Multiple actions of doxorubicin on the sphingolipid network revealed by flux analysis

Justin M. Snider, Magali Trayssac, Christopher J. Clarke, Nicholas Schwartz, Ashley J. Snider, Lina M. Obeid, Chiara Luberto, and Yusuf A. Hannun

Molecular and Cellular Biology and Biochemistry and Structural Biology Graduate Program, Departments of Medicine, Physiology and Biophysics, Biochemistry, Pharmacology, and Pathology, and Stony Brook Cancer Center, Stony Brook University, Stony Brook, NY; and Northport Veterans Affairs Medical Center, Northport, NY

ORCID IDs: 0000-0003-3349-3369 (Y.A.H.)

Abstract Sphingolipids (SLs) have been implicated in numerous important cellular biologies; however, their study has been hindered by the complexities of SL metabolism. Furthermore, enzymes of SL metabolism represent a dynamic and interconnected network in which one metabolite can be transformed into other bioactive SLs through further metabolism, resulting in diverse cellular responses. Here we explore the effects of both lethal and sublethal doses of doxorubicin (Dox) in MCF-7 cells. The two concentrations of Dox resulted in the regulation of SLs, including accumulations in sphingosine, sphingosine-1-phosphate, dihydrosphingosine, and ceramide, as well as reduced levels of hexosylceramide. To further define the effects of Dox on SLs, metabolic flux experiments utilizing a d17 dihydrosphingosine probe were conducted. Results indicated the regulation of ceramidases and sphingomyelin synthase components specifically in response to the cytostatic dose. The results also unexpectedly demonstrated dose-dependent inhibition of dihydrosphingosine desaturase and glucosylceramide synthase in response to Dox. Taken together, this study uncovers novel targets in the SL network for the action of Dox, and the results reveal the significant complexity of SL response to even a single agent. This approach helps to define the role of specific SL enzymes, their metabolic products, and the resulting biologies in response to chemotherapeutics and other stimuli. —Snider, J. M., M. Trayssac, C. J. Clarke, N. Schwartz, A. J. Snider, L. M. Obeid, C. Luberto, and Y. A. Hannun. Multiple actions of doxorubicin on the sphingolipid network revealed by flux analysis. J. Lipid Res. 2019. 60: 819–831.

Supplementary key words high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry • chemotherapeutic • metabolism

Sphingolipids (SLs) such as ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S1P) are important bioactive lipids, and they are involved in numerous cellular biologies, including cell proliferation, migration, inflammation, and apoptosis (1, 2). In cancer cells, Cer functions as a tumor-suppressor lipid, whereas S1P acts as a tumor-promoter lipid, and this has led to increased interest in modulating SL levels for cancer therapeutics (3–6). However, studies translating the effects on SL metabolism into signaling components have been hindered by the interconnected nature of the SL network. Indeed, SL metabolism represents a dynamic network in which a single stimulus can result in diverse responses due to the metabolic interconversion of the various bioactive SLs, leading to multiple potential biologies. For example, Cer, the central hub of SL metabolism, can be generated in response to chemotherapeutics, cytokines, growth factors, and UV radiation, and once generated, Cer then functions to enhance/mediate apoptosis, senescence, and/or cell-cycle arrest (7). However, the catabolism of Cer leads to Sph, which is subsequently phosphorylated to S1P, a potent prosurvival signal. Cer can also be phosphorylated to ceramide-1-phosphate, which has been associated with breast cancer invasiveness (8). Similarly, the activation of glucosylceramide synthase (GCS) can neutralize Cer accumulation and reduce cell death (9). Moreover, one stimulus can result in the

Abbreviations: ACER2, alkaline ceramidase 2; CDase, ceramidase; Cer, ceramide; CerS, ceramide synthase; C12dhCer-PYR, β-erythro-C12-dihydro pyridinium ceramide; DeS, dihydrosphingosine desaturase; dhCer, dihydroceramide; dhSph, dihydrosphingosine; Dox, doxorubicin; FB1, fumonisin B1; GCS, glucosylceramide synthase; GluCer, glucosylceramide; HexCer, hexosylceramide; MPB, mobile phase B; NBD C6-Cer, N-[6-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino][6-erythro-sphingosine; SK, sphingosine kinase; SL, sphingolipid; SMS, sphingomyelin synthase; Sph, sphingosine; SIP, sphingosine 1-phosphate.

1To whom correspondence should be addressed.
2e-mail: yusuf.hannun@stonybrookmedicine.edu (Y.A.H.);
3chiara.luberto@stonybrookmedicine.edu (C.L.)
4The online version of this article (available at http://www.jlr.org) contains a supplement.
regulation of more than one enzyme of SL metabolism. Consequently, the dissection of each component of SL metabolism is essential for understanding the alterations in cellular SL levels in response to specific stimuli. This context is particularly important if therapeutic targeting of the SL network is to be effective.

Anthracyclines are among the most potent chemotherapeutics and provide the best coverage of different types of cancer (10). Doxorubicin (Dox), a hydroxylated derivative of daunorubicin, the first anthracycline discovered, is widely used in the treatment of breast cancer. However, due to significant side effects, Dox is often used in combination treatment or at lower than optimal doses (11). Studies in multiple cell types have shown that the accumulation of Cer is integral to the cytotoxicity of Dox (12). However, it has also been demonstrated that Cer is generated at sublethal concentrations of Dox, which can lead to growth arrest in cancer cells (13, 14). Still, it remains unclear from current studies whether these biologies are driven via different pools of SLs or by the activation of multiple and possibly interfering pathways of SL metabolism. Indeed, there are over 20 enzymes in the SL network that can synthesize or catabolize Cer alone, and the perturbation of some of these enzymes has been shown to affect Dox signaling (15–18). Studies have shown that neutral SMases (19), acid SMases (20), GCS (19), ceramide synthases (CerSs) (18), sphingosine kinases (SKs) (17), and ceramidases (CDases) (21) all have roles in breast cancer sensitivity to Dox. However, the interplay between SL enzymes and their substrates is often more complex than static lipid levels reveal. Consequently, analyzing the flux in the SL network is necessary for understanding both the dynamics and cellular effects of Dox-induced Cer production.

