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SHORT COMMUNICATION

Novel D-erythro *N*-octanoyl sphingosine analogs as chemo- and endocrine-resistant breast cancer therapeutics

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Abstract
Purpose Resistance to endocrine and chemotherapies remains the primary cause of breast cancer treatment failure. We have synthesized four novel D-erythro *N*-octanoyl sphingosine analogs and catalogued their activity in drug-sensitive (MCF-7), endocrine-resistant (MDA-MB-231) and chemoresistant (MCF-7TN-R) breast cancer cells.
Methods 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability; colony assay was performed to determine effects on clonogenic survival and ^{1}H NMR, ^{13}C NMR, HPLC spectra and elemental analytical data analyses were used to determine analog identity and purity.
Results All four analogs inhibited both viability and clonogenic survival, with analog C exhibiting a log-fold improvement in anti-survival activity compared to the parent compound.
Conclusion With resistance to current breast cancer chemotherapies on the rise, the development of novel therapeutic targets is of growing importance. Our results show that lipid analogs have therapeutic potential in treating chemo- and endocrine-resistant breast cancer.

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Introduction

Breast cancer remains the most commonly diagnosed cancer in women resulting in 192,370 estimated new cases and 40,610 deaths in 2009 [1]. Resistance to endocrine and chemotherapy is the major cause of treatment failure in the clinic. Dysregulation of sphingolipid metabolism has been shown to be an important mechanism of drug resistance in breast cancer, specifically decreased endogenous and chemotherapy-induced levels of the lipid ceramide [2–5].

Ceramide is known to mediate a wide range of cellular activities including proliferation, apoptosis, differentiation, senescence and survival in many human cancers, including breast and prostate cancer [6–10]. From a clinical perspective, development of therapeutic agents aimed at the sphingolipid pathway may improve patient response rates in chemoresistant and endocrine therapy-resistant breast cancer. Common breast cancer chemotherapies, such as paclitaxel, doxorubicin and etoposide, all increase ceramide as a mechanism of inducing apoptosis and inhibiting cancer growth. As a result, pharmacological targeting of the active sphingolipid ceramide is of growing interest.

Recently, our laboratory showed that specific alterations in the backbone of D-erythro *N*-octanoyl sphingosine analogs, also known as C8-ceramide, increased the potency of C8-ceramide and increased its effectiveness as a breast cancer therapeutic [11, 12]. In this study, we used the short-chain ceramide D-erythro *N*-octanoyl sphingosine as the lead compound in developing novel breast cancer therapeutics. Endogenous ceramide is metabolized into

sphingosine, which is then rapidly phosphorylated into sphingosine-1-phosphate, a prosurvival lipid [11]. To prevent ceramide from being degraded into sphingosine, modifications have been carried out at the amide group where the ceramidase-catalyzed hydrolysis reaction occurs. Two strategies were used for the modification of the ceramide structure. Strategy I: Cycloalkyl group (analog A) or branched alkyl group (analog D) were introduced as substituents on the amide group replacing the flexible heptyl chain to enhance the rigidity and steric hindrance and shield this hydrolysis site. Strategy II: The amide functional group was replaced with an amine functional group (analog B) or an imine functional group (analog C) to prevent amide hydrolysis by the enzyme ceramidase.

Materials and methods

Cell culture

Human cancer cell lines derived from breast, MCF-7, MDA-MB-231 and MCF-7TN-R were cultured in 75-cm^2 culture flasks in DMEM (Invitrogen, Co.) supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD, USA), basic minimum MEM essential (50×, Invitrogen Co.) and MEM non-essential (100×, Invitrogen, Co.) amino acids, sodium pyruvate (100× Invitrogen Co.), antimycotic–antibiotic (10,000 U/ml penicillin G sodium; 10,000 μg/ml streptomycin sulfate; 25 μg/ml amphotericin B as Fungizone®) and human recombinant insulin (4 mg/ml, Invitrogen Co). Culture flasks were maintained in a tissue culture incubator in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

