer stem cells^{2, 20–23}, was found to be depleted from MDA-MB-231 cells in response to gug+bex treatment. Our data suggest that gug+bex-induced ceramide generation leads to robust reduction of BCRP due to its association and secretion with exosomes. We reasoned that this mechanism will sensitize breast cancer cells and breast cancer stem-like cells to doxorubicin-induced cell death.

Materials and Methods

Materials

MDA-MB 231 cells were obtained from Dr. John Cowell, Georgia Regents University Cancer Center, who tested these cells by a comparative genomic hybridization (CGH) analysis. The cells revealed copy number changes characteristic of this cell line²⁴. DMEM and penicillin/streptomycin were from Cellgro (Manassas, VA, USA) and Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville,

GA, USA). BCRP antibody was from Enzo Life Sciences (Farmingdale, NY, USA). Anti-ceramide rabbit IgG was generated in our laboratory as described previously²⁵. Anti-ceramide mouse IgM (MAS00014) was from Glycobiotech (Kuekels, Germany). Z-guggulsterone (gug) was from Steraloids, Inc (Newport, RT, USA). Bexarotene (bex) and anti-actin mouse IgG were from Santa Cruz Biotechnology (Dallas, TX, USA). Doxorubicin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit, and Fluoroshield supplemented with DAPI were from Sigma-Aldrich (St. Louis, MO, USA). Cy3-conjugated goat anti-rat IgG, Cy5-conjugated donkey anti-mouse IgM, μ -chain specific, Alexa fluor 647-conjugated goat anti-mouse IgG γ -chain specific were from Jackson ImmunoResearch (West Grove, PA, USA). The *in situ* terminal dUTP nick-end labeling (TUNEL) fluorescence staining kit was from Roche (Indianapolis, IN, USA). The sulforhodamine fluorochrome inhibitor of caspases (SR-FLICA) *in vitro* poly caspase kit was from Immunochemistry Technologies (Bloomington, MN, USA). The aldehyde dehydrogenase (ALDH) activity (Aldefluor) assay kit was from Stemcell Technologies (Durham, NC, USA). Total Exosome Isolation Reagent was from Life Technologies (Grand Island, NY, USA).

Methods

Cultivation and treatment of MDA-MB-231 cells—MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were treated with gug, bex and doxorubicin at various concentrations in DMEM medium supplemented with 2% serum for 24–48 h.

Exosome isolation—To harvest exosomes, serum-free media from control cells or cells treated with gug, bex, gug+bex, or C6 ceramide were centrifuged (4°C) at 300×g for 10 min and 20,000×g for 30 min to remove cellular debris and larger vesicles. The supernatant was then centrifuged (4°C) at 110,000×g for 2 h, and EV pellets were resuspended in SDS sample buffer for Western blot analysis or used for further exosome purification by sucrose density gradient centrifugation. For density gradient centrifugation EV pellets were resuspended in PBS, layered on the top of a sucrose density gradient (0.3/0.55/0.8/1.05/1.3/1.55/1.80/2.05 M of sucrose in 20 mM Hepes, pH 7.2) and then centrifuged (4°C) at 110,000g for 16 h. Each fraction was transferred to a new tube, diluted with PBS, and centrifuged (4°C) at 110,000g for 90 min to harvest exosomes. Exosomes were resuspended in SDS sample buffer for Western blot analysis. We also purified exosomes from conditioned cell culture media using the Total Exosome Isolation Reagent from Life Technologies following the manufacturer's protocol.

MTT assay—Cells were seeded in 96-well plates (5,000–10,000 cells per well) and treated with vehicle, gug, bex, and/or doxorubicin at various concentrations in DMEM medium supplemented with 2% serum for 24 h. 20 μ l of 5 mg/ml MTT stock solution was added, and incubated for 4 h at 37°C in a culture hood. After the supernatants were discarded, 100 μ l of acidic (HCl) isopropanol was added into each well and incubated for 1 h at 37 °C. Finally, absorbance was measured at 570 nm with a reference filter of 650 nm using a Benchmark

plus microplate spectrophotometer. All experiments were performed in triplicate with at least 3 independent samples of cells.