In this study, we applied our recently developed methodology (22) using d17 dihydrocerphospho-sphingosine (dhSph) as a probe to monitor the effects of Dox on metabolic flux through the de novo and hydrolytic SL pathway in combination with a gene expression array analysis of the SL network. Both dihydroceramide desaturase (DeS) and GCS were identified as major nodes of regulation by Dox. Moreover, a spatial context was given to Sph generated in response to the cytostatic Dox dose as the kinetics of d17Cer metabolism into d17Sph indicated activation of CDases in the Golgi. We also performed chase experiments utilizing d17dhSph and verified hydrolytic contributions to Dox-induced Cer generation. Importantly, this study used a recently developed technique to probe de novo synthesis (after serine palmitoyltransferase), complex SL generation, and hydrolytic pathways, and the results obtained are a proof of concept of how this technique can be applied to decipher the regulation of key nodes in SL metabolism in response to specific stimuli, in our case the chemotherapeutic Dox.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Synthetic SL reference standards [13C16Cer, 13C22Cer, d17Sph, d17dhSph, d17S1P, Cer d17:1/16:0, Cer d17:1/24:1, SM d18:1/18:1, SM d18:1/17, hexosylceramide (HexCer) d18:1/18:1, HexCer d18:1/C17, Sph, dhSph, S1P, Cer d18:1/16:0, Cer d18:1/24:1, Cer d18:1/24:1Sph, Cer d18:1/16:0, Cer d18:1/17, SM d18:1/16:0, SM d18:1/24:1, glucosylceramide (GlucCer) d18:1/16:0, and GlucCer d18:1/24:1] were either synthesized at the Medical University of South Carolina Lipidomics Shared Resource Core or acquired from Avanti Polar Lipids, Inc. (Alabaster, AL). 6-Ethyl-6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanal containing SLs (NBD-C6Cer and NBD-C6SM) were acquired from Avanti Polar Lipids, Inc. Doxorubicin was acquired from Sigma-Aldrich (St. Louis, MO). Analytical-grade solvents from Burdick and Jackson (Muskegon, MI) were used unless otherwise noted. Extraction solvent A consisted of 2-propanol/ethyl acetate (15:85; v/v) and was utilized in the extraction of media samples, while extraction solvent B consisted of ethyl acetate/2-propanol/water (60/30/10; v/v) and was utilized for cell/tissue extractions.

**Cell lines and cell culture**

MCF-7 human breast adenocarcinoma cells were obtained from ATCC (Manassas, VA) and maintained in RPMI media (Invitrogen, Carlsbad, CA) containing 10% (v/v) FBS. Cells were maintained in a humidified incubator at 37°C with 5% CO2.

**Dox treatment**

MCF-7 cells were plated in a 10 cm dish at approximately 30% confluence (400,000 cells) for 24 h prior to Dox treatment. Media were changed to RPMI containing 2% FBS 1 h prior to Dox treatment. Cells were treated with either DMSO, 500 nM Dox, or 1,000 nM Dox. Cells were harvested 24 h posttreatment as follows (direct extraction technique): cells were washed twice with room-temperature PBS, 2 ml extraction solvent B was added, and after 1 min the cell extract was transferred from the plate to a 15 ml conical tube and stored at −80°C to be extracted following the extraction protocol described in Bielawski et al. (23).

**Pulse labeling with d17dhSph**

MCF-7 cells were labeled as previously described (22). Briefly, MCF-7 cells were plated in a 10 cm dish at approximately 30% confluence (400,000 cells) for 48 h prior to labeling with d17dhSph, and the media were changed to RPMI containing 2% FBS at 23 h prior to labeling. For samples undergoing Dox treatment, Dox was added 24 h prior to labeling. Label was added to a final concentration of 500 nM from a 10 µM stock in ethanol. Cells were incubated to the indicated time points and harvested using the direct extraction technique.

**Chase for d17dhSph label**

Chase parameters were dependent on the label attaining a pseudo-steady state for incorporation into complex SLs, as this assay was designed to monitor the hydrolytic pathways that generate Cer from these metabolites. In MCF-7 cells, optimal steady-state conditions occurred between 10 and 12 h of labeling. Therefore, MCF-7 cells were plated similarly to pulse experiments and labeled for 12 h with 500 nM d17dhSph prior to changing the media to 2% FBS. After 1 h, cells were then treated with Dox similarly to pulse conditions and harvested at designated time points using the direct extraction technique.

**DeS activity assay**

MCF-7 cells were grown and treated with Dox in similar conditions as those described above. For the in situ assay (24), cells were labeled with 500 nM C12dhCer-PYR for 2 h, at which point they were harvested via direct extraction. For in vitro assays, 200 µg (~200 µl volume) of cell lysate (in 50 mM sucrose, 5 mM HEPES,
pH 7.4) was utilized. Lysate was incubated with 1 µM C12dhCer-
pyr and 2 mM NAPDH for 20 min at 37°C and harvested via direct
extraction. All samples were then subjected to HPLC/MS/MS
analysis utilizing the conditions described by Kraveka et al. (25).
The percentage of the conversion of α-erythro-2-N-[12′-(1″-pyridinium)dodecanoyl]sphingosine is depicted in the figures.

**NBD-C6Cer fluorescence assay**

NBD labeling protocols were the same as for d17dhSph,
although at 24 h Dox treatment, 1 µM NBD-C6Cer was added and a single time point was collected after 1 h of labeling. NBD was
diluted from a 0.5 mM stock in ethanol. Label incorporation into complex SLs was assessed with an Agilent 1200 series HPLC/
fluorescence detector utilizing a Peak Scientific C8 column (3 µm
particle; 4.6 × 150 mm) column under similar gradient and col-
umn conditions to the 13C methods listed below. NBD fluorescence was detected using excitation and emission wavelengths of
470 and 530 nm, respectively. The fluorescent peaks for NBD SLs
were identified by comparing their retention times with those of
standards for NBD-C6Cer and NBD-C6SM.

**Cell-cycle analysis**

Following 24 h of Dox treatment, MCF-7 cells were washed
once with ice-cold PBS, scraped in ice-cold PBS, and pelleted by
centrifugation for 5 min at 1,000 g. Cells were fixed by adding cold
70% ethanol dropwise and incubating overnight at 4°C. Follow-
ing fixation, cells were resuspended in a staining solution contain-
ing propidium iodide, RNAse (Cell Signaling Technology,
Danvers, MA), and 0.2% Triton X-100. Samples were analyzed by
flow cytometry using the FACS Calibur system and ModFit software.

