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Role of P-glycoprotein inhibitors in ceramide-based therapeutics for treatment of cancer

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Abstract

The anticancer properties of ceramide, a sphingolipid with potent tumor-suppressor properties, can be dampened via glycosylation, notably in multidrug resistance wherein ceramide glycosylation is characteristically elevated. Earlier works using the ceramide analog, C6-ceramide, demonstrated that the antiestrogen tamoxifen, a first generation P-glycoprotein (P-gp) inhibitor, blocked C6-ceramide glycosylation and magnified apoptotic responses. The present investigation was undertaken with the goal of discovering non-anti-estrogenic alternatives to tamoxifen that could be employed as adjuvants for improving the efficacy of ceramide-centric therapeutics in treatment of cancer. Herein we demonstrate that the tamoxifen metabolites, desmethyltamoxifen and didesmethyltamoxifen, and specific, high-affinity P-gp inhibitors, tariquidar and zosuquidar, synergistically enhanced C6-ceramide cytotoxicity in multidrug resistant HL-60/VCR acute myelogenous leukemia (AML) cells, whereas the selective estrogen receptor antagonist, fulvestrant, was ineffective. Active C6-ceramide-adjuvant combinations elicited mitochondrial ROS production and cytochrome c release, and induced apoptosis. Cytotoxicity was mitigated by introduction of antioxidant. Effective adjuvants markedly inhibited C6-ceramide glycosylation as

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Chemical compounds studied in this article:

C6-ceramide (CID=5702613); Tamoxifen-HCl (CID=2733526); N-Desmethyl tamoxifen-HCl (CID=24200434); Zosuquidar-3HCl (CID=153997); Tariquidar (CID=148201); Fulvestrant (CID=104741); Cyclosporin A (CID=5284373); Verapamil-HCl (CID=62969); Didesmethyltamoxifen (CID=3036172)

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well as conversion to sphingomyelin. Active regimens were also effective in KG-1a cells, a leukemia stem cell-like line, and in LoVo human colorectal cancer cells, a solid tumor model. In summary, our work details discovery of the link between P-gp inhibitors and the regulation and potentiation of ceramide metabolism in a pro-apoptotic direction in cancer cells. Given the active properties of these adjuvants in synergizing with C6-ceramide, independent of drug resistance status, stemness, or cancer type, our results suggest that the C6-ceramide-containing regimens could provide alternative, promising therapeutic direction, in addition to finding novel, off-label applications for P-gp inhibitors.

Graphical abstract

Keywords

leukemia; colon cancer; sphingolipid metabolism; ceramide; P-glycoprotein inhibitors

1. Introduction

Ceramide, a potent tumor-suppressor sphingolipid [1–4], can be generated *in situ* by an array of anticancer drugs or administered exogenously, most prominently in the form of a short-chain ceramide, C6-ceramide [5, 6]. Whereas both avenues of enhancing ceramide levels are utilized, the sphingolipid-metabolizing machinery of cancer cells can function to dampen the tumor-censoring impact of this lipid. For example, metabolism of ceramide to glucosylceramide (GC) by glucosylceramide synthase (GCS) is a main route utilized by cancer cells to diminish ceramide-driven apoptosis- and autophagy-inducing responses [7, 8]. In addition, ceramide hydrolysis by ceramidases is an effective mode of ceramide elimination; however, this avenue can be problematic as sphingosine, produced via ceramidase activity, can be phosphorylated by sphingosine kinase (SK) to yield sphingosine 1-phosphate (S1-P), a mitogenic sphingolipid with an important role of its own in cancer biology [9, 10]. Maintaining a balance between ceramide and S1-P is thought paramount in maintaining the tumor-suppressor properties of ceramide. To this end, a number of pharmacologic and molecular approaches have been explored to improve ceramide's anticancer properties, approaches that encompass use of antisense oligonucleotides [11] as well as inhibitors of ceramide glycosylation and hydrolysis [12–16]. Of further importance, ceramide can be phosphorylated by intracellular ceramide kinase yielding ceramide 1-phosphate. This sphingolipid is also mitogenic and anti-apoptotic [17–19], properties that would as well limit the tumor-suppressor actions of ceramide.

In several prominent studies of ceramide metabolism, GCS inhibitors have demonstrated efficacy and supported the idea that inhibition of ceramide glycosylation is an effective means to drive ceramide-orchestrated cancer cell death [1]. These inhibitors, often referred to as “P-drugs” include agents like D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-propanol (PPMP), 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP), and derivatives thereof [20]. One distinct agent, structurally and functionally divorced from the P-drugs that blocks GC synthesis in cancer cells is tamoxifen, a front-line breast cancer drug that functions as an estrogen receptor antagonist. In addition to inhibition of ceramide glycosylation [21], tamoxifen also exhibits a number of estrogen receptor-independent actions, including circumvention of multidrug resistance, downregulation of survivin, inhibition of Acyl-CoA: cholesterol acyl transferase (ACAT) [22], and downregulation of acid ceramidase [15]. The capacity to block ceramide glycosylation has made tamoxifen an object of myriad investigations into its use as an adjuvant with ceramide-centric therapies, including 4-HPR [23], short-chain ceramides [24], and short-chain ceramides in combination with paclitaxel [25]. Although tamoxifen is not a direct inhibitor of GCS, it limits intracellular production of GC by blocking GC transport into the Golgi, a process that requires Golgi-resident P-gp [22]. This interesting action well complements the long, enduring history of tamoxifen as a first generation P-gp inhibitor and modulator of multidrug resistance in cancer; tamoxifen interacts directly with P-gp but itself is not a substrate transport [26, 27].

Although tamoxifen and desmethyltamoxifen (DMT) have been shown effective in combination with C6-ceramide in acute myeloid leukemia (AML) [28, 29], herein our aim was to discover alternatives to tamoxifen that would be void in antiestrogen activities. Additionally, having effective alternatives to tamoxifen would broaden the utility of ceramide as a cancer therapeutic.

