Bioorg. Med. Chem. 32 (2021) 116011

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of *erythro*- B13 enantiomers and stereospecific action of full set of B13-isomers in MCF7 breast carcinoma cells: Cellular metabolism and effects on sphingolipids

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ARTICLE INFO

Keywords:
B13
Chiral center
Sphingolipid
Metabolites
Ceramide

ABSTRACT

B13 is an acid ceramidase (ACDase) inhibitor. The two chiral centers of this aromatic amido alcohol lead to four stereoisomers, yet we have little knowledge about its *erythro*- enantiomers, (1R, 2S) and (1S, 2R). In this paper, for the first time, the synthesis of two *erythro*- enantiomers is described, and the compounds are evaluated along with two *threo*- enantiomers, (1R, 2R) and (1S, 2S). The key metabolites and sphingolipid (SL) profile of the full set of B13 stereoisomers in MCF7 breast carcinoma cells are presented. The results demonstrated that the *erythro*- enantiomers were more effective than the *threo*- enantiomers on growth inhibition in MCF7 cells, although there were no statistically significant differences within the *threo*- and *erythro*- series. Measurement of intracellular levels of the compounds indicated that the *erythro*- seemed a little more cell permeable than the *threo*- enantiomers; also, the (1R, 2S) isomer with the same stereo structure as natural ceramide (Cer) could be hydrolyzed and phosphorylated in MCF7 cells. Furthermore, we also observed the formation of C16 homologs from the full set of B13 isomers within the cells, indicating the occurrence of de-acylation and re-acylation of the amino group of the aromatic alcohol. Moreover, the decrease in the Cer/Sph ratio suggests that the growth inhibition from (1R, 2S) isomer is not because of the inhibition of ceramidases. Taken together, (1R, 2S) could be developed as a substitute of natural Cer.

1. Introduction

B13, N-((1R, 2R)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl)tetradecanamide, also named D-NMAPPD, was first synthesized in 1992, and it was later found to inhibit acid ceramidase (ACDase).[1–2] B13 is a typical aromatic sphingolipid (SL) analog, contains all features that natural SL contains, except that the two chiral centers are (1R, 2R) [*threo*- isomer], which differs from natural ceramide [Cer, (2S, 3R), *erythro*- isomer]. (1R, 2R) is an ACDase inhibitor, and its *in vitro* IC_{50} can achieve 20 μM when the cell lysate is used as enzyme source under acidic assay conditions.[3] Notably, (1R, 2R) has been shown to induce apoptosis in many cancer cell lines. Moreover, *in vivo* studies have shown that tumor growth was completely prevented when nude mice were treated with (1R, 2R) while bearing aggressive human colon cancer cells that had already metastasized to the liver.[4] Also, in other animal studies, (1R, 2R) has been used in combination with other therapies to achieve better tumor shrinkage. Since then, one publication mentioned chiral centers of B13,[5] however, that study only examined two *threo*- enantiomers, and found that (1R, 2R) was more effective in LNCaP whereas (1S, 2S) was more potent in PC3 prostate cancer cells. LNCaP cells are androgen-sensitive prostate adenocarcinoma cells and have low metastatic potential, whereas PC3 does not respond to androgen deprivation, glucocorticoids or fibroblast growth factors, and has high metastatic potential. Therefore (1S, 2S) seems a more preferable option to shrink a highly metastatic prostate tumor. Indeed, that paper also presented preliminary *in vivo* studies, showing that (1S, 2S) sensitized tumors to

Abbreviations: B13, N-((1R, 2R)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl) tetra-decanamide; ACDase, acid ceramidase; CDase, ceramidase; SL, sphingolipid; PCC, pyridinium chlorochromate; Cer, ceramide; Sph, sphingosine; UHPLC-MS/MS, ultra performance liquid chromatography - tandem mass spectrometer; D-e-MAPP, N-((1S, 2R)-1-hydroxy-1-phenylpropan-2-yl)tetradecanamide; DES, dihydroceramide desaturase; TLC, thin layer chromatography; ESI, electrospray ionization; MRM, multiple reaction monitoring.