MTT viability assay

The effect of ceramide and ceramide analogs on cell growth was determined using MTT viability assays. MCF-7, MDA-MB-231 or MCF-7TN-R cells were plated at 7.5×10^5 cells per well in a 96-well plate in phenol-free DMEM supplemented with 5% FBS and allowed to adhere overnight. Cells were then treated with analogs A, B, C or D (ranging from 0.1 to 100 μM) for 24 h. Following treatment, 20 μl of MTT (5 mg/ml) reagent was added to each well prior to incubation for 4 h. Cells were then lysed with 20% SDS in 50% dimethylformamide. The pH and absorbances were read on an ELx808 Microtek plate reader (Winooski, VT, USA) at 550 nm, with a reference wavelength of 630 nm.

Clonogenic survival assay

MCF-7, MDA-MB-231 or MCF-7TN-R cells were plated in 6-well plates at 1,000 cells per well. Twenty-four hours later, cells were treated with varying concentrations of ceramide analog and then monitored microscopically for colony growth. Ten days later, the cells were fixed with 3% glutaraldehyde. Following fixation for 15 min, the plates were washed and stained with a 0.4% solution of crystal violet in 20% methanol for 30 min, washed with PBS and air-dried. Colonies of ≥30 cells were counted as positive. Results are normalized to percent clonogenic survival from untreated control cells. Statistical analysis of IC_{50} values was calculated from concentration–response curves using GraphPad Prism 5.0 (Graphpad Software, San Diego, CA, USA) and the equation:

$$Y = \text{bottom} + (\text{top} - \text{bottom})/1 + 10\,\text{logEC50} - X.$$

Statistical analysis

Statistical analysis of IC_{50} values was calculated from concentration–response curves using GraphPad Prism 5.0 (Graphpad Software, San Diego, CA, USA), using the equation:

$$Y = \text{bottom} + (\text{top} - \text{bottom})/1 + 10\text{logEC50} - X.$$

Assuming a standard slope, where the response goes from 10 to 90% of maximal as X increases over two log units. Differences in IC_{50} were compared using Student's unpaired t test with $P < 0.05$ as the limit of statistical significance. Experiments comparing multiple concentrations to the control were tested with one-way ANOVA with Bonferroni post-test to compare individual concentrations. All statistical analysis were performed using GraphPad Prism 5.0.

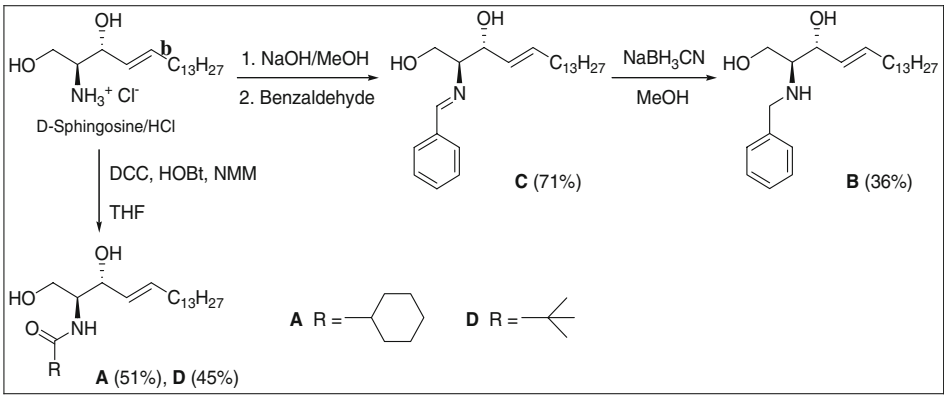
Results

The synthesis of analogs A–D is depicted in Fig. 1. At 0°C, coupling reactions of D-sphingosine/HCl with cyclohexanecarboxylic acid and pivalic acid gave *N*-((2S,3R,E)-1,3-dihydroxyoctadec-4-en-2yl)cyclohexanecarboxamide (analog A) and *N*-((2S,3R,E)-1,3-dihydroxyoctadec-4-en-2-yl)pivalamide (analog D) in 51 and 45% yields, respectively. In the presence of sodium hydroxide, D-sphingosine/HCl went through a condensation reaction with benzaldehyde in methanol giving (2S,3R,E)-2-((E)-benzylideneamino) octadec-4-ene-1,3-diol (analog C) in 71% yield, which was then reduced by sodium cyanoborohydride to give (2S,3R,E)-2-(benzylamino)octadec-4-ene-1,3-diol (analog B) in 36% yield. These results show that all of these reactions occurred in mild conditions, and ceramide analogs were produced with acceptable yields.