The present work relates the discovery of a number of agents that are effective in combination with C6-ceramide and reveals commonalities in structure-function and in mechanism of action. Specifically, the most efficacious C6-ceramide-adjuvant-containing regimens blocked the metabolism of C6-ceramide via the glycosylation route and elicited the generation of reactive oxygen species (ROS). Importantly, these data suggest that specific P-gp inhibitors such as zosuquidar and tariquidar may find new utility when paired with ceramide-centric therapies as opposed to combining with standard, cytotoxic chemotherapies such as daunorubicin and vinblastine. In addition, that DMT is effective in combination with C6-ceramide is noteworthy, as this predominant tamoxifen metabolite in humans exerts < 1% of the antiestrogenic activity of parent tamoxifen [30], indicating that traditional anti-estrogen pathways are not involved in cellular responses. Of clinical relevance, we have previously shown that the C6-ceramide-tamoxifen combination is non-toxic in peripheral blood mononuclear cells, indicative of a cancer-selective action [28].

2. Materials and Methods

2.1. Materials

C6-ceramide (*N*-hexanoyl-D-*erythro*-sphingosine) and C6-NBD-lactosylceramide (*N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-lactosyl- β 1-1'-sphingosine) were

obtained from Avanti Polar Lipids, Alabaster, AL. C6-NBD-ceramide (*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingosine) was from Cayman Chemical Company, Ann Arbor, MI. *N*-Hexanoyl-NBD-glucosylceramide and *N*-hexanoyl-NBD-galactosylceramide were purchased from Matreya, State College, PA. NBD-X (6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoic acid) and NBD-C6-ceramide complexed to bovine serum albumin (BSA) were from Invitrogen, Carlsbad, CA. NBD-C6-sphingomyelin [*N*-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl]sphingosine phosphocholine] was from Setareh Biotech, Eugene, OR. High performance TLC (HPTLC) glass plates, 10 cm × 10 cm, silica gel 60 matrix polymeric binder with fluorescent indicator (product number Z740223) were from Millipore-Sigma, Darmstadt, Germany. Calibrated micropipets were from Drummond Science Company, Broomall, PA. Tamoxifen-HCl, *N*-Desmethyltamoxifen-HCl, propidium iodide solution (PI), and Vitamin E (alpha-tocopherol) were purchased from Sigma, St. Louis, MO. Hank's Balanced Salt Solution (HBSS) was purchased from Thermo Scientific, Waltham, MA. MitoSOX™ Red Mitochondrial Superoxide Indicator for measuring ROS was purchased from Life Technologies, Carlsbad, CA. The pan-caspase inhibitor Z-VAD-fmk was a product R&D Systems, Minneapolis, MN. The Cell Titer 96 Aqueous One Solution Assay Kit (known as MTS) for determining cell viability was purchased from Promega (Madison, WI). LY335979 (Zosuquidar-3HCL), was from ApexBio, Houston, TX. Tariquidar (XR9576) and Fulvestrant (Faslodex) were purchased from AdooQ BioScience, Irvine, CA. Cyclosporin A and verapamil-HCl were purchased from Enzo Life Sciences, Farmingdale, NY. All experimental drugs were dissolved in 100% DMSO (Life Technologies, Carlsbad, CA) and stored as stock solutions (10 mM) at -20 °C. Plastic tissue culture items were from Falcon and Corning, and purchased from various suppliers.

Microwave (MW) irradiation experiments were carried out in a CEM Corporation (Matthews, NC) Discover monomode microwave operating system at a frequency of 2.45 GHz. The reactions were carried out in 10 mL glass tubes, sealed with a Teflon septum. ^1H NMR spectra were recorded on a 500 MHz Bruker spectrometer (Boston, MA).

2.2. Cell culture

The human, vincristine-resistant (multidrug resistant) AML cell line, HL-60/VCR, was provided by A.R. Safa (Indiana University School of Medicine, Indianapolis, IN); cells were grown in medium containing 1.0 µg/mL vincristine sulfate (LC Laboratories, Woburn, MA). The KG-1a human AML cell line and the human colorectal cancer (CRC) cell line, LoVo, were obtained from the American Type Culture Collection (ATCC), Manassas, VA. Cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), (Atlanta Biologicals, Atlanta, GA), and 100 units/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Carlsbad, CA). The cell lines were not tested or authenticated over and above documentation provided by the ATCC, which includes antigen expression, DNA profile, short tandem repeat profiling, and cytogenic analysis. Cells were grown in humidified conditions in a tissue culture incubator with 95% air and 5% CO₂, at 37 °C. Confluent LoVo cells were subcultured using 0.05% trypsin/0.53 mM EDTA solution (Invitrogen Corp, Carlsbad, CA). For experiments with HL-60/VCR cells, vincristine was removed from the medium.

2.3. Synthesis of triphenylbutene and didesmethyltamoxifen

But-1-ene-1,1,2-triyltribenzene (triphenylbutene, TØb, **3**) was prepared as reported by Pathe and Ahmed [31] in good yields (Fig. 1A). Briefly, to a freshly prepared Zn-SnCl₄ complex under N₂ atmosphere, a mixture of benzophenone (**1**) and propiophenone (**2**) in THF was added slowly at same room temperature. Progress of the reaction was monitored by TLC and the reaction mixture was quenched with 10% aqueous NaHCO₃ solution and extracted with ethyl acetate. After usual work up and column chromatography, the desired product **3** was obtained as yellow semi solid in 70% Yield, ¹H NMR (CDCl₃, 500 MHz) δ 7.38 (t, 2H, *J*=8.0Hz), 7.32-7.26 9m, 3H), 7.21-7.18 (m, 5H), 7.06-6.98 (m, 3H), 6.93-6.89 (m, 2H), 2.51 (q, 2H, CH₂, *J*=7.5 Hz), 0.97 (t, 3H, CH₃, *J*= 7.5Hz) (E/Z) N,N-Didesmethyltamoxifen (DiDMT, **6**) was synthesized as reported [32] with minor modification (Fig. 1B). The key intermediate in the synthesis (E,Z)-1-(4-hydroxyphenyl)-1,2-diphenylbut-1-ene (**5**) was prepared from 4-hydroxybenzophenone (**4**) using super-base metalated propylbenzene [33]. Powdered potassium hydroxide (2.5 mmol) was added to a stirred solution of (E,Z)-1-(4-hydroxy)-1,2-diphenyl-1-ene (**5**) (0.5mmol) in dry toluene/dioxane (6:1, 7 ml) and mixture was irradiated in microwave at 90 °C (maximum power 250W) for 10 min. 2-Chloroethylamine hydrochloride (1.0 mmol) was then added and the mixture was irradiated in microwave at 90°C (maximum power 250W) for additional 30 min. After the usual work up as reported [29], the desired compound **6** was obtained in 68% yield as a mixture of E & Z isomers (30:70); ¹H NMR (CDCl₃, 500 MHz) 7.37 (t, 1H, *J*=7.5 Hz), 7.31-7.24 (m, 3H), 7.23-7.09 (m, 10H), 7.05-6.98 (m, 3H), 6.94-6.86 (m, 4H), 6.80 (d, 1H, *J*=8.5Hz), 6.58 (d, 1H, *J*=9.0Hz), 4.052 (t, 1.38H, Z-CH₂-NH₂, *J*=5.0 Hz), 3.89 (t, 0.62H, E-CH₂-NH₂, *J*=5.0 Hz), 3.15 (br s, 1.38H, Z-NH₂), 3.05 (br s, 0.62H, E-NH₂), 2.53 (q, 1.40H, Z-CH₂-CH₃), 2.47 (q, 0.60H, E-CH₂-CH₃), 0.97 (t, 3H, CH₃, *J*=9.0 Hz).