**Immunoblotting**

After Dox treatment, cells were washed and scraped on ice with
PBS. After centrifugation, pellets were resuspended in RIPA buffer
containing phosphatase inhibitor cocktails P5726 and P0044
(Sigma-Aldrich) and protease inhibitor cocktail tablet 04935159001
(Roche Diagnostics, Indianapolis, IN) and sonicated. After cen-
trifugation at 12,000 rpm for 10 min at 4°C, protein concentra-
tion was determined using a BCA protein assay kit (Bio-Rad,
Hercules, CA). Equal amounts of proteins (20 µg) were prepared
in Laemmli buffer (Amresco, Solon, OH), boiled for 10 min, sep-
arated utilizing a 4% to 20% SDS-PAGE gel (Novex Tris-Glycine;
Invitrogen), and blotted onto a PVD membrane (Bio-Rad). After
blocking with 5% nonfat milk in 0.1% Tween PBS for 2 h at room
temperature, the following primary antibodies were used over-
night at 4°C: anti-DeS1 (1:2000; Abcam), anti-poly(ADP-ribose)
polymerase (1:1000; Cell Signaling Technology), and anti-β-actin
(1:5000; Sigma-Aldrich). The protein levels of DeS1, full-length/cleaved poly(ADP-ribose) polymerase, and β-actin were visualized
by chemiluminescence using peroxidase-conjugated secondary
antibodies (Jackson Immunoresearch Laboratories, West Grove,
PA) and a Pierce ECL protein detection kit (Thermo Fisher Sci-
entific, Waltham, MA). ImageJ software was utilized to quantify
Western blots by densitometry.

**Quantitative RT-PCR mRNA array analysis**

To assess the gene expression of the SL network, we utilized a custom PCR array published previously (26). For these experi-
ments, MCF-7 cells were treated with vehicle (DMSO) or the Dox
doses shown for 24 h. Following stimulation, mRNA was extracted
using the Purelink RNA extraction kit (Life Technologies, Carls-
bad, CA), and 1 µg RNA was converted to cDNA with the Super-
script II kit for first-strand synthesis (Life Technologies). To run
the array, a mastermix of 637.5 µl SYBR-green, 51 µl cDNA, and
586.5 µl dH2O was prepared for each cDNA template, and 25 µl
was loaded per well. The RT-PCR protocol consisted of 10 min at
95°C for polymerase activation followed by 40 cycles of a 15 s melt
at 95°C and 60 s at 60°C for annealing and extension. After the
cycles were run, a melt curve was performed to confirm a single
product for each primer pair. Data were analyzed utilizing the
Data Analysis Center at www.qiagen.com. Major gene changes
from the array were validated by Taqman assays. Briefly, reac-
tions were performed in triplicate in 96-well plates, with each
reaction containing 10 µl 2× iTAG mastermix, 5 µl diluted cDNA,
1 µl 20× Taqman gene-specific primer probe, and 4 µl water.
The following probes were purchased from Life Technologies:
AC1B (Hs01060665_g1), SMPD3 (Hs00920534_m1), ACER2
(Hs01892094_g1), CERK (Hs00368483_m1), and UGGC
(Hs00916612_m1). The RT-PCR protocol consisted of 2 min at
95°C for polymerase activation followed by 40 cycles of a 10 s melt
at 95°C and 60 s at 60°C for annealing and extension.

**Analysis by HPLC/MS/MS**

Chromatographic separation was achieved utilizing a Thermo
Accela HPLC system (Thermo Fisher Scientific). Operational condi-
tions were optimized using a Peak Scientific C8 column (3 µm
particle; 4.6 × 150 mm). A column temperature of 45°C maximized
the intensity and integrity of analytes while maintaining baseline separa-
tion. Mobile phase A consisted of MS-grade water containing 0.2%
formic acid and 1 mM ammonium formate (pH 5.6), and mobile phase
B (MPB) consisted of MS-grade methanol containing 0.2%
formic acid and 1 mM ammonium formate (pH 5.6). Chromato-
graphic conditions were as follows. Upon sample injection the gradi-
ent was constant for 2 min at 82% MPB, then increased to 90% MPB
by 4 min, and then increased to 98% MPB by 10 min; 98% MPB
was sustained until 28 min, at which point MPB was reduced to 82%
by 30 min and then reequilibrated for 5 min for a total gradient of 35
min. For SM analysis, an abbreviated gradient was utilized. Briefly,
upon sample injection the gradient was increased from 90% to 99%
MPB over the first 7 min and then maintained at 99% MPB until 17
min into the gradient, at which point the gradient was returned to
90% MPB within 1 min and allowed to equilibrate for the remainder
of the 20 min method. Detection was accomplished utilizing a
Thermo Scientific Quantum Access triple quadrupole mass spec-
trometer equipped with an ESI source operating in positive-ion mul-
tiple reaction monitoring mode. The ESI source was operated at
400°C vaporizer temperature and 300°C capillary temperature in
positive ionization mode with a spray voltage of 3,500 V. Gases were
set at 40, 5, and 10 for sheath, ion sweep, and auxiliary gases, respec-
tively. MS detection of labeled lipids was accomplished using transi-
tions described in Snider et al. (22).

**Statistical analysis**

One-way ANOVA, two-way ANOVA, and student's t-test were
utilized for statistical analysis in GraphPad Prism (GraphPad Soft-
ware, San Diego, CA). Bonferroni's posttest was applied for mul-
tiple comparisons for both one- and two-way ANOVA analysis.