The four analogs were screened for their effects on viability of drug-sensitive (MCF-7), endocrine therapy-resistant (MDA-MB-231) and chemotherapy-resistant

Fig. 1 **a** Structures of D-erythro *N*-octanoyl sphingosine analogs. The IC_{50} values (μM) for **b** viability assays and **c** clonogenic survival assays were calculated from experiments shown in Fig. 2. All values were statistically significant with P values <0.05

a



b

Analog	MDA-MB-231	MCF-7TN-R	MCF-7
A	5.4	2.4	22.6
B	3.7	3.0	6.3
C	4.8	3.6	9.0
D	8.0	4.2	13.5

c

Analog	MDA-MB-231	MCF-7TN-R	MCF-7
A	1.6	0.7	1.5
B	1.1	0.6	1.5
C	1.4	0.1	1.7
D	0.5	0.6	1.5

(MCF-7TN-R) breast cancer cells (Fig. 2a). Cells were treated with various concentrations of an analog (0.1–100 μM) for 24 h, treated with MTT for 4 h and then solubilized with SDS in DMF. As seen in Fig. 1b, all four analogs displayed biological activity in both drug-resistant and drug-sensitive breast cancers. Interestingly, no analog had the lowest IC_{50} in all three cell lines. Analog A was the most effective in MCF-7TN-R with an IC_{50} value of 2.4 μM. Analog C was the most potent of the analogs in MCF-7 and MDA-MB-231 cells, with IC_{50} values of 6.3 and 3.7 μM, respectively.

The low micromolar efficacy of these analogs on viability led us to examine the effects of the analogs on long-term clonogenic survival (Fig. 2b). Cells were plated and treated with varying concentrations of analogs (0.1–100 μM) for 10 days and then monitored microscopically for colony growth. Cells were fixed with 3% glutaraldehyde and stained with a 0.4% solution of crystal violet in 20% methanol before colonies were counted. The four analogs displayed increased efficacy in blocking survival compared to viability in all three breast cancer cell lines. Interestingly, the analogs were most effective in the chemoresistant MCF-7TN-R cell line, with analog C displaying an IC_{50} of 0.1 μM.

In summary, we found that the four novel ceramide analogs were effective in diminishing chemoresistant and endocrine-resistant breast cancer cell viability and survival, with analogs A and C displaying the lowest IC_{50} values for these assays, respectively. Analog C is substantially more effective than the other analogs in decreasing chemoresistant breast cancer cell survival. This increased efficacy may be due to the presence of the imine functional group in analog C. This functional group is believed not to be hydrolyzed by the enzyme ceramidase, effectively blocking the production of sphingosine and preventing ceramide analog depletion. Compared to previously published ceramide analogs in our laboratory with varying backbones, these analogs display increased efficacy compared to their counterparts [11]. The clonogenic survival IC_{50} values of analogs A, B, C and D were significantly lower than their counterparts with similar substitutions but amide backbones, suggesting that the structural changes to the short-chain ceramide backbone in these analogs confer an increased efficacy in drug-resistant breast cancer cell lines