2.4. Cell viability assays

For viability by propidium iodide (PI), HL-60/VCR cells were seeded in black-wall 96-well plates at 100,000 cells/well and treated with indicated drugs in 0.2 mL 5% FBS RPMI-1640 medium. After addition of agents, cells were incubated at 37 °C, 5% CO₂ for 24 h, and viability was determined using PI as follows. A positive cell control was first permeabilized by addition of 10 µL of 1 mg/mL digitonin and incubated at 37 °C, 5% CO₂ for 20 min, followed by the addition of 0.1 mL of a 3X PI solution in 1X PBS for a final well concentration of 5 µM PI. The plate was then incubated for an additional 20 min, and viability was calculated as the mean (n = 4 or n = 6) fluorescence (minus permeabilized vehicle control) at 530 nm excitation and 620 nm emission, using a Bio-Tek Synergy H1 microplate reader, BIO-TEK Instruments (Winooski, VT). The effect of the pan-caspase inhibitor Z-VAD-fmk on HL-60/VCR cell viability in response to C6-ceramide-P-gp inhibitor regimens was evaluated by pre-incubating cells for 2 h with the inhibitor (20, 40, 50 µM) prior to addition of drugs.

LoVo cell viability was assessed using the CellTiter 96 One Solution Cell Proliferation Assay Kit (MTS) following manufacturer instructions. Cells were seeded in 96-well plates at 10,000 cells/well in 0.1 mL complete medium and allowed to attach at 37 °C, 5% CO₂ for 24 h before adding drugs. Drugs were diluted freshly into culture medium containing 1% FBS and added to wells to a total volume of 0.2 mL, thus the final concentration of FBS

during treatment of LoVo cells was 5.5%. Viability was calculated as the mean ($n = 4$ or $n = 6$) absorbance (minus vehicle control) at 490 nm, using a Bio-Tek Synergy H1 microplate reader.

2.5. Apoptosis assays

Apoptosis was detected by flow cytometry using the ApoDETECT Annexin V-FITC Kit (Life Technologies, Carlsbad, CA), following the manufacturers protocol. Briefly, cells were seeded in 6-well plates at 5×10^5 cells/mL in 5 mL of RPMI-1640 medium containing 5% FBS. Cells were treated with the indicated drugs for 18 h, collected by centrifugation and washed with PBS and stained with Annexin V using 1X Annexin binding buffer (provided in the kit), and the percent of Annexin V-positive cells was determined by flow cytometry. Cell acquisition was performed on a Becton Dickinson FACSCalibur. Analysis was performed using FCS Express 4 from De Novo Software (Glendale, CA). Apoptosis was also determined by flow cytometric analysis of DNA fragmentation following our previously published protocol [34].

2.6. Measurement of mitochondrial reactive oxygen species

Mitochondrial superoxide was assayed using MitoSOXTM Red. Cells (5×10^5 cells/mL RPMI-1640, 5% FBS medium) seeded in 6-well plates, were treated with the indicated drugs for 18 or 24 h and then collected (adherent cells were collected using trypsin) and washed in HBSS and incubated in 0.25 mL staining buffer (HBSS containing 5 μ M MitoSOX) for 15 min at 37°C, protected from light. Cells were washed again in HBSS, resuspended at 1×10^6 viable cells/mL in HBSS, and a 0.1 mL aliquot was added to the wells of black-walled 96-well plates. Fluorescence was measured at 510 nm excitation and 580 nm emission, using a Bio-Tek Synergy H1 microplate reader. For photomicrographs, a 0.1 mL aliquot of cells was added to black-walled 96-well plates, and after centrifugation images were captured using fluorescence microscopy.

2.7. Cytochrome c release

Cytochrome c release from mitochondria was assessed as described [28, 35, 36]. Briefly, 2×10^6 cells/2 mL RPMI-1640 medium, 2.5% FBS medium, were seeded in 6-well plates and treated with selected agents for 18 h, after which 1×10^6 cells were removed and placed on ice in 0.1 mL of digitonin (Sigma, St. Louis, MO) (100 μ g/mL in PBS, 100 mM KCl) for 3–5 min, until 95% of the cells were permeabilized (stain positive with 0.2% trypan blue). At this point, 0.1 mL of 4% paraformaldehyde was immediately added to the cells. After centrifugation, cells were then fixed at room temperature in 4% paraformaldehyde in PBS for 20 min, washed with PBS and resuspended in blocking buffer (PBS, 3% BSA, 0.05% saponin); the saponin (Sigma, St. Louis, MO) was freshly prepared. Cells were then incubated for 30 min at 4°C in a 1 : 100 dilution of FITC conjugate (6H2) anti-cytochrome c antibody (Life Technologies, Carlsbad, CA) in blocking buffer, washed, and levels of cytochrome c were determined by flow cytometry as described in the above sections.

2.8. Vitamin E experiments

Vitamin E was prepared fresh before each experiment. A 250 mM stock solution of vitamin E in 100% ethanol was diluted to 25 mM in 1.0 M HEPES buffer, pH 7.3; this working solution was used in experiments. To assess the effect of vitamin E, cells were preincubated in culture medium containing 250 μ M vitamin E for 2 h prior to addition of test agents.