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https://doi.org/10.1016/j.bmc.2021.116011
Received 30 October 2020; Received in revised form 22 December 2020; Accepted 28 December 2020
Available online 8 January 2021

radiation in a xenografted PC3 mouse model, resulting in reduction of both tumor volume and tumor weight.

Our group and others have made various structural modifications on B13;[6–8]; nevertheless, changes were all made based on the B13′s *threo*- enantiomers: (1R, 2R) or (1S, 2S). Therefore, in this paper, we focus on the synthesis of B13′s *erythro*- enantiomers: (1R, 2S) and (1S, 2R), and the evaluation of full set of B13 stereoisomers' SL profile and cytotoxicity in MCF7 cells.

2. Results and discussion

2.1. Synthesis of erythro- B13

The synthesis of *threo*- B13 was published previously.[1] The general approach for synthesis of *erythro*- B13 starts from *threo*- B13. Therefore, (1R, 2S) B13 was synthesized from (1S, 2S) B13, and (1S, 2R) B13 was synthesized from (1R, 2R) B13 (1, Scheme 1). In detail, trityl chloride was used to protect the primary hydroxyl group. Then (1R, 2R)-3-O-trityl B13 (2a) was oxidized by pyridinium chlorochromate (PCC) to produce (1R, 2R)-3-O-trityl-1-oxo-B13 (3a). Subsequently, (1R, 2R)-3-O-trityl-1-oxo-B13 (3a) was reduced by sodium borohydride to generate (1R, 2R)-3-O-trityl-B13 (2a) and (1S, 2R)-3-O-trityl-B13 (4a). After flash column chromatography separation, purified (1S, 2R)-3-O-trityl B13 (4a) was de-protected under acidic condition to yield (1S, 2R) B13 (5a). NMR, optical rotation, and MS data were used to confirm all newly synthesized isomers. After 2 times flash column chromatography separation, (1S, 2R) and (1R, 2S) B13 isomers were purified as waxy white solids, with a total yield of 11.3% and 12.3% and with no additional synthetic optimization.

2.2. Inhibitory effects of the full set of B13 isomers on MCF7 cell growth

First, we determined the profile of B13 isomers' cytotoxicity in MCF7 cells. The results indicated that there was little difference within *threo*- [(1R, 2R) *vs* (1S, 2S)] and *erythro*- [(1S, 2R) *vs* (1R, 2S)] enantiomers; but they did show 3–5 fold differences between *threo*- and *erythro*- enantiomers, with *erythro*- isomers being more effective than *threo*- isomers on MCF7 cells growth inhibition (MTT assay, Fig. 1 and Table 1). The sequence of IC_{50} values in MCF7 cells is (1S, 2S) > (1R, 2R) ≫ (1S, 2R)> (1R, 2S).

2.3. Cell uptake and key metabolites of full set of B13 isomers in MCF 7 cells

Next, we measured the cellular level of full set of B13 isomers to investigate whether the difference in cytotoxicity was related to the difference in their cell permeability. Our results showed that after 1 h incubation, the cellular level of all isomers increased in a dose dependent pattern, especially for *erythro*- enantiomers at the high dose [Fig. 2**A**]. Interestingly, when we prolonged the incubation time (24 h), three of the four isomers were maintained at a level similar to 1 h, yet the cellular level of (1R, 2S) B13 dropped dramatically [Fig. 2**B**].

Because (1R, 2S) B13 has a stereo-structure comparable to Cer [Fig. 3**A and B**], we then explored whether (1R, 2S) B13 also functioned as a substrate for ceramidases (CDases) and therefore could be hydrolyzed and further metabolized in cells. This pathway has been demonstrated with exogenous C6-Cer in A549 cells.[9] In MCF7 cells, treatment with 17C6-Cer results in the formation of the free sphingoid base, 17Sph, indicating the operation of CDase. This is also accompanied by