**RESULTS**

**Dose-dependent effects of Dox on SL metabolism in breast cancer cells**

The MCF-7 breast cancer cell line is a hormone receptor-
positive cell line commonly used to study early-stage breast
cancer. For cell studies, 1 µM Dox in MFC-7 cells induces
cell death, thus acting as an equivalent to therapeutic
concentrations in vivo (12, 18, 27). However, in therapeu-
tic uses, Dox levels spike directly after treatment and then
normalize to lower levels prior to the next treatment (28).
Consequently, the concentrations in patients’ blood can reach 3 µM during treatment but may only reach suboptimal concentrations in solid tumors during treatments (29). Therefore, to define the effects of Dox on SL metabolism, MCF-7 breast cancer cells were treated with two doses: one that results in cell-cycle arrest (500 nM) and one that results in cell death (1,000 nM), as established and verified in supplemental Fig. S2A and B (13, 15, 18). A preliminary analysis of SL gene expression indicated that the dynamic regulation of gene expression was associated with these Dox doses (supplemental Table S1), with alterations in the genes for CDases, SMases, and GCS being among the major changes observed and validated (supplemental Fig. S1). SL levels were examined using MS, with a focus on the most abundant species of dihydroceramide (dhCer), Cer, HexCer, SM, sphingoid bases, and sphingoid base phosphates. Discrete patterns in sphingoid base regulation emerged at the cytostatic dose, with Sph and S1P levels increasing 2- and 3-fold, respectively (Fig. 1A, B). On the other hand, there were no significant increases in either SL at the higher cytotoxic dose (1,000 nM) of Dox (Fig. 1A, B). An analysis of Cer species showed that both dhCer d18:0/16:0 and Cer d18:1/16:0 increased more than 2-fold at the cytotoxic dose (Fig. 1C, D). The very long chain dhCer d18:0/24:1 was significantly increased at the cytotoxic dose, but in contrast, Cer d18:1/24:1 demonstrated no such increase, although the saturated Cer d18:1/24:0 species was significantly decreased at both doses of Dox (Fig. 1C, D). In regard to complex SLs, HexCers demonstrated a dose-dependent decrease regardless of the length of the N-acyl chain, the most impressive of which was a 76% decrease in HexCer d18:1/24:0 at the higher dose of Dox (Fig. 1E). In contrast, SM levels remained largely unchanged, with a trend toward a decrease at the cytostatic dose and an increase at the cytotoxic dose, although neither was significantly different from the vehicle control (Fig. 1F). Taken together, these results show that low-versus high-dose Dox had differential effects on most SL species analyzed with dhCer, Cer, and HexCer levels, suggesting the regulation of de novo synthesis (CerS) and of complex sphingolipids (HexCer).

Dox has dynamic effects on de novo SL flux

To define the effects of Dox treatment on key steps and enzymes in the de novo pathway and to provide context and explanation to the observed changes in the

![Fig. 1](image-url). Endogenous SL profile following Dox treatment. MCF-7 cells were treated with either DMSO (vehicle) or 500 or 1,000 nM Dox for 24 h, and cellular SLs were analyzed by HPLC/MS/MS. Sph (A), S1P (B), dhCer (C), Cer (D), HexCer (E), and SM (F) levels were normalized to nmol total lipid phosphate. Data are means ± SDs, n = 4. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 compared with vehicle; #P < 0.05, ##P < 0.01, and ###P < 0.001 compared with 500 nM Dox.
endogenous lipid levels, d17dhSph labeling was utilized to perform a flux analysis. Previously established pulse parameters (22) were utilized to distinguish the time-dependent incorporation of label phases of CerS, DeS, CDase, SK activity, and HexCer and SM synthesis (Scheme 1). MCF-7 cells were labeled with 500 nM d17dhSph for up to 120 min after treatment for 24 h with either vehicle or 500 or 1,000 nM Dox, and the resulting labeled metabolites were assessed using LC/MS. By calculating a best-fit line to the labeled product of a given enzymatic reaction during previously established time phases (Scheme 1) (22), we were able to generate rates (slope) for that enzyme under different treatment conditions. The levels of labeled precursor (d17dhSph) in the cells remained unaffected by Dox treatment (supplemental Fig. S3). d17dhSph incorporation into total dhCer remained largely unchanged during the CerS Phase (0–15 min) of d17dhCer generation (Fig. 2A). While there was no significant change in the rates for dh-Cer d17:0/24:0 or dhCer d17:0/24:1 (Fig. 2C, D), there was a moderate but significant increase in Cer activity that was responsible for dhCer d17:0/16:0 production in response to the cytotoxic Dox dose (Fig. 2B), demonstrating a rate of 0.32 pmol labeled lipid/nmol lipid Pi/min with vehicle that increased to 0.54 pmol labeled lipid/nmol lipid Pi/min at the high Dox dose (Fig. 2B). These results argue against a generalized activation of CerSs and rather support distinct regulation of a specific CerS, most likely CerS5 and/or 6, at only the cytotoxic dose.

The next step in de novo SL flux is the DeS phase, which was monitored between 20 and 60 min via the formation of d17Cer, the product of DeS activity. Dox administration exerted a dose-dependent reduction in the rate of DeS activity by 34% and 80% at cytostatic and cytotoxic Dox, respectively (Fig. 3A, Scheme 2). This decrease in DeS activity resulted from significantly decreased very long chain ceramides, while Cer d17:1/16:0 showed no statistically significant change in response to Dox (Fig. 3B–D). Further confirmation of DeS inhibition was observed at later time points of the pulse; after 60 min, the DeS substrate accumulated while the product was largely reduced in response to Dox doses (supplemental Fig. S4). Taken together, these data establish for the first time DeS inhibition as the first major node of de novo SL flux regulated in response to Dox.

Inhibition of DeS activity by Dox

Because the bioactive potential for dhCer is poorly understood and the effects of Dox on DeS have not been reported, we chose to probe DeS function at both doses of Dox to corroborate these results and to possibly gain an understanding of the signaling potential for DeS activity. First, to define the contribution of each of the DeS isozymes, we utilized a direct substrate for DeS activity, C12dhCer-PYR (24). The product of DeS1, d-erythro-2-N-[12’-(1’”-pyridinium)dodecanoyl] sphingosine, could then be monitored using LC/MS. Moreover, utilizing the mass transition of 577.4/79.8 m/z at 90 CE, the hydroxylated product of DeS2 activity could be also monitored. The results showed significant inhibition of both cellular enzymatic activities in response to Dox (Fig. 3E, F). DeS1 inhibition by Dox was further confirmed utilizing an in vitro assay (supplemental Fig. S5). Finally, consistent with the minimal effect of Dox on the gene expression of DEGS1, Dox had no effects on the DeS1 protein (Fig. 3G, supplemental Table S1). Therefore, further interrogation of this understudied enzyme will be required to determine whether posttranslational modifications, translocation, or cofactor availability are responsible for this decrease in activity.