2.9. C6-ceramide metabolism assays in intact cells

A modified method of a previous procedure was followed [37]. Briefly, control and 18 h inhibitor-pretreated HL-60/VCR cells (1×10^6 cells/mL RPMI-1640 medium containing 5% FBS, 6-well plates) were assessed for viability using trypan blue and seeded into 96-well strip wells at 100,000 viable cells/45 μ L serum-free RPMI-1640 containing 1% BSA. LoVo cells were seeded at 100,000/well, 96-well plate, and allowed to attach overnight before pretreatment with inhibitor for 18 h. LoVo cells were also seeded in parallel to assess viability after pretreatment with the inhibitors. The enzyme reaction (inhibitors were present during the assay) was initiated by addition of 5 μ L NBD-C6-ceramide complexed to BSA (25 μ M final substrate concentration) and placed in a tissue culture incubator for 2 h. Samples were then placed on ice, and the cells were transferred to 1-dram glass vials for lipid extraction [38]. The lower, lipid-containing chloroform phase was evaporated to dryness under a stream of nitrogen. Total lipids were dissolved by addition of 40 μ L chloroform/methanol (5:1, v/v), vortex mixed, and 5 μ L was applied to the origin of a HPTLC plate. Commercial lipid standards were spotted in lateral lanes. Lipids were resolved in a solvent system containing chloroform/methanol/ammonium hydroxide (80:20:2, v/v/v). Products were analyzed directly on the HPTLC plates using the BioRad ChemiDoc Touch and quantified with Image Lab software by BioRad (Hercules, CA).

2.10. Data Analysis

Results are expressed as means $\pm$ SEM and were analyzed by ANOVA. Differences among the treatment groups were assessed by Tukey post hoc test. Differences were considered significant at *P 0.05, **P 0.01, ***P 0.001. Drug-induced cytotoxic synergy was analyzed by CalcuSyn® software from Biosoft (Great Shelford, Cambridge, United Kingdom). Each condition (single agent and combination) was tested in replicates of six and repeated at least twice. The mean proliferative index for each compound at the indicated concentrations was entered into the CalcuSyn program for dose–effect analysis. By this method, a combination index (CI) is determined based on the Chou–Talalay method [39] where a CI of 0.9–1.10 indicates an additive effect, and CI values of 0.3–0.7 and 0.1–0.3 indicate synergism and strong synergism, respectively.

3. Results

3.1. Chemical structures of agents investigated with C6-ceramide

The chemical structures of tamoxifen, tamoxifen metabolites, and the other agents utilized in this study are shown in Fig. 2. Tamoxifen contains a TØb nucleus and an *N*-dimethylethanolamine function. The corresponding desmethylated metabolites, DMT and Di-DMT contain one and no methyl groups, respectively. TØb, the aromatic nucleus, is

devoid of the *N*-dimethylethanolamine moiety. Although an antiestrogen, tamoxifen has been widely utilized as a P-gp inhibitor in clinical studies [22] and is also a component of the Dartmouth regimen for treatment of melanoma [40]. It is termed a first generation P-gp inhibitor. In addition to tamoxifen, we studied two other first generation P-gp inhibitors, the nonpolar, cyclic oligopeptide immunosuppressive, cyclosporin A, and the vasodilator and calcium channel blocker, verapamil (Fig. 2). Zosuquidar, a cyclopropyldibenzosuberane, and the anthranilamide derivative, tariquidar (Fig. 2), are third generation P-gp inhibitors and despite the diverse chemical structures and origins, these agents demonstrate high potency and specificity for the P-gp transporter, and typically third generation inhibitors do not inhibit other ABC transporters, such as the multidrug resistance protein, MRP1 (ABCC1). Fulvestrant (trade name Faslodex), a synthetic estrogen receptor antagonist, like tamoxifen, is composed of a steroid nucleus and is thus chemically distinct from the other agents under evaluation.

3.2. The effect of tamoxifen, tamoxifen metabolites, and other P-gp inhibitors on cytotoxic response to C6-ceramide in HL-60/VCR and KG-1a cells

As shown in Fig. 3A, after a 24 h exposure, HL-60/VCR cells were refractory to C6-ceramide. In turn, tamoxifen and metabolites DMT, DiDMT, and the tamoxifen nucleus, TØb, imposed limited cytotoxicity when administered singly. However, sensitivity to C6-ceramide was strongly enhanced by addition of either tamoxifen or the tamoxifen metabolites. For example, combination C6-ceramide-tamoxifen, -DMT, and -DiDMT exposure resulted in 60%, 70% and 75% cell death, respectively, whereas the tamoxifen nucleus, TØb, was completely devoid of C6-ceramide-enhancing activity, and thus served as an appropriate negative control. Tariquidar, cyclosporin A, and zosuquidar each demonstrated efficacy when co-administered with C6-ceramide (Fig. 3B); when administered singly however, these agents were only moderately cytotoxic. For example, whereas tariquidar elicited approximately 20% cell death over control, the C6-ceramide-tariquidar regimen elicited >50% cell death. C6-ceramide-cyclosporine A and C6-ceramide-zosuquidar combinations produced like cytotoxic responses in HL-60/VCR cells. Much in contrast, verapamil and fulvestrant were devoid of C6-ceramide-enhancing cytotoxicity (Fig. 3C). To determine possible synergy, we investigated one of the nontamoxifen-related compounds, zosuquidar. Based on the Chou-Talalay method [39], combination indexes (CI) were determined. By this method, a CI of 1.0 indicates an additive effect, and CI's of 0.7–0.85, 0.3–0.7, and 0.1–0.3 indicate moderate synergism, synergism, and strong synergism, respectively. As shown in Fig. 3D, (left, isobologram; right, in tabulated data) all combinations, with the exception of number 1, demonstrated synergy as denoted by the CI's. Combination 6, yielding a CI of 0.3, was strongly synergistic. We next sought to determine the efficacy of C6-ceramide-adjuvant regimens in KG-1a, a human AML cell line that exhibits characteristics of CD34⁺CD38[−] leukemia stem cells [41–43], which are considered to be the principal reason for relapse. As shown in Fig. 3E, tamoxifen and zosuquidar were effective in reducing KG-1a cell viability when administered with C6-ceramide, albeit these assays were conducted over 72 h as opposed to the 24 h exposures used in the HL-60/VCR experiments.