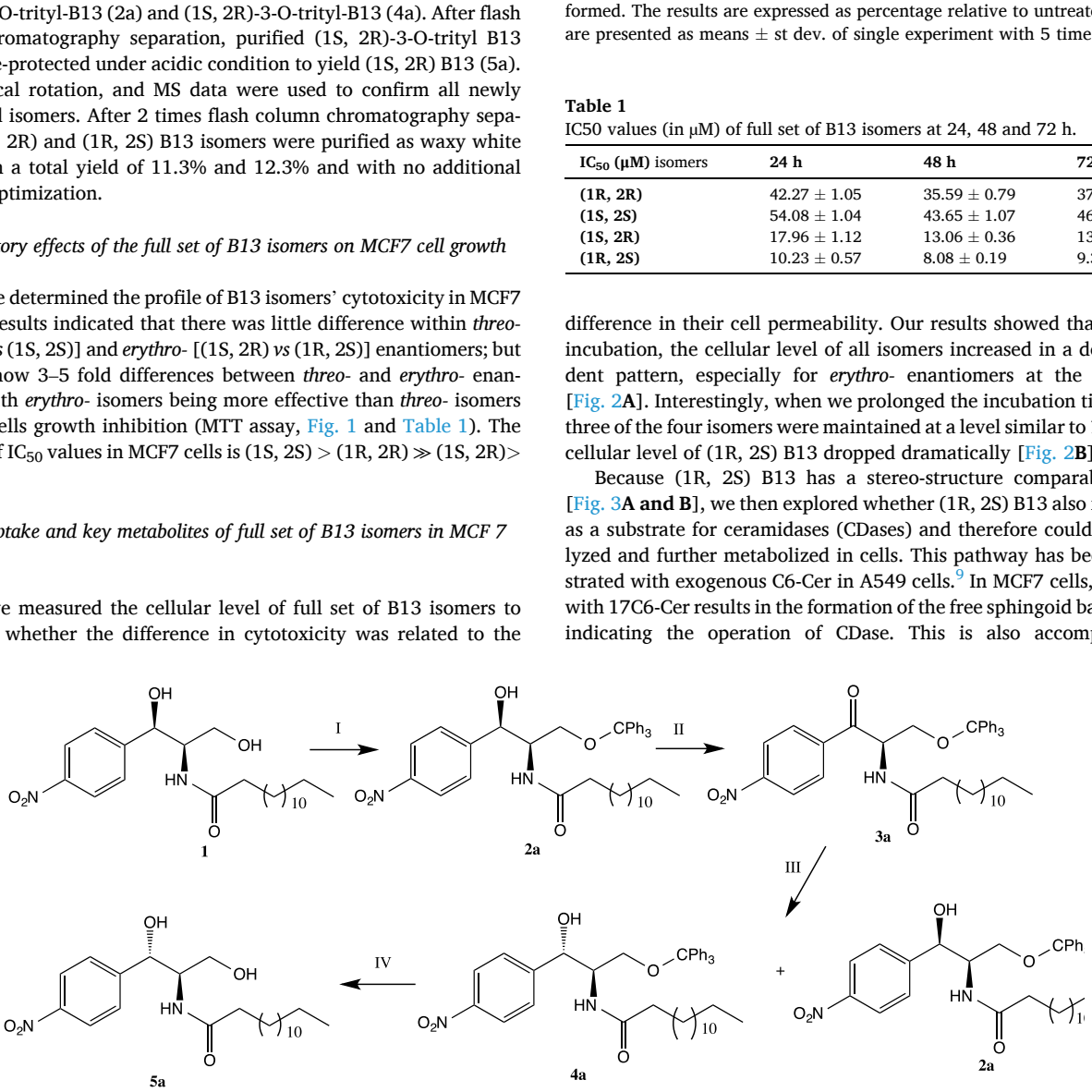

Fig. 1. Cytotoxicity of full set of B13 isomers in MCF7 viable cell analysis at 48 h. MCF7 cells were treated with vehicle, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100uM of different B13 isomers for 48 h and then MTT assays were performed. The results are expressed as percentage relative to untreated cells and are presented as means ± st dev. of single experiment with 5 time replicates.

Table 1
IC50 values (in μM) of full set of B13 isomers at 24, 48 and 72 h.