Regulation of complex sphingolipid generation in response to Dox

As established by earlier studies, the delayed incorporation of label into complex SLs requires the ER to Golgi transfer of Cer (30, 31). Synthesis rates for complex SLs (SM and HexCer) were monitored between 60 and 120 min. The rate of d17HexCer flux was reduced by 67% at the cytostatic dose of Dox and further reduced by 85% at the cytotoxic dose (Fig. 4A, Scheme 2). Unlike HexCer, d17SM synthesis rates were reduced at low-dose Dox by 52% of the initial rate but only 42% at the cytotoxic dose (Fig. 4). As seen previously, SM d17:1/16:0 accounted for the majority of the data (not shown) (22, 32).

Monitoring the flux through both of these synthetic pathways relies on DeS activity in the ER to generate the d17Cer substrate. As our data demonstrated a significant inhibition of DeS, it became important to tease out the effects of Dox on de novo synthesis past the DeS step. To this end, we used NBD-C6Cer, a direct substrate for the generation of complex SLs, to corroborate dose-dependent responses of these pathways independently from the activity of DeS. HexCer synthesis demonstrated a dose-dependent inhibition with a maximum effect (79%) at the cytotoxic dose (Fig. 4C). The formation of NBD-C6SM, on the other hand, exhibited no changes at the cytotoxic dose but was inhibited at the cytostatic dose (Fig. 4D), demonstrating a major component of SL metabolism that is differentially regulated between cytostatic and cytotoxic Dox. These results suggest Dox exerts separate and distinct effects on Cer formation and complex SLs synthesis.

Next, rates for sphingoid bases and phosphates were monitored in response to Dox under pulse conditions.

Scheme 1. SL enzymatic phases for incorporating d17dhSph into SL metabolism. Cells were pulse-labeled with 500 nM d17dhSph, and the incorporation into SL metabolites was assessed using HPLC/MS/MS. CerS phase: CerS activity was assessed within the first 15 min of label addition by monitoring the formation of d17dhCer. DeS phase: DeS activity was monitored by measuring the formation of d17Cer between 20 and 60 min into the pulse. HexCer or SM synthesis phase: HexCer or SM synthesis was monitored by measuring the formation of d17HexCer or d17SM, respectively, between 60 and 120 min into the pulse.

Regulation of the sphingolipid network by doxorubicin 823
Label incorporation into d17dhS1P, the result of SK activity, increased rapidly at both doses and remained steady at roughly double the concentration as the vehicle in both doses throughout the pulse (Fig. 5A). This is not surprising, as the previous work (22) establishing the pulse parameters indicated that SK activity occurred very quickly, and thus the pseudo-steady state of labeled d17S1P could be utilized to estimate SK flux, which in this case suggests the activation of SK in the presence of Dox.

In response to Dox, d17Sph formation increased significantly during later time points (60–120 min), but this was significant only in the cytostatic dose (Fig. 5B). Because the formation of Sph requires prior formation of Cer from dh-Sph, these results suggest the activation of a CDase by Dox that was matched by the induction of alkaline ceramidase 2 (ACER2) (supplemental Table S1).

Hydrolytic pathways are activated by the cytotoxic dose of Dox

The acute labeling experiments described above assessed flux through components of the de novo SL pathway into complex SLs. However, hydrolytic pathways of SL metabolism have also been shown to generate Cer in response to cytotoxic stimuli (33). The expression of genes for both SMases and CDases, enzymes central to hydrolytic pathways, indicated regulation under both Dox doses (supplemental Table S1), consistent with prior studies (13, 34).

Accordingly, a chase utilizing d17dhSph label was performed to probe the flux through the hydrolytic pathways. Here, MCF-7 cells were pulse-labeled for 12 h to obtain a pseudo-steady state of intracellular d17-labeled complex SLs. At this point, media were changed, and Dox was added 1 h later. d17 SL metabolites were then monitored utilizing LC/MS/MS at relevant time points. The C16:0 species of SLs are presented in Fig. 6, as they were the major Nacyl species of SLs altered in response to Dox treatment. Within 30 min of chase (replacement of labeled media with unlabeled media), both d17dhSph and d17dhCer species were depleted to below detection limits (data not shown), suggesting that the turnover and incorporation of these metabolites are very rapid.

The chase protocol revealed a biphasic loss of SM and HexCer. There was an initial drop in all d17 SL metabolites over the first 12 h (Fig. 6A, B). Dox did not significantly affect the rate of hydrolysis of complex SLs during this period, although as demonstrated in the literature, labeled SM pools were relatively stable, with an estimated half-life of 30 h for SM compared with 22 h for HexCer (calculated utilizing time points between 0 and 32 h from the vehicle) (Fig. 6) (35). In response to Dox, a major peak of Cer d17:1/16:0 as well as d17Sph levels occurred between 24 and 32 h post-Dox treatment, suggesting a hydrolytic pathway as the source of this Cer and Sph (Fig. 6C, D). In turn, this suggests the activation of SMases and CDases by Dox.
which corresponds with mRNA data for these enzymes (supplemental Table S1).

DISCUSSION

Due to the complexity of the SL network, specific molecular tools are needed to probe the interconversion of SL metabolites to determine the importance of discrete phases in synthesis and the localization of subcellular pools of specific species in response to various stimuli, including chemotherapeutics. Here, we deployed d17dhSph in a nearly comprehensive analysis of de novo SL flux at cytostatic and cytotoxic doses of Dox in MCF-7 breast cancer cells. While Dox-induced changes in SL mass, particularly Cer, have been implicated in apoptosis (18, 34), growth arrest (13, 15), and drug resistance (9, 36), our method adds flux dimensions and potentially some spatial context to Dox-induced alterations in SL metabolism, serving to clarify and unify prior results in SL mass measurements as well as reconcile

Fig. 3. DeS inhibition is dose-dependent. MCF-7 cells were treated with either DMSO (vehicle) or 500 or 1,000 nM Dox. After 23.5 h of treatment, 500 nM 17CdhSph was added to the cells. Cells were harvested at relevant time points and analyzed with HPLC/MS/MS. E, F: In situ DeS activity assay in treated cells for 1 h. DeS activity was analyzed with HPLC/MS/MS. G: Western blot analysis from MCF-7 cells treated with Dox. Data are means ± SDs, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 compared with vehicle; #P < 0.05 and ####P < 0.0001 compared with 500 nM Dox.
SL metabolic flux rates in response to Dox. Rates are the calculated slope of label incorporation into the product analyte for a given enzymatic reaction during the time frames established in Figs. 2–5. The CDase enzymatic rates represent time points of the pulse concurrent with label metabolism in the Golgi (60–120 min). Treatments are indicated as follows: vehicle is black, cytostatic dose is blue, and cytotoxic dose is red. Units are pmol labeled product/min. Percentage vehicle is also given. *Rates that are significantly different from vehicle. CERT, ceramide transport protein; VT, vesicular trafficking.