3.3. Influence of C6-ceramide-adjuvant on mitochondrial ROS, impact of antioxidant, and effects on cytochrome c

Mitochondria play an important role in cancer cell biology. In addition, generation of mitochondrial ROS can have a potential therapeutic role in AML [44]. Thus, we next sought to investigate the effects of the C6-ceramide-adjuvant on the production of mitochondrial ROS. The data in Fig. 4A demonstrate a positive correlation between effective cytotoxic regimens and ROS generation. For example, whereas the C6-ceramide-verapamil regimen did not influence ROS levels, over control, the tamoxifen-, DMT-, and zosuquidar-containing combinations imparted approximate 1.7-, 3.7-, and 2.5-fold increases over control in ROS levels. Thus generation of ROS appears to be a common denominator in the cytotoxic action of the effective regimens. Noteworthy, short-time, 12 h exposure to combination C6-ceramide-DMT (10 + 10 μ M), promoted a 3-fold increase in ROS levels, while cell viability remained at 80% of control (data not shown). Thus, temporally, ROS generation precedes the full brunt of this regimen's cytotoxic impact. To determine whether antioxidant could protect HL-60/VCR cells from C6-ceramideadjuvant-induced insult and thus implicate ROS in the underlying cytotoxic mechanism, we evaluated the effects of vitamin E. The data in Fig. 4B show that cytotoxicity imparted by adjuvants that were effective in combination with C6-ceramide, tamoxifen, DMT, DiDMT, cyclosporin A, and zosuquidar, was mitigated by pre-exposure to vitamin E. For example, pretreatment of cells with Vitamin E diminished C6-ceramide-DiDMT-induced cytotoxicity from approximately 65 to 20% cell death, compared to drug-free control. Vitamin E protection from combination C6-ceramide-adjuvant (-tamoxifen, -DMT, -zosuquidar) was verified using the MTS cell viability assay method (data not shown). Further mitochondrial targeting was evidenced by evaluating cytochrome c release, a measure of mitochondrial injury and apoptotic signaling; both tamoxifen- and zosuquidar-containing C6-ceramide regimens promoted release of cytochrome c (Fig. 4C, flow cytometry histogram, left; quantitation, right).

3.4. The influence of P-gp inhibitors on C6-ceramide metabolism in intact cells

Tamoxifen has previously been shown an effective inhibitor of ceramide glycosylation in cancer cells [21]. As several of the P-gp inhibitors tested coactively enhanced C6-ceramide cytotoxicity, it was of interest to determine whether there would be a correlation between this enhancement and the effects of the inhibitors on C6-ceramide metabolism. Firstly, the thin-layer chromatogram in Fig. 5A, control lane, shows that glycosylation (generation of NBD-C6-GC) and conversion to sphingomyelin (NBD-C6-SM) are the major pathways of NBD-C6-ceramide metabolism in HL-60/VCR cells, whereas lower levels of metabolism via hydrolysis were observed (NBD-hex, hexanoic acid, indicative of ceramidase activity). It is important to note that the NBD-C6-ceramide substrate spot is prominent because it consists of both extracellular and intracellular NBD-C6-ceramide; in these experiments the total incubation mixture, cells plus media, was extracted. Figure 5A also clearly demonstrates the impact of various P-gp inhibitors on NBD-C6-ceramide metabolism. Noteworthy, tamoxifen, DMT, and zosuquidar, all of which were synergistic with C6-ceramide (see Fig. 3), inhibited NBD-C6-GC production by 75, 63, and 83%, respectively (Fig. 5B, quantitation of chromatogram in Fig. 5A), whereas Fulvestrant was less effective, 28% inhibition. Verapamil had no significant inhibitory effects, whereas T06b, the tamoxifen nucleus, was slightly stimulatory. Additionally, tamoxifen, DMT, and verapamil caused only a modest

reduction in the conversion of NBD-C6-ceramide to NBD-C6-SM, 24, 21, and 22% inhibition, respectively; however, zosuquidar blocked synthesis by 67% (Fig. 5A, B). Fulvestrant also inhibited NBD-C6-SM synthesis by 53%, similar with zosuquidar. Of note, zosuquidar, a specific, high affinity P-gp inhibitor (K_i 60 – 80 nM), at 0.1 μ M significantly depressed NBD-C6-GC synthesis; the inhibition was dose-dependent (Fig. 5C). Zosuquidar, which is cytotoxic in combination with C6-ceramide in KG-1a cells (see Fig. 3E), also strongly inhibited NBD-C6-ceramide glycosylation and NBD-C6-ceramide conversion to NBD-C6-SM by 85 and 100%, respectively, in KG-1a cells (Fig. 5D).

3.5. Effect of C6-ceramide-P-gp inhibitor combinations on apoptosis

We next investigated several of the agents that were co-actively effective with C6-ceramide to determine whether apoptosis was a factor underlying cytotoxic responses. The data in Fig. 6A, flow cytometry histograms, show that combination C6-ceramide-tamoxifen, -DMT, and-zosuquidar effectively induced apoptosis that was well above control values. All combinations, with the exception of the verapamil-containing regimen, elicited apoptosis that measured 55–65%, a significant increase above the 10% in control cultures (Fig. 6B). With the C6-ceramide-verapamil combination, apoptosis measured approximately 20% over control values. To confirm apoptotic responses, we utilized flow cytometry to evaluate DNA fragmentation. Results by this method verified that the C6-ceramide-P-gp inhibitor regimens elicited apoptotic cell death (data not shown). We then employed the pan-caspase inhibitor, Z-VAD-fmk to determine whether apoptosis was caspase-dependent. Results demonstrated that inclusion of caspase inhibitor at 20, 40, 50 μ M failed to reverse cytotoxic responses, suggesting that these drug regimens induce caspase-independent apoptosis, a topic of relevance in cancer therapy [45–47].

3.6. Efficacy of C6-ceramide-zosuquidar in colorectal cancer cells

The effect of zosuquidar on cytotoxic response to C6-ceramide in LoVo cells was investigated in order to determine whether this ceramide-based pharmacological approach could be of broader utility. Firstly, the thin-layer chromatogram in Fig. 7A, left, and quantitation, Fig. 7, right, demonstrate that zosuquidar inhibited NBD-C6-ceramide glycosylation in a dose-dependent manner in LoVo cells. For example, exposure to 1 μ M zosuquidar inhibited glycosylation by >20%. Also noteworthy, zosuquidar inhibited the synthesis of NBD-C6-SM, dose-dependently (Fig. 7A). Accordingly, combination C6-ceramide-zosuquidar sturdily reduced LoVo cell viability, whereas exposure to the single agents had limited impact (Fig. 7B). Closer examination reveals that the combination regimens, at the concentrations shown (Fa, fraction affected), affected 60, 80, 87, and 79% of the cell populations, as detected by isobologram analysis (Fig. 7C, left); combinations 1, 2, 3, and 4 produced in CI's ranging from 0.390 to 0.265, indicating synergism and strong synergism (Fig. 7C, right). Analysis of NBD-C6-ceramide levels and metabolites in LoVo cells, after cells had been washed free of extracellular substrate, provided a better assessment of intracellular only events. These experiments revealed that approximately 50% of the ceramide that had been taken up was metabolized to GC and sphingomyelin (SM) (Fig. 7D, control lane), effectively lowering the intracellular concentration of NBD-C6-ceramide (control lane, top spot); however, in the presence of either tamoxifen or zosuquidar, this metabolic conversion was largely blocked, as shown by the reduction in intensity of NBD-