Ceramide analysis—Total lipids were extracted from cells by sonicating cell pellets in a mixture of chloroform:methanol (2:1, vol/vol). After 1 h of extraction under agitation using a magnetic stirrer, methanol was added to the solution yielding a mixture of chloroform:methanol (1:2, vol/vol). The solution was centrifuged at 3,000×g for 15 min. The supernatant was transferred to a clean glass tube, solvent was evaporated in a stream of nitrogen, and lipids in the dried residue were resuspended in a small volume of methanol normalized to the protein content. Lipids were resolved by high-performance thin layer chromatography (HPTLC) using the running solvent chloroform:methanol:acetic acid (95:5:0.5, vol/vol/vol) for the separation of ceramide. Individual bands were visualized by staining with 3% cupric acetate in 8% phosphoric acid and identified by comparing them to the migration distance of standard ceramide²⁶.

RT-PCR—Total RNA was prepared from each experimental group of cells using TRIzol reagent following the manufacturer's protocol (Invitrogen). First-strand cDNA was synthesized using an iScript cDNA Synthesis kit according to the manufacturer's instructions (Bio-Rad Hercules, CA). The amount of template from each sample was adjusted until PCR yielded equal intensities of amplification for β -actin mRNA. The following primers were used for RT-PCR:

hBCRP-1 -(sense): 5'-CACCTTATTGGCCTCAGGAA-3'

hBCRP-1-(antisense): 5'-CCTGCTTGGAAGGCTCTATG-3'

hBCRP-2-(sense): 5'-AGATGGGTTTCCAAGCGTTCAT-3'

hBCRP-2-(antisense): 5'-CCAGTCCCAGTACGACTGTGACA-3'

nSMase2-(sense): 5'-GCGAGCAGCCACCAAATTGAAAGA-3'

nSMase2-(antisense): 5'-CTGCGGCCCTCCTCACTCTC-3'

β -actin (sense) 5'-CAT CGA GCA CGG CAT CGT CA-3'

β -actin (antisense) 5'-TAG CAC AGC CTG GAT AGC AAC-3'

siRNA transfection—ON-target plus SMPD3 siRNA SmartPool, ON-target plus non-targeting control pool and siGLO RISC-free control siRNA were purchased from Thermo Scientific-Dharmacon (Waltham, MA, USA). Using Oligofectamine (Invitrogen), cells were transfected with SMPD3 siRNA SmartPool (50 nM), control pool (50 nM), and siGLO RISC-free control siRNA (5–50 nM). Cells were harvested after 72 h and analyzed for expression of BCRP.

Aldefluor, FLICA, and Hoechst exclusion assays—The fluorescent aldehyde dehydrogenase (Aldefluor) activity assay was used to identify ALDH(+) cells as cancer stem-like cells²⁷. Cells on glass cover slips (living cells) were incubated for 1 h with Aldefluor working solution following the manufacturer's protocol (StemCell Technologies). The Aldefluor staining was monitored by confocal fluorescence microscopy using the 488

nm excitation channel. The fluorochrome inhibitor of caspases (FLICA) assay was used to detect apoptosis. According to the manufacturer's instructions (Immunochemistry Technologies), 200 μ l of FLICA solution was added to each cover slip and incubated for 1 h at 37 °C. After incubation, cells were washed with normal media and fixed with 4% PFA/PBS. FLICA staining was monitored by confocal fluorescence microscopy using the 543 nm excitation channel.

Hoechst 33342 dye was used for the Hoechst exclusion assay²⁸. Cells were incubated with 5 μ g/ml Hoechst 33342 dye for 15 to 30 min at 37°C in a culture hood. The Hoechst staining was monitored by epifluorescence microscopy using the 405 nm excitation channel.