SL changes with gene expression data. In particular, data demonstrated the regulation of sphingomyelin synthase (SMS) and CDase activities only at the cytosstatic dose of Dox. The results also disclosed novel effects of Dox on DeS activity and the de novo synthesis of HexCer and SM, thus providing insight into the regulation of the SL network in response to cytostatic and cytotoxic concentrations of Dox.

Significant research has been devoted to the effects of Dox on SL metabolism with the goal of targeting the SL network to increase the efficacy of Dox and/or reduce off-target effects. However, as these studies are a patchwork of different doses in different cell types, understanding the broader effects of Dox on SL metabolic pathways is difficult. Here, our initial analysis of endogenous SL mass suggested that Dox affected SL metabolism at multiple points (Fig. 1). Notably, some of these changes are consistent with prior studies and concordant with expression array data (supplemental Table S1). Thus, for example, increased generation of S1P and Sph have both been documented in response to the cytosstatic dose of Dox (Fig. 1A, B) (21, 34, 37). Similarly, the accumulation of Cer and induction of neutral SMase2 mRNA levels at the cytosstatic dose are consistent with studies performed by Shamseddine et al. (13). In contrast to this, results with cytotoxic Dox doses were less clear. Although we observed the generation of Cer at higher Dox doses, consistent with prior literature (15, 18), results of gene expression data at cytotoxic doses were less pronounced than the coordinated signaling events occurring at cytostatic doses (supplemental Table S1).

Consequently, results did not immediately suggest a specific regulatory node in the SL network. From this, we concluded that a more dynamic assessment of SL metabolism was required to unify gene expression data with static lipid mass measurements. To this end, we took advantage of our recently developed d17dhSph pulse assay, which, in a single experiment, allows for the dissection of de novo SL metabolism from the point of initial dhCer synthesis through the generation of complex SLs. Results of these pulse experiments revealed that Dox provoked a more complex regulation of the SL network in both the ER and Golgi than previously appreciated.

The incorporation of d17dhSph into d17dhCer during the CerS Phase revealed distinct N-acyl-chain profiles, suggesting differential regulation of the CerS enzymes in response to Dox. Flux of d17dhSph into dhCer d17:0/16:0 exhibited a minor but significant increase in the rate of CerS activity at the cytotoxic dose, likely through the activities of CerS5 and CerS6, which exhibit high preference for C16 fatty acyl chains (38). On the other hand, dhCer d17:0/24:0 and dhCer d17:0/24:1 flux indicated no changes in response to Dox (Fig. 2, Scheme 2). This increase in long-chain CerS activity supports published data indicating a Dox-induced upregulation of CerS5/6 in breast cancer cells (39), although the role of CerSs activation in response to Dox is not clearly defined. Moreover, expression of the CerS genes in our MCF-7 cell line was unchanged in response to Dox (supplemental Table S1), which would point to potential posttranslational activation. Notably, in HL-60 cells at a similar Dox dose, Bose et al. observed that Cer generated during Dox treatment was fumonisin B1 (FB1; CerS inhibitor)-dependent, demonstrating that CerS activity is important for apoptosis (18, 40). However, in the same cell line, Jaffrézou et al. attributed biologic responses to Cer generated from hydrolytic pathways because FB1 failed to reduce Dox-induced Cer and apoptosis (15). These seemingly contradictory results would indicate that de novo-generated Cer is context-dependent and that the activity of CerSs may only partially contribute to the complex generation of Cer in response to Dox. On the other hand, sufficiently prolonged FB1 treatment would suppress the generation of Cer from all SL sources, including hydrolytic pathways, by causing a drop in the mass of SM and HexCer (and hence would suppress Cer generated from their hydrolysis). In such cases, FB1 would not provide a clear distinction between the primary effects of Dox on the activation of CerSs from simply demonstrating that CerSs are required for the generation of SM and glycoSLs whose hydrolysis would result in the accumulation of Cer. Thus, the use of FB1 would help implicate SLs but not define a role for the activation of CerS. It should also be noted that at the time of those studies, they could distinguish dhCer from Cer. In this context, the pulse experiments are useful in establishing a regulatory effect of Dox on CerSs, with current results showing only modest induction of dhCer formation and accompanied by reduced overall de novo Cer formation.

GCS has been identified as playing a large role in Dox resistance because its expression is increased in resistant
Regulation of the sphingolipid network by doxorubicin

The current results demonstrate dose-dependent decreases in endogenous HexCer (Fig. 1E). In a study on GCS involvement in Dox resistance, Liu et al. (9) reported no significant change in GluCer levels in response to 1.7 µM Dox treatment in MCF-7 cells at 24 h, although there was a trend toward decreased GluCer at 48 h. This discrepancy in HexCer levels in response to Dox may arise from the differences in Dox concentration utilized as well as from different methodology: [3H]palmitate labeling resolved by TLC versus LC/MS. In the current study, the inhibition of GCS was apparent in HexCer mass, d17dhSph flux, and mRNA levels in response to Dox may arise from the differences in Dox concentration utilized as well as from different methodology: [3H]palmitate labeling resolved by TLC versus LC/MS. In the current study, the inhibition of GCS was apparent in HexCer mass, d17dhSph flux, and mRNA levels at both cytostatic and cytotoxic levels of Dox (Figs. 1E and 4A, E, supplemental Table S1). Even though for d17dhSph flux measurements DeS inhibition observed at both concentrations of Dox (Fig. 3) could potentially limit labeled substrate from reaching downstream metabolic components such as GCS, the inhibition of GCS activity measured with NBD-C6Cer in intact cells following treatment with Dox (41) confirmed the independent inhibitory effects of the drug on this SL enzyme (Fig. 4C). Furthermore, these results support the d17dhSph flux method in its ability to accurately monitor GCS rates, even with the perturbations of upstream metabolic components.