C6-GC and NBD-C6-SM spots (Fig. 7D). This resulted in markedly higher levels of free, intracellular NBD-C6-ceramide, 77 and 79% of total metabolites in tamoxifen- and zosuquidar-treated cells, respectively (Fig. 7D). Thus, in cells with P-gp inhibitors, higher levels of free ceramide (NBD-form) are maintained. Lastly, in LoVo as in HL-60/VCR cells, C6-ceramide-tamoxifen and C6-ceramide-zosuquidar combinations were effective generators of ROS (Fig. 7E, photomicrographs, left; bar graph quantitation, right).

4. Discussion

Although tamoxifen has previously been shown to enhance ceramide-driven cancer cell death [24], little is known regarding the structure-activity relationship (SAR) supporting this response, nor, for purposes of broadening therapeutic application, have alternatives to tamoxifen been explored. Knowledge of the SAR underlying these responses would provide a useful guide for the rational engineering of potent ligands based on the chemical structure of tamoxifen. Results herein illustrate the close relationship of P-gp-interacting drugs with enhancement of C6-ceramide-elicited cytotoxicity and with inhibition of C6-ceramide metabolism. Although it is possible that the effects of P-gp inhibitors on C6-ceramide cytotoxicity are related to inhibition of efflux, we have previously demonstrated that C6-ceramide is not a substrate for P-gp and that P-gp inhibitors do not influence retention of C6-ceramide [48]. This work documents discovery of alternatives to tamoxifen that synergistically enhance ceramide-driven apoptosis and reveals insight into the mechanism of action underlying the cytotoxic responses.

Golgi-resident P-gp functions as a SM and GC transmembrane flippase [49, 50]. This finding spawned the idea that P-gp played a role in sphingolipid metabolism, and thus could be involved in regulating ceramide levels and therefore, ceramide sensitivity. Studies of Shabbits and Mayer [51] and Smyth et al [52] bolstered this notion. The idea that P-gp protects cells from ceramide cytotoxicity was supported by studies in HeLa cells that conditionally express P-gp. Those works showed that P-gp-expressing cells were resistant to ceramide, whereas the P-gp-devoid counterpart was ceramide-sensitive [48]

With regard to tamoxifen and metabolites, that DMT also enhanced response to C6-ceramide in HL-60/VCR cells is of added clinical relevance, as this tamoxifen metabolite is a poor antiestrogen [30] and would therefore not impact estrogen receptor-related biology. The efficacy of DMT also demonstrates that responses are not linked to traditional antiestrogen pathways. Further, that fulvestrant, a specific estrogen receptor antagonist, was without effect, also underscores that the C6-ceramide-enhancing activities of tamoxifen and metabolites are divorced from estrogen receptor jurisdiction. DiDMT is also a metabolite of tamoxifen in humans [53, 54] ; however, its efficacy as a modulator of MDR is not known. Our work is the first to demonstrate that DiDMT enhances ceramide's pro-apoptotic effects. The tamoxifen nucleus, TØb, was devoid of C6-ceramide-enhancing activity; this highlights structural specificity and requirement for the dimethylethanolamine moiety, although the methyl groups do not appear to be a requirement. Perhaps the slight increases in activity noted with DMT and DiDMT (see Fig. 3A, tamoxifen, DMT, DiDMT), compared to tamoxifen, result from enhanced uptake of these desmethylated tamoxifen metabolites.

The non-tamoxifen-related drugs, cyclosporin A, tariquidar, and zosuquidar, were effective enhancers of C6-ceramide cytotoxicity. Cyclosporin A, a substrate for P-gp, blocks the pumping of drugs in a competitive manner [55] and inhibits drug-activated and basal ATPase activity of P-gp [56]. Tariquidar, a potent inhibitor of P-gp [57, 58], but also a substrate and inhibitor for breast cancer resistance protein (BCRP/ABCG2) [59], shows a noncompetitive interaction with P-gp substrates and inhibits the ATPase activity of P-gp [60]; it could thus be considered to have an allosteric effect on substrate recognition or ATP hydrolysis. There is also a report showing that tariquidar inhibits P-gp drug efflux by blocking transition to the open state during the catalytic cycle [61]. Tariquidar has been evaluated in Phase I and in Phase II studies [62, 63]. Zosuquidar, a high affinity ($K_i = 60\text{--}80\text{ nM}$) P-gp competitive inhibitor does not inhibit other members of the ATP-drug binding transporter family, such as MRP and BCRP [64], and lacks pharmacokinetic interactions often seen with other MDR inhibitors that alter the plasma concentration of co-administered oncolytic agents. Zosuquidar restores drug sensitivity in P-gp-expressing AML cells [65], and is generally well tolerated, as evaluated in Phase I trials in patients with advanced malignancies [66, 67]. The agent can be given safely to patients with AML in combination with induction doses of conventional cytotoxic drugs [68]; however, zosuquidar did not improve outcome in older AML in part because of the presence of P-gp-independent mechanisms of resistance [69]. Of note, however, results in a study by Lancet et al [70] in P-gp positive patients indicated that pre-administration of zosuquidar followed by continuous infusion, prior to daunorubicin administration was well tolerated and able to completely inhibit P-gp function.

Verapamil and fulvestrant were ineffective in combination with C6-ceramide. Verapamil, a calcium channel blocker and a well-known, much utilized first generation P-gp modulator, is a P-gp substrate that inhibits drug transport in a competitive manner; it also stimulates P-gp ATPase activity [29, 71]. Fulvestrant is a pure estrogen receptor antagonist, which unlike tamoxifen, works both by down-regulating and by degrading the estrogen receptor; it binds competitively to the estrogen receptor in cells. The agent has been shown to inhibit P-gp function and subsequently reverse P-gp-mediated drug resistance in a breast cancer model [72]; however, its history as MDR modulator is truly limited.