ABC transporter activity assays—MDA-MB-231 cells were seeded in 24-well plates and treated with vehicle control, gug, and/or bex for 24 h in DMEM medium supplemented with 2% serum. Doxorubicin was added and incubated for an additional 24 h. After incubation, cells were washed twice with ice-cold PBS, dissociated by trypsinization, and transferred into 96-well plates. 100 μ l of methanol was added into each well and the intensity of the signal for doxorubicin fluorescence was measured by cytofluor multi-well plate reader (excitation: 485 nm, emission: 585 nm)²⁹.

TUNEL, immunocytochemistry, and image analysis—For immunocytochemistry, cells were grown on glass cover slips and treated with vehicle, gug, and/or bex. Cells were fixed with 4% PFA/PBS for 15 min and then permeabilized by incubation with 0.2% Triton X-100 in PBS for 10 min at room temperature. Nonspecific binding sites were blocked with 3% ovalbumin/10% donkey serum/PBS for 1 h at 37°C. Primary and secondary antibodies were diluted in 0.1% ovalbumin/PBS. After washing, cover slips were mounted using Fluoroshield with DAPI mounting solution to visualize the nuclei. Confocal fluorescence microscopy was performed using a Zeiss LSM510 confocal laser scanning microscope (Zeiss, Jena, Germany) equipped with a two-photon argon laser at 488 nm (Cy2), 543 nm (Cy3), or 633 nm (Cy5, Alexa Fluor 647), respectively. LSM 510 Meta 3.2 software was used for image acquisition. Images obtained with secondary antibody only were used as negative controls representing the background intensity in a particular laser channel. Antigen-specific immunostaining was quantified using ImageJ by counting cells (double-blinded) that showed signals twofold or more above background fluorescence¹⁹. TUNEL staining was performed according to the manufacturer's (Roche) instructions.

Miscellaneous—For immunoblot analysis, protein concentrations were determined using the RC/DC protein assay, in accordance with the manufacturer's (Bio-Rad) instructions. Equal amounts of protein were loaded onto a 4–20% gradient gel, and SDS-PAGE was performed using the Laemmli method. For immunoblotting, membranes were first blocked with 5% dry milk in PBST (PBS containing 0.1% Tween-20) and incubated with primary antibodies (BCRP antibody, 1:500) diluted in the blocking buffer overnight at 4°C. Membranes were then washed three times with PBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. After washing, bands were detected using chemiluminescence (ECL) reagent and exposure to X-ray film. Membranes were then stripped and re-probed, as described above, with anti- β -actin (1:1,000) to confirm equal loading.

Statistical analysis—The mean, standard deviation, and statistical significance (Student's *t*-test) of control and treatment samples were calculated in Microsoft Excel 2010. A value of $p < .05$ was considered statistically significant. The statistical significance for differences between treatment groups was confirmed by one-way ANOVA.

Results

Exogenous and endogenous ceramide induce secretion of exosome-associated BCRP

Our studies and those from other groups have shown that ceramide is instrumental for the formation and secretion of exosomes^{9–10, 30}. In addition, it has been shown that secreted EVs can carry ABC transporters to increase MDR of recipient cells^{4, 6, 31–32}. Our approach differed from these previous studies by hypothesizing that ceramide-induced secretion of EV-associated ABC proteins will primarily reduce MDR of the donor cells. To test this hypothesis, we determined the level of BCRP in MDA-MB-231 cells incubated with 5 μ M C6 ceramide and in EVs isolated from the medium supernatant. Long-term incubation (>72 h) with short-chain ceramides has been shown to up-regulate multidrug resistance proteins (MRPs) such as MDR1³³, while short-term incubation (24 h) has been found to increase secretion of exosomes¹⁰, potentially associated with MRPs. Figure 1A and B and Suppl. Fig. 1A and B show that exogenous C6 ceramide reduced the level of intracellular BCRP to 30% of vehicle-treated control cells, while the amount of EV-associated BCRP was increased by 3-fold. EV association of BCRP was confirmed by sucrose density gradient centrifugation showing that BCRP and the exosome markers Alix1 and TSG101 were co-distributed to fractions with the same sucrose density (Fig. 1C). Interestingly, the two bands at 72 kDa observed for the BCRP signal on immunoblots were distributed to different sucrose density fractions, suggesting that the two BCRP isoforms, possibly the glycosylated and non-glycosylated forms of BCRP³⁴, were associated with distinct EV populations. To test if C6 ceramide-induced EVs contain a proportion of apoptotic bodies that may also carry BCRP, we removed apoptotic bodies by filtrating (0.22 μ m) medium supernatants prior to EV preparation by the Total Exosome Isolation Reagent. Supplemental Fig. 2A and B shows that filtration reduced the level of BCRP by 40%, suggesting that about 60% of BCRP was associated with EVs that were distinct from apoptotic bodies or large microvesicles.