As previous studies have indicated the involvement of a hydrolytic component to Cer generation in response to Dox (13, 15), a d17dhSph pulse/chase was utilized. There was a significant production of d17Cer and d17Sph between 24 and 28 h after Dox treatment (corresponding to 25–29 h into the chase) that did not correspond to decreases in both complex SL pathway (Fig. 6). Therefore, we were unable to definitively assign the d17Cer generated in the chase by Dox to a particular hydrolytic pathway. Shamseddine et al. (13) implicated neutral SMase2 in MCF-7 growth arrest at the cytostatic dose of Dox, showing a significant increase in neutral SMase2 activity, confirmed here by a 70-fold increase in neutral SMase2 expression at cytostatic doses. On the other hand, Licht et al. (42) coexpressed glucocerebrosidase 1 (GBA1) with MDR1 in PA317

Fig. 4. Dynamic regulation of complex SL synthesis by Dox. MCF-7 cells were treated with DMSO (vehicle) or 500 or 1,000 nM Dox. After 23.5 h of treatment, 500 nM 17CdhSph (A, B) was added to the cells and harvested at relevant time points and analyzed with HPLC/MS/MS, and 1 µM NBD-C6Cer (C, D) was added to the cells for 1 h and detected using excitation and emission wavelengths of 470 and 530 nm, respectively. Data are means ± SDs, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with vehicle.

cells (36), and the knockdown of GCS has been shown to resensitize resistant cells to Dox-induced apoptosis (9).
packaging cells and observed increased sensitivity to Dox, raising the possibility that increased flux in the catabolism of complex glycoSLs could be a component of SL signaling activated to generate cytotoxic Cer. Increased turnover of complex glycoSLs is a distinct possibility, as d17HexCer increased (Fig. 6A), albeit insignificantly, at the same time points and with the same pattern as the d17Cer during the chase. Therefore, the hydrolytic mechanisms responsible for Cer generation in response to Dox will require further investigation, but they likely involve the combined regulation of both of these pathways.

In addition to validating and extending prior findings in a broader metabolic context, this study also demonstrates the utility of our methodology as a tool for discovering overlooked or unknown points of regulation in the SL network. In this context, a clear and novel finding from this

---

Fig. 5. Regulation of sphingoid base flux by Dox. MCF-7 cells were treated with either DMSO (vehicle) or 500 or 1,000 nM Dox. After 23.4 h of treatment, 500 nM 17CdhSph was added to the cells. Cells were harvested at relevant time points and analyzed with HPLC/MS/MS. Data are means ± SDs, n = 3. *P < 0.05 compared with vehicle.

Fig. 6. The effects of Dox on SL hydrolytic pathways. MCF-7 cells were labeled for 12 h with 500 nM 17CdhSph, the media were then changed, and after 1 h either DMSO (vehicle) or 500 or 1,000 nM Dox was added. Cells were harvested at relevant time points and analyzed with HPLC/MS/MS. Data are means ± SDs, n = 3. *P < 0.05, **P < 0.01, and ****P < 0.0001 compared with vehicle.
cytostatic dose (supplemental Fig. S3B) while, rather corroborated a significant reduction in SMS activity at the Golgi and allows in situ SMS activity (48), corroborating the inhibition of SMS enzymes in response to Dox. This is likely not well documented, as endogenous SM levels are not significantly altered by Dox treatment (Fig. 1F), although in a recent study in which Molina-Mora et al. (47) utilized hybrid mathematical modeling of NBD SL incorporation, the label incorporation at 500 nM Dox most closely aligned with the SMS inhibitor D609. Similarly, we observed the inhibition of SM synthesis occurring at the cytostatic dose and much less so at the cytotoxic dose, demonstrating 40-fold ACER2 induction at cytoplastic doses of Dox (supplemental Table S1). As activation of the cDase is only significant at the cytostatic Dox dose, this may represent a pathway for the clearance of apoptotic Cer that is greatly diminished at the cytotoxic dose. Furthermore, as there is a phase of d17Sph generation that is temporally concurrent with d17Sph generation, this suggests that Dox regulates the action of a Golgi Cer (Fig. 5A), very consistent with the Golgi localization of ACER2. Thus, in a single experiment, this approach can be used to determine the increased metabolic rate of a specific Golgi pool of SLs. In the chase phase, the data also suggested the activation of CDases with the formation of d17Sph between 24 and 32 h, although the nature and location of these CDases could be distinct from the CDase activity identified in the pulse.

In conclusion, these studies demonstrate the use of a novel methodology in probing the regulation of SL flux in response to the widely used chemotherapeutic Dox, examining its effects on the dynamics of the SL network and adding context to alterations in SL flux at cytoplastic and cytotoxic doses. In addition to validating prior findings pertaining to CerS, nSMase2, and ACER2, flux data also defined a heretofore unappreciated Dox-regulated SL node at the level of DeS. As this regulation is posttranslational in nature, it would not have been evident from gene expression data. Moreover, the broader effects of Dox on SL metabolism would not have been clear from a static analysis of endogenous lipids. Therefore, while this novel methodology can act to integrate mRNA and endogenous lipid data, it also functions as a powerful new tool for the discovery of overlooked regulatory nodes in SL metabolism.

The authors thank the staff and personnel of the Stony Brook University Lipidomics Core Facility (John Haley, Iztok Miletic, and Rob Rieger) and the Stony Brook University Flow Cytometry Core Facility.
REFERENCES

1. Obeid, L. M., C. M. Linardic, L. A. Karolak, and Y. A. Hannun. 1993. Programmed cell death induced by ceramide. Science. 259: 1769–1771.

2. Van Broeckhoven, J. R., M. J. Lee, R. Menzeleev, A. Oliveira, L. Edsall, O. Cuvillier, D. M. Thomas, P. J. Coopman, S. Thangada, C. H. Liu, et al. 1998. Dual actions of sphingosine-1-phosphate: extracellular through the G1-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. J. Cell Biol. 142: 229–240.