The agents demonstrating efficacy with C6-ceramide are inhibitors of P-gp function; verapamil, at the concentration employed, was the exception. It is perhaps noteworthy that the C6-ceramide-verapamil regimen also failed to activate ROS generation (see Fig. 4A) and weakly induced apoptosis when compared to the other combination regimens (see Fig. 6B). Another commonality among agents that demonstrated synergy with C6-ceramide was the capacity to inhibit C6-ceramide glycosylation (see Fig. 5), in this instance tamoxifen, DMT, and zosuquidar were potent inhibitors. Thus, we propose that the P-gp inhibitors amplify the ceramide effect in part by contributing to preserve high levels of intracellular C6-ceramide, via metabolic blockade. This effect is clearly illustrated in LoVo cells (see Fig. 7E) where both conversion to GC and SM were severely compromised, a maneuver that may implement and enforce ROS generation and cellular demise. That cytotoxicity was reversed by exposure to a ROS scavenger, vitamin E, demonstrates that oxidative injury may play a vital role in the cytotoxic response.

We have recently demonstrated that combination C6-ceramide-tamoxifen promotes a decrease in mitochondrial membrane potential and inhibits complex I respiration in KG-1 AML cells [73]. As damaged mitochondria stimulate increased ROS production [74], we propose that the active combinations presented herein share mitochondria as a common target and employ ROS. The capacity of ROS to drive tumor cell death has been exploited as an avenue in cancer therapy. Along these lines, leukemia stem cells (LSC) [75], thought to play a pivotal role in relapse and in the refractory nature of AML, become an attractive target. Taking into account the unique nature of self-renewing LSCs, the ceramide-containing-P-gp-inhibitor-containing regimens may be of unique utility. Firstly, LSCs express high levels of P-gp, and these cells usually reside in a quiescent state, making agents that target cell cycle ineffective. As well apropos in the current context, oxidative stress inhibits self-renewal in LSCs [76]. Thus, agents that simultaneously enhance the apoptotic impact of ceramide via P-gp modulation and induce oxidative stress should target LSCs effectively. Our results in KG-1a cells indicate that the C6-ceramide-containing combinations could be effective against LSC. That the C6-ceramide-P-gp inhibitor regimens were also effective in LoVo, a colon cancer model, broadens the potential therapeutic utility of such an approach.

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Abbreviations

C6-ceramide	N-hexanoyl-d-erythrosphingosine
GC	glucosylceramide
GCS	glucosylceramide synthase
SK	sphingosine kinase
S1-P	sphingosine 1-phosphate
PPMP	D-threo-1-phenyl-2-decanoylamino-3-morpholino-propanol
PPPP	1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol
ACAT, Acyl-CoA	cholesterol acyl transferase
4-HPR	fenretinide
P-gp	P-glycoprotein
DMT	N-Desmethyltamoxifen-HCl
AML	acute myeloid leukemia
ROS	reactive oxygen species
PI	propidium iodide

Vitamin E	alpha-tocopherol
HBSS	Hank's Balanced Salt Solution
LY33597	Zosuquidar
Fulvestrant	Faslodex
Tariquidar	XR9576
MW	microwave
CRC	colorectal cancer cells
ATCC	American Type Culture Collection
TØb	But-1-ene-1,1,2-triyltribenzene, triphenylbutene
DiDMT	(E/Z) N,N-didesmethyltamoxifen
FBS	fetal bovine serum
MTS	CellTiter 96 One Solution Cell Proliferation Assay Kit
SEM	standard error of the mean
CI	combination index
NBD-C6-GC	N-Hexanoyl-NBD-glucosylceramide
NBD-C6-SM	NBD-C6-sphingomyelin
NBD-C6-ceramide	C6-NBD-ceramide (N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-erythro-sphingosine)
NBD-hex	NBD hexanoic acid
SM	sphingomyelin
SAR	structure-activity relationship
LSC	leukemia stem cells
Fa	fraction affected
DMSO	dimethyl sulfoxide

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Fig. 1. Chemical synthesis of but-1-ene-1,1,2-triylbenzene (triphenylbutene, TØb, **3**) and (E/Z) *N,N*-didesmethyltamoxifen (DiDMT, **6**). (A,B) TØb (**3**) and DiDMT (**6**), respectively, were chemically synthesized, purified, and analyzed as detailed in Materials and methods.

Fig. 2.
Chemical structures of tamoxifen and tamoxifen metabolites and other agents used in the study, cyclosporin A, verapamil, tariquidar, zosuquidar, and fulvestrant.

Fig. 3.

The influence of tamoxifen, DMT, DiDMT, cyclosporin A, tariquidar, zosuquidar, fulvestrant, and verapamil on the cytotoxic effects of C6-ceramide in HL-60/VCR and KG-1a cells. (A-C) Influence of C6-ceramide (10 μM) and combinatorial regimens (10 + 10 μM) on HL-60/VCR cell viability. (D) Evaluation of C6-ceramide-zosuquidar regimen in HL-60/VCR cells by combination index (CI) and isobologram. Left, isobologram; right, tabulated results. Regimens 2–6 yielded CI's denoting synergism (0.3–0.7). For (A-C), cells (100,000/well) were seeded in 96-well plates, and after a 2 h equilibration period, cells were treated with the agents indicated for 24 h. (E) Effect of tamoxifen and zosuquidar on C6-ceramide cytotoxicity in KG-1a cells. Cells (50,000/well, 96-well plates) were seeded, and after a 2 h equilibration period, cells were exposed to the agents indicated at the concentrations shown, for 72 h. Alamar Blue was used to measure cell viability.

Experimental details and methods of statistical analyses are provided in Materials and methods. Data are presented as the mean $\pm$ SEM of three independent experiments in triplicate. Asterisks indicate a significant difference (*P 0.05; ***P 0.001). C6-cer, C6-ceramide; Tam, tamoxifen; DMT, N-desmethyldamoxifen; DiDMT, didesmethyl tamoxifen; TØb, triphenylbutene; Tarq, tariquidar; Cyc A, cyclosporin A; Zos, zosuquidar; Ver, verapamil; Ful, fulvestrant; Fa, fraction affected; CI, combination index; SEM, standard error of the mean

Fig. 4.

The effect of vitamin E on cytotoxicity of combination regimens, and the influence of these regimens on mitochondrial ROS generation and cytochrome c release in HL-60/VCR cells.

(A) Effect of various combination regimens on mitochondrial ROS generation.