To determine the physiological significance of C6 ceramide-induced secretion of EVs, we tested if elevation of endogenous ceramide also increased secretion of EV-associated BCRP. We and others have shown that elevation of endogenous ceramide by activation of nSMase2 increases exosome formation or secretion^{9–10, 30}. Further, we have demonstrated that in breast cancer cells, this activation is achieved by incubation with the FXR antagonist guggulsterone (gug)¹⁹. Figure 2A and B shows that the combination of gug with bexarotene (bex), an agonist of the FXR heterodimer binding partner RXR, increased ceramide levels in MDA-MB-231 cells 4-fold. Ceramide elevation by gug, bex, or gug+bex did not alter the BCRP mRNA levels (Suppl. Fig. 3A). However, Fig. 2C and Suppl. Fig. 1C show that ceramide elevation was concurrent with reduction of the cellular BCRP protein level by 50% with gug (lane 3), which was dramatically enhanced (>80%) when using the gug+bex combination (lane 4). Interestingly, gug+bex also reduced the protein levels of FXR and RXR (Suppl. Fig. 3B), consistent with previous studies suggesting that nuclear receptors are

proteolytically degraded when occupied by single agonists³⁵. Proteolytic degradation of RXR was confirmed by partial inhibition of gug+bex-induced degradation with the proteasome inhibitor MG132 (lane 7 in Suppl. Fig. 3C). In contrast, gug+bex-induced reduction of the BCRP level was not prevented by MG132 (lane 7 in Suppl. Fig. 3D), but by inhibiting nSMases with GW4869 (lane 5 in Fig. 2C). This effect was specific for nSMase2 since knockdown using siRNA reduced mRNA for nSMase2 and restored BCRP protein levels in gug+bex-treated cells (Fig. 2D and E and Suppl. Fig. 1D and E). Therefore, our results suggested that gug+bex treatment reduced BCRP by activation of nSMase2 and elevation of endogenous ceramide. Figure 2F and G and Suppl. Fig. 1F and G show that reduction of cellular BCRP was concurrent with increased secretion of EV-associated BCRP, suggesting that elevation of endogenous ceramide by gug+bex has a similar effect as incubation with exogenous ceramide (Fig. 1A and B). Gug+bex incubation also increased Alix1 levels in the medium-derived ultracentrifugation pellet, suggesting that EVs were of exosomal origin (Fig. 2G, lane 4). In addition, BCRP and Alix were concentrated in the pellet of the Total Exosome Isolation Reagent assay (Fig. 2H, lane 2), a specific isolation method for exosomes from cell culture media, further suggesting that elevation of EV-associated BCRP was due to increased secretion of exosomes. To exclude that a proportion of BCRP was associated with apoptotic bodies, we used the filtration method as described for C6 ceramide-induced EV secretion. Supplemental Fig. 2A and B shows that >90% of BCRP was recovered from the EV pellet after centrifugation of the medium filtrate, suggesting that BCRP was associated with EVs that were distinct from apoptotic bodies and larger microvesicles. We and others reported that inhibition of sphingomyelin synthase enhances exosome secretion, probably by elevation of ceramide due to prevention of its conversion to sphingomyelin^{30, 36}. Consistent with ceramide-induced exosome secretion, the sphingomyelin synthase inhibitor MS-209 (Dofequidar)³⁷ also reduced cellular BCRP levels by elevating secretion of EV-associated BCRP (Fig. 2G, lanes 2 and 3). In addition, inhibition of nSMase2 with GW4869 reduced the level of BCRP and Alix in the Total Exosome Isolation Reagent pellet (Fig. 2H, lane 3), supporting the hypothesis that elevation of ceramide by gug+bex-induced activation of nSMase2 triggers secretion of EV-associated BCRP.