3. Delgado, A., G. Fabrías, C. Bedía, J. Casas, and J. L. Abad. 2012. Sphingolipid modulation: a strategy for cancer therapy. Anticancer Agents Med. Chem. 12: 285–302.

4. Canals, D., and Y. A. Hannun. 2013. Novel chemotherapeutic drugs in sphingolipid cancer research. Handb. Exp. Pharmacol. 215: 211–238.

5. Dimanche-Boitrel, M. T., and A. Rebillard. 2013. Sphingolipids and response to chemotherapy. Handb. Exp. Pharmacol. 216: 73–91.

6. Hannun, Y. A., and C. M. Linardic. 1993. Sphingolipid breakdown products: anti-proliferative and tumor-suppressor lipids. Biochem. Biophys. Acta. 1154: 223–236.

7. Hannun, Y. A., and L. M. Obeid. 2008. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell Biol. 9: 139–150.

8. Sotowich, B., C. Rhein, I. Mileva, R. Ahmad, C. J. Clarke, J. Snider, L. M. Obeid, and Y. A. Hannun. 2018. Identification of an acid sphingomyelinase ceramide kinase pathway in the regulation of the chemokine CCL5. J. Lipid Res. 59: 1219–1229.

9. Liu, Y. Y., T. Y. Han, A. E. Giuliano, and M. C. Cabot. 1999. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. J. Biol. Chem. 274: 1140–1146.

10. Weiss, R. B. 1992. The arachidonoyl-cyclooxygenases: will we ever find a better doxorubicin? Mol. Cancer Res. 41: 1015–1023.

11. Diéras, V. 1997. Review of docetaxel/doxorubicin combination in metastatic breast cancer. Oncology (Williston Park). 11 (Suppl. 8): 31–33.

12. Laurent, G., and J. P. Jaffrézou. 2001. Signaling pathways activated by doxorubicin. Blood. 98: 913–924.

13. Shamseddine, A. A., C. J. Clarke, B. Carroll, M. V. Airola, S. Mohammed, A. Rella, L. M. Obeid, and Y. A. Hannun. 2015. P53-dependent upregulation of neutral sphingomyelinase-2: role in doxorubicin-induced growth arrest. Cell Death Dis. 6: e1947.

14. Vyas, D., G. Laput, and A. K. Vyas. 2014. Chemotherapy-enhanced inflammation may lead to the failure of therapy and metastasis. Onco Targets Ther. 7: 1015–1023.

15. Jaffrézou, J. P., T. Levade, A. Bettaieb, N. Andrieu, C. Bezombes, N. Maestre, S. Vermeersch, A. Rouse, and G. Laurent. 1996. Double actions of sphingomyelinase: extracellular and intracellular. C. R. Acad. Sci. Paris. 323: 229–240.

16. Bielawski, J., J. S. Pierce, J. Snider, B. Rembiesa, Z. M. Szulc, and A. J. Bielawski. 2007. Involvement of dihydrolipoyl dehydrogenase 1 regulated sensitivity to cisplatin is associated with the activation of CREB3L1. Oncotarget. 8: 12952–12960.

17. Boland, M. P., S. J. Foster, and L. A. O’Neill. 1997. Daunorubicin accumulation in p53- deficient cells and associated tumor-derived microvesicles. Oncotarget. 7: 18159–18170.

18. Mullen, T. D., S. Spassieva, R. W. Jenkins, K. Kitatani, J. Bielawska, Y. A. Hannun, and L. M. Obeid. 2016. CHK1 regulates NF-kappaB signaling upon DNA damage in p53-deficient cells and associated tumor-derived microvesicles. Oncotarget. 7: 18440–18457.

19. Lavie, Y., H. Cao, A. Volner, A. Lucci, T. Y. Han, V. Geffen, A. E. Giuliano, and M. C. Cabot. 1997. Agents that reverse multidrug resistance: discovery of complex interregulation of sphingolipid metabolism. J. Lipid Res. 38: 1152–1166.
41. Boldin, S., and A. H. Futerman. 1997. Glucosylceramide synthesis is required for basic fibroblast growth factor and laminin to stimulate axonal growth. *J. Neurochem.* **68**: 882–885.

42. Licht, T., J. M. Aran, S. K. Goldenberg, W. D. Vieira, M. M. Gottesman, and I. Pastan. 1999. Retroviral transfer of human MDR1 gene to hematopoietic cells: effects of drug selection and of transcript splicing on expression of encoded P-glycoprotein. *Hum. Gene Ther.* **10**: 2173–2185.

43. Fabrias, G., J. Munoz-Olaya, F. Cingolani, P. Signorelli, J. Casas, V. Gagliostro, and R. Ghidoni. 2012. Dihydroceramide desaturase and dihydrosphingolipids: debutant players in the sphingolipid arena. *Prog. Lipid Res.* **51**: 82–94.

44. Siddique, M. M., Y. Li, B. Chaurasia, V. A. Kaddai, and S. A. Summers. 2015. Dihydroceramides: from bit players to lead actors. *J. Biol. Chem.* **290**: 15371–15379.

45. Bielawska, A., H. M. Crane, D. Liotta, L. M. Obeid, and Y. A. Hannun. 1993. Selectivity of ceramide-mediated biology: Lack of activity of erythro-dihydroceramide. *J. Biol. Chem.* **268**: 26226–26232.

46. Ternes, P., S. Franke, U. Zahringer, P. Sperling, and E. Heinz. 2002. Identification and characterization of a sphingolipid delta 4-desaturase family. *J. Biol. Chem.* **277**: 25512–25518.

47. Molina-Mora, J. A., M. Kop-Montero, I. Quiros-Fernandez, S. Quiros, J. L. Crespo-Marino, and R. A. Mora-Rodriguez. 2018. A hybrid mathematical modeling approach of the metabolic fate of a fluorescent sphingolipid analogue to predict cancer chemosensitivity. *Comput. Biol. Med.* **97**: 8–20.

48. Bhabak, K. P., A. Hauser, S. Redmer, S. Banhart, D. Heuer, and C. Arenz. 2013. Development of a novel FRET probe for the real-time determination of ceramidase activity. *ChemBioChem.* **14**: 1049–1052.