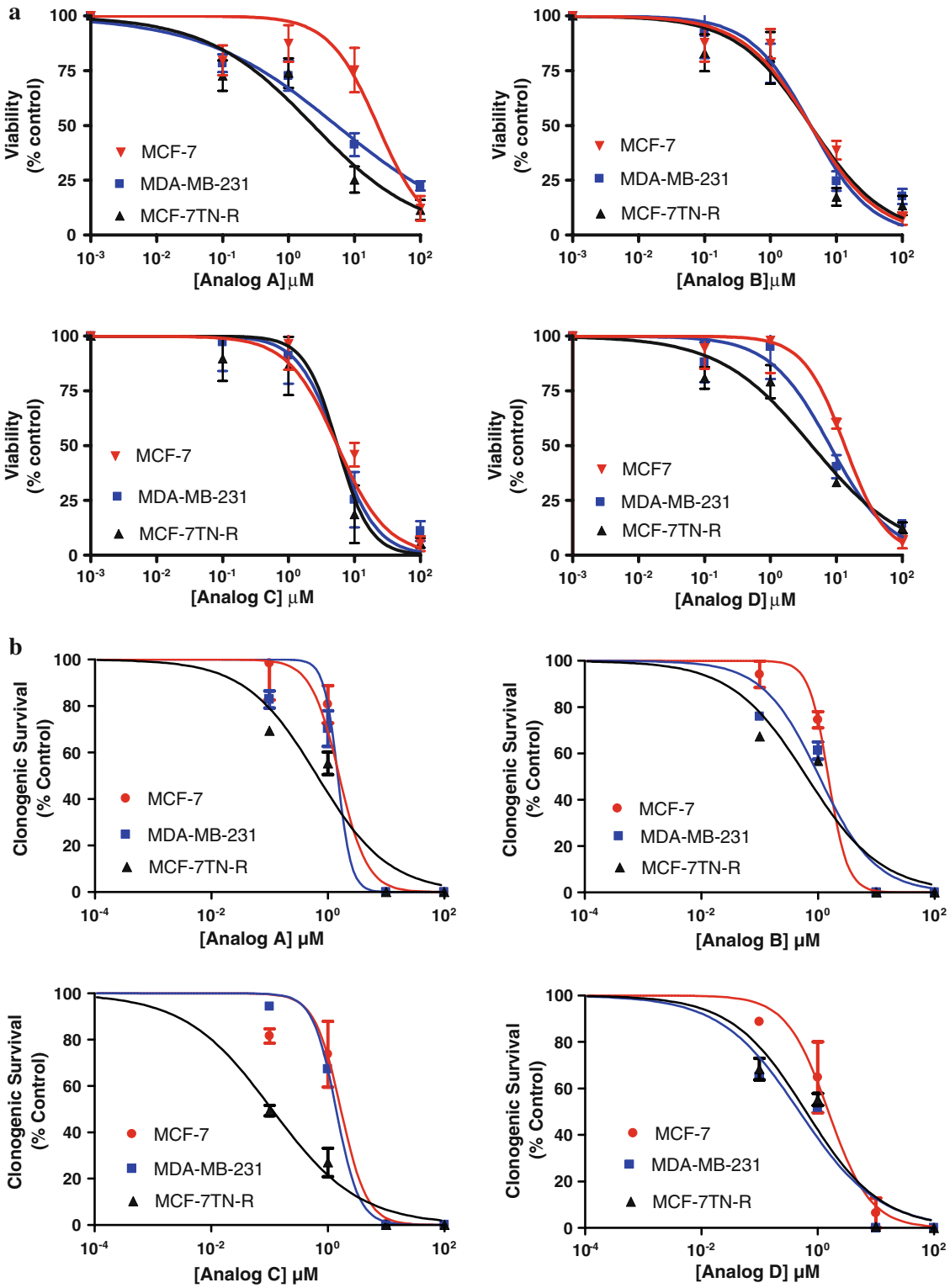


Fig. 2 **a** Effect of analogs on breast cancer viability. MCF-7, MDA-MB-231 and MCF-7TN-R cells were treated with varying concentrations of analog (0.1–100 μM) for 24 h. The values are the mean of ±SE of four independent experiments. **b** Effect of ceramide analogs on breast cancer clonogenic survival. MCF-7, MDA-MB-231 and MCF-7TN-R cells were treated with varying concentrations of analog (0.1–100 μM) for 10 days. The values are the mean of ±SE of three independent experiments

[11]. The major cause of treatment failure in breast cancer is the development of resistance to first-line and second-line treatment regiments. These novel analogs suggest that ceramide-based therapy has potential in treating drug-resistant forms of breast cancers.

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Conflict of interest statement None.

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