Gug+bex-induced ceramide elevation is concurrent with down-regulation of BCRP and induction of apoptosis in cancer and cancer stem-like cells

To determine if cells with elevated ceramide contain less BCRP and whether ceramide elevation by gug+bex treatment down-regulates BCRP, we performed immunocytochemistry using antibodies against ceramide and BCRP. Apoptotic cells were detected using TUNEL assays. Figure 3A and Suppl. Fig. 4A and B show that in gug+bex-treated MDA-MB-231 cells, higher levels of ceramide signals were colocalized with lower signals for BCRP. A quantitative image analysis showed that gug + bex treatment increased the ceramide signal by about 30% and reduced the BCRP signal by about 40% (Suppl. Fig. 4C). There appeared to be a discrepancy to the analysis of ceramide by HPTLC (4-fold increase, Fig. 2A and B) and BCRP by immunoblot (80% reduction, Fig. 2C), suggesting that the latter two methods are more accurate when determining actual quantities.

In a proportion of cells (ca. 20%), ceramide elevation was also correlated with TUNEL labeling or DAPI staining for condensed nuclei (Fig. 3A and B), which was indicative of apoptosis. In TUNEL(+) cells, ceramide elevation was concurrent with dramatic loss of BCRP labeling (Suppl. Fig. 5A), consistent with the observation that ceramide elevation was correlated with down-regulation of BCRP and induction of cell death (Fig. 3A). It is known that BCRP is most elevated in breast cancer stem cells, thereby mediating drug resistance in a cell type that is highly tumorigenic and metastatic^{2, 20–22, 38}. Since our previous study showed that gug induces ceramide generation and apoptosis in cancer stem-like 4T1 cells, we tested if gug+bex also elevated ceramide and reduced BCRP in cancer stem-like MDA-MB-231 cells¹⁹. Figure 3B and Suppl. Fig. 5B show that BCRP reduction induced by gug +bex treatment was prominent in Aldefluor(+) cancer stem-like cells, which amounted to about 10% of the total cell population consistent with previous reports on the proportion of Aldefluor(+) cancer stem-like cells in MDA-MB-231 cell culture³⁹. In control cells, the Aldefluor(+) signal was concurrent with low ceramide labeling (Suppl. Fig. 6), suggesting that prior to gug+bex treatment, cancer stem-like cells were not prone to ceramide-induced apoptosis. Figure 3C (control) and D (gug+bex-treated) shows that gug+bex treatment induced ceramide elevation, which was concurrent with activation of caspases (FLICA assay). Further, gug+bex treatment abolished Hoechst exclusion in cancer stem-like cells, indicating that BCRP mediating efflux of its substrate Hoechst 33342 dye was down-regulated in these cells. Based on the observation that gug+bex treatment almost completely abolished BCRP staining on immunoblots (Fig. 2C, F), but induced apoptosis in <20% of the cells, we concluded that ceramide-induced BCRP reduction concurrent with apoptosis was most prominent in the proportion of cancer stem-like cells.