Mitochondrial superoxide was assayed using MitoSOX™ Red as described in Materials and methods. Cells were treated with the indicated agents (10 μ M) for 24 h, and response was measured in populations of viable cells (MitoSOX does not react in non-viable cells) by fluorescence spectroscopy. (B) Effect of vitamin E on cytotoxicity. Cells (100,000/well) were seeded in 96 well plates, equilibrated at 37°C for 2 h and where indicated pretreated with 250 μ M vitamin E (prepared freshly) for 2 h before addition of the combination regimens (all agents administered at 10 μ M). Viability was measured after 24 h, as in Fig. 3, using Alamar Blue. (C) Influence of C6-ceramide combination regimens on cytochrome c release. Cells were treated with the indicated agents (10 μ M) for 18 h before evaluation of cytochrome c release by flow cytometry. Left panel, flow cytometry histogram; right panel,

bar graph quantitation. Data are presented as the mean $\pm$ SEM of three independent experiments in triplicate. ** P 0.01; ***P 0.001 Abbreviations: C6-cer, C6-ceramide; Tam, tamoxifen; Vit E, vitamin E; DMT, N-desmethyldamoxifen; DiDMT, didesmethyl tamoxifen; Cyc A, cyclosporin A; Zos, zosuquidar; ROS, reactive oxygen species; Ver, verapamil; SEM, standard error of the mean

Fig. 5.

Influence of tamoxifen and other agents on NBD-C6-ceramide metabolism in HL-60/VCR and in KG-1a cells. (A) TLC chromatogram illustrating qualitative effect of agents on production of NBD-C6-GC, NBD-hexanoic acid (denotes ceramidase activity), and NBD-C6-SM from NBD-C6-ceramide, in HL-60/VCR cells. NBD-C6-ceramide complexed to BSA was utilized as substrate, thus products are designated as NBD-containing. Whole cell incubations were carried out as detailed in Materials and methods. Note: Total incubation extracts (cells plus media) were applied to the origin of the TLC plate, thus the NBD-C6-cer spot is comprised of both extracellular and intracellular compound. Visualization was by

fluorescence. (B) Quantitative analysis of NBD-C6-GC and NBD-C6-SM from the TLC chromatogram in panel (A). Quantitation was carried out as described in Materials and methods. (C) Effect of low-dose zosuquidar on NBD-C6-ceramide glycosylation in HL-60/VCR cells. Cells were exposed to the agent for 24 h before initiating the 2 h incubation with NBD-C6-ceramide. Cellular NBD-C6-GC was resolved by TLC and quantitated as described above. (D) Effect of zosuquidar on NBD-C6-ceramide metabolism in KG-1a cells. The 2 h incubations contained 100,000 and 25 μ M substrate as detailed in Materials and methods. Data are presented as the mean $\pm$ SEM of three independent experiments in triplicate. *P 0.05, **P 0.01, ***P 0.001. Abbreviations: Tam, tamoxifen; DMT, N-desmethyldamoxifen; Zos, zosuquidar; Ver, verapamil; TØb, triphenylbutene; Ful, Fulvestrant; NBD-C6-cer, NBD-C6-ceramide; NBD-C6-GC, NBD-C6-glucosylceramide; NBD-hex, NBD-hexanoic acid; NBD-C6-SM, NBD-C6-sphingomyelin SEM, standard error of the mean

Fig. 6.

C6-ceramide-P-gp inhibitor combinations induce apoptosis in HL-60/VCR cells. (A) Flow cytometry dot-plot demonstrating the degree of Annexin V binding in control and treated cells. Cells were treated with the combinations indicated (10 μ M each agent) for 18 h. (B) Bar graph quantitation of apoptosis from data in panel (A). Cell treatment protocol and flow cytometry methods were conducted as described in Materials and methods. Data are presented as the mean $\pm$ SEM of three independent experiments in triplicate. *** P 0.001. Abbreviations: C6-cer, C6-ceramide; Tam, tamoxifen; DMT, N-desmethyltamoxifen; Zos, zosuquidar; Ver, verapamil; SEM, standard error of the mean

Fig. 7.

The effect of zosuquidar and tamoxifen on NBD-C6-ceramide metabolism, cytotoxicity, and ROS generation in the human colorectal cancer cell line LoVo. (A) TLC chromatogram, left; bar graph quantitation, right, showing effects of zosuquidar on synthesis of NBD-C6-GC and NBD-C6-SM from NBD-C6-ceramide. Cells, 100,000/reaction, were incubated for 2 h with 25 μ M NBD-C6-ceramide substrate. Lipid extraction and TLC were conducted as described in Materials and methods. (B) Effect of zosuquidar on C6-ceramide cytotoxicity in LoVo cells. Cells (10,000/well) were seeded into 96-well plates in RPMI-1640 medium containing 10% FBS and the following day treated for 24 h with C6-ceramide and zosuquidar as indicated (formulated in RPMI-1640 medium containing 1% FBS). LoVo cell viability was determined by MTS reagent. (C) Assessment of drug regimen efficacy by CI and Fa isobologram, left; tabulated results of combination regimens 1–4, right. Results yielded CIs denoting synergism (CI= 0.3–0.7) and strong synergism (CI= 0.1–0.3). (D) Effect of tamoxifen and zosuquidar on NBD-C6-ceramide metabolism in LoVo cells. After a 2 h incubation, cell monolayers were rinsed free of extracellular NBD-C6-ceramide substrate followed by extraction of cellular lipids. (E) Effect of drug regimens on ROS generation. Mitochondrial superoxide was assayed using MitoSOX™ Red as described in

Materials and methods. Cells were treated with the indicated agents for 18 h, and response was measured by fluorescence photomicrograph and spectroscopy. Left, photomicrographs denoting mitochondrial ROS levels (red fluorescence) in control and treated cells. Right, bar graph quantitation of ROS levels in control and treated cells after 18 h treatment with indicated agents. Concentrations (μM) of the agents used are noted in the bars. Data are presented as the mean $\pm$ SEM of three independent experiments in triplicate. *** $P < 0.001$. Abbreviations: NBD-C6-cer, NBD-C6-ceramide; NBD-C6-GC, NBD-C6-glucosylceramide; NBD-C6-SM, NBD-C6-sphingomyelin; Zos, zosuquidar; GCS, glucosylceramide synthase; C6-cer, C6-ceramide; Fa, fraction affected; CI, combination index; Tam, tamoxifen; ROS, reactive oxygen species; SEM, standard error of the mean.