IC_{50} (μM) isomers	24 h	48 h	72 h
(1R, 2R)	42.27 ± 1.05	35.59 ± 0.79	37.01 ± 0.91
(1S, 2S)	54.08 ± 1.04	43.65 ± 1.07	46.63 ± 1.30
(1S, 2R)	17.96 ± 1.12	13.06 ± 0.36	13.51 ± 2.49
(1R, 2S)	10.23 ± 0.57	8.08 ± 0.19	9.39 ± 0.40

Scheme 1. Synthetic outline for *erythro* B13. Reagents and conditions: (i) trityl chloride, DMAP, Et3N, CH2Cl2, rt; (ii) CrO3.Py, CH2Cl2, 4 °C to rt; (iii) NaBH4, CH3OH, 4 °C to rt; (iv) acetic acid, rt.

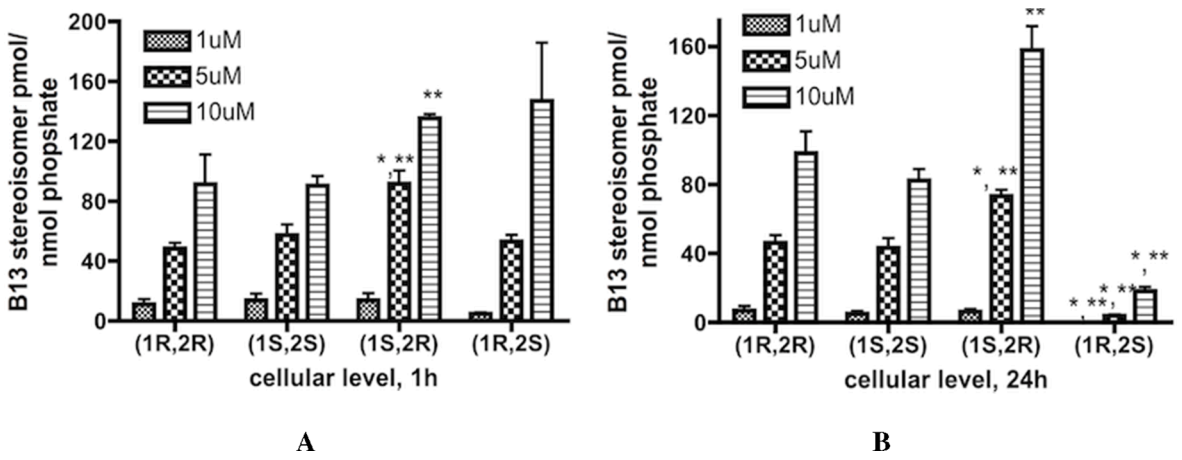

Fig. 2. Intracellular level of full set of B13 isomers after 1 h (**A**) and 24 h (**B**) incubation in MCF7 cells. Cells were treated with 1, 5 and 10uM of different B13 isomers for indicated time before cell pellets were isolated. Cellular levels of each isomer were then quantified by UHPLC-MS/MS analysis of lipid extract from cell pellets. Results are presented as means ± st dev. of triplicates. (*$p < 0.05$ (*vs* (1R, 2R)); ** $p < 0.05$ (*vs* (1S, 2S)).

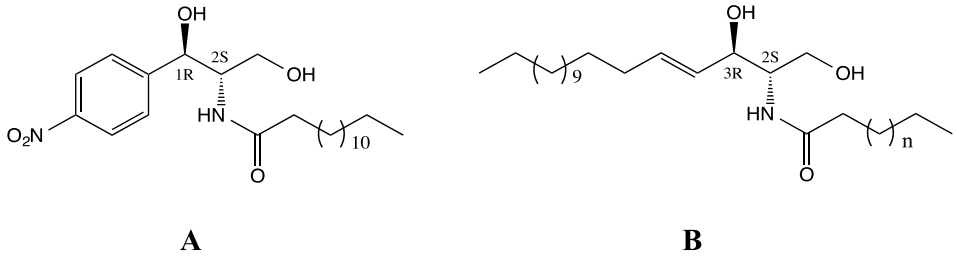

Fig. 3. Structure of (1R, 2S) B13 (**A**) and natural Ceramide (**B**).

formation of Cer with various *N*-acyl species on the 17 sphingoid backbone, with 17C16, 17C24 and 17C24:1-Cer being the three major metabolites produced within