Gug+bex-induced BCRP down-regulation sensitizes MDA-MB-231 cells toward doxorubicin-induced cell death

Next, we tested if reduced BCRP levels were correlated with decreased MDR. BCRP mediates MDR to a variety of drugs, among which some are known to induce apoptosis in breast cancer cells by increasing the level of ceramide^{40–41}. We focused on doxorubicin because: a) it has been reported to elevate ceramide levels^{40–41}, and b) chemotherapy of triple (estrogen receptor, Her2/neu, progesterone receptor) negative breast cancer cells with this drug is severely hampered by BCRP-mediated MDR⁴². To test if gug+bex incubation increased the drug sensitivity of MDA-MB-231 cells, we monitored the increase of intracellular fluorescence that is associated with retention of doxorubicin in cells with reduced MDR. Figure 4 shows that incubation with gug or gug+bex led to a 3-fold increase of doxorubicin retention in cells (Fig. 4A), concurrent with increased fluorescence signal and dramatically altered morphology of the cells (Fig. 4B). Doxorubicin retention was also found in Aldefluor (+) cells (Suppl. Fig. 7), indicating that ceramide-mediated BCRP down-regulation (Fig. 3B–D) reduced MDR in cancer stem-like cells. Consistent with enhanced sensitivity of MDA-MB-231 cells toward doxorubicin, cell survival was reduced to less than 20% in gug+bex and doxorubicin-treated cells, while doxorubicin by itself, the gug+bex combination without doxorubicin, or the combination of doxorubicin with either gug or bex only moderately affected survival (Fig. 5A and B and Suppl. Fig. 8A). Induction of apoptosis in gug+bex and doxorubicin –treated cells was inhibited by GW4869 (Suppl. Fig. 8B), consistent with the critical function of nSMase2 in sensitization of MDA-MB-231 cells

to doxorubicin and induction of apoptosis. In summary, our results suggest that gug+bex enhances or restores sensitivity to doxorubicin chemotherapy, which leads to induction of cell death.

Discussion

Anthracyclines such as daunomycin and doxorubicin are among the most commonly used chemotherapeutics in a variety of cancers, including leukemia and breast cancer. However, cancer cells often acquire MDR, compromising drug efficacy and ultimately, severely increasing mortality in patients undergoing chemotherapy. There are many adaptive responses mediating MDR including down-regulation of pro-apoptotic factors, up-regulation of cell survival factors, alteration of cell cycle regulatory factors and probably the most problematic, expression or activation of ABC proteins, which are mainly comprised of pumps that neutralize the drug by transporting it out of its target compartment or the entire cell.

The family of ABC transporters consists of 19 members, of which BCRP/ABCG2 is highly expressed in cancer stem-like cells^{21–22, 43–44}. BCRP was cloned in 1998 and comprises a 72 kDa protein that forms disulfide bridge-stabilized homodimers or homomultimers in the plasma membrane and can be N-glycosylated²¹. Most recently, plasma membrane-derived vesicles termed microvesicles, microparticles, or ectosomes have been found to transfer ABC transporters from drug-resistant to drug-sensitive cancer cells, thereby rendering the recipient cells drug resistant^{4–7}. EVs have also been reported to alter target tissue for migrating tumor-initiating cells, thereby generating a hospitable microenvironment for metastasis^{45–46}. Our approach differed from previous studies in that we tested whether induced secretion of EV-associated ABC transporters can deplete the resistant cells of MDR, which ultimately makes them sensitive to chemotherapeutics such as doxorubicin. Moreover, we tested if this mechanism is critical for drug sensitization of cancer stem-like cells.

Breast cancer stem-like cells are often found within a side population of cells that exclude Hoechst 33342, a BCRP substrate and DNA intercalating dye^{20, 28, 47}. Breast cancer stem-like cells are considered a model for cancer stem cells found in tumors of breast cancer patients that are highly metastatic. Accordingly, elevated BCRP expression is also found in cancer stem cells of the most aggressive (invasive) and doxorubicin-resistant tumors in humans⁴³. Therefore, finding treatment strategies that down-regulate or eliminate BCRP and potentially, other ABC transporter proteins in cancer cells and cancer stem-like cells will tremendously strengthen chemotherapy for breast cancer.

Based on our previous studies showing that cancer stem-like cells (4T1 cells) increase ceramide levels when incubated with gug¹⁹, we tested whether this will induce secretion of EVs. Interestingly, combining gug with bex further elevated ceramide levels and the secretion of EV-associated BCRP. Bex is a retinoid agonist of RXR, a nuclear receptor that forms heterodimers with FXR, the receptor for gug⁴⁸. Bex (Targretin) is already in clinical use as a chemotherapeutic to treat cutaneous T-cell lymphoma, has undergone a clinical phase II study for treatment of advanced breast cancer, and has been suggested for treatment

of Alzheimer's disease^{49–50}. Gug or guggu lipid is a sterol antagonist of FXR, is available as a dietary supplement, and has been used in numerous clinical trials demonstrating its cholesterol-lowering activity⁵¹. Gug has also been shown to induce apoptosis in a variety of cancer cells⁵².

In previous studies, gug or bex have been found to lower multidrug resistance, including that against doxorubicin in breast cancer cells^{53–54}. However, these previous studies did not combine gug and bex, did not test the effect on ceramide and cancer stem like cells, and did not invoke EV-associated secretion of BCRP and other ABC transporters as a potential mechanism. In particular, the effect of gug+bex-induced activation of nSMase2 on EV-associated secretion of BCRP is intriguing, since nSMase2 is known to be instrumental for exosome formation^{9–10, 30, 36}. ABC transporters have previously been detected on cell membrane-derived microvesicles or microparticles³¹. There is very little information on the secretion of BCRP or other ABC transporters in exosomes, which are derived from the multivesicular endosome. To our knowledge, our study shows, for the first time, that BCRP is associated and secreted with EVs induced by nSMase2. The result that EV secretion is dependent on sphingomyelinase-generated ceramide is supported by the observation that MS-209, an anti-cancer drug that has been reported to inhibit sphingomyelin synthase³⁷, also induces secretion of EV-associated BCRP. Our laboratory and other groups have reported that inhibition of sphingomyelin synthase is a potent method to enhance exosome formation and secretion, most likely due to elevation of ceramide that is not converted to sphingomyelin^{10, 30}. Therefore, activation of nSMase2 and inhibition of sphingomyelin synthase may be promising new approaches to reduce levels of cellular ABC transporters by inducing their association and secretion with exosomes.

To date, however, it is not clear how gug+bex activates nSMase2 and generates ceramide for EV association of BCRP. Our previous study suggested that gug down-regulates VEGF receptor (Flk-1) expression and, in turn, Flk-1-mediated inhibition of nSMase2¹⁹. Alternatively, alteration of the cellular lipid metabolism or redox potential by gug or gug+bex may trigger activation of nSMase2 and elevation of ceramide levels. This may also explain the reduction of BCRP levels, which we have previously suggested to be up- and down-regulated by sphingomyelin and ceramide, respectively¹⁷. The precise mechanism of this regulation by the interplay of sterols or other nuclear receptor ligands (such as gug and bex) and sphingolipids (such as sphingomyelin and ceramide) is currently being investigated in our laboratory.

The gug+bex-mediated down-regulation of BCRP, concomitant with tremendously enhanced apoptosis induced by doxorubicin, suggests that gug+bex are promising drugs for adjuvant chemotherapy of breast cancer. Of particular importance is the observation that reduction of BCRP levels and induction of apoptosis was prominent in breast cancer stem-like cells. Considering the high metastatic potential of cancer stem-like cells, gug+bex adjuvant therapy may prevent metastasis in breast cancer. Therefore, our results suggest that adjuvant lipid therapy using the gug+bex combination may offer an efficient approach to restore or increase sensitivity of drug-resistant cancer cells in anti-metastatic breast cancer chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and impact statement

Reduction of BCRP by ceramide-induced exosome secretion is a novel observation, which is likely to be of impact for potential adjuvant breast cancer therapy.

Figure 1. Incubation of MDA-MB-231 cells with exogenous C6 ceramide reduces cellular BCRP protein levels and induces secretion of EV-associated BCRP

Cells were incubated with 5 μ M C6 ceramide for 24 h and the cellular (A) and EV-associated (B) BCRP levels determined by immunoblot with protein derived from cell lysates and ultracentrifuged medium supernatant, respectively. C. BCRP, Alix, and TSG101 levels in subsequent sucrose density fractions show that the 72 kDa BCRP isoform is co-distributed with exosomal marker proteins.

Figure 2. Treatment with gug+bex increase ceramide levels by activation of nSMase2 and reduces BCRP protein levels

A. Ceramide level in MDA-MB-231 cells incubated for 24 h with 50 µM gug, 5 µM bex, or a combination of the two reagents (GB). Lipids were isolated and separated by thin layer chromatography. B. Quantification of ceramide as shown in A. N = 3, ** $p < 0.01$ for gug + bex (GB) vs. control (Con). C. Immunoblot using protein from MDA-MB-231 cells. Cells were incubated for 24 h with 50 µM gug, 5 µM bex, or a combination of the two reagents (GB). In addition, cells were pre-incubated with 10 µM GW4869 prior to gug+bex

treatment. D, E. mRNA level of nSMase2 (D) and protein level of BCRP (E) in cells with knocked down nSMase2. Cells were transfected with scrambled control or nSMase2-specific siRNA 72 h prior to incubation with gug+bex for another 48 h. N = 3. F,G. Cellular (F) and EV-associated (G) BCRP after incubation for 24 h with MS209 (lanes 2 (20 μ M) and 3 (40 μ M)) or gug+bex. H. EV-associated BCRP and Alix after incubation with gug+bex (GB, lane 2), 20 μ M GW4869 and gug+bex (GW+GB, lane 3), and 20 μ M GW4869.

Figure 3. Gug+bex-induced elevation of ceramide level is concurrent with down-regulation of BCRP and apoptosis in cancer cells and cancer stem-like cells

Cells were incubated for 24 h with gug+bex and then immunocytochemistry or live cell enzyme assays performed. A. Immunocytochemistry (confocal fluorescence microscopy) using antibodies against ceramide and BCRP, and TUNEL assay. DIC, differential interference contrast. B. Immunolabeling of BCRP after Aldefluor assay for cancer stem-like cells. C,D. Epifluorescence image of live cells of untreated control (C) or after incubation with gug+bex (D) and FLICA analysis for apoptosis, Aldefluor, and Hoechst 33342 (Hoechst exclusion) assays. Note that after gug+bex treatment, Aldefluor (+) cancer

stem-like cells show activation of caspases (FLICA (+)) and lose the ability to exclude Hoechst 33342 dye. FLICA, fluorescence labeled inhibitor of caspases; PC, phase contrast.

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Figure 4. Gug+bex treatment leads to retention of doxorubicin due to loss of MDR
Cells were incubated for 24 h with 50 μ M gug, 5 μ M bex, or a combination of the two reagents (GB), followed by treatment with doxorubicin (dox) for another 24 h. A. Doxorubicin retained within the cells was extracted with methanol and the relative fluorescence determined using a cytofluor fluorescence reader. $**p < 0.01$ for gug+bex vs. control and gug vs. control. $*p < 0.05$ for gug+bex vs. gug. B. Epifluorescence image showing the effect of doxorubicin on morphology and fluorescence of gug+bex-treated cells.

Note the robust retention of doxorubicin and aberrant morphology of gug+bex-treated cells, which was not found when cells were only incubated with doxorubicin.

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Figure 5. Gug+bex treatment sensitizes MDA-MB-231 cells to doxorubicin-inducible cell death
Cells were pre-treated for 24 h with gug+bex and then for another 24 h with 1 μ M doxorubicin (dox). A. Cell survival of MDA-MB-231 cells analyzed using the MTT assay. *** $p < 0.001$ for gug+bex+dox (GBD) vs. control, GB, and Dox. B. Phase contrast image of cells treated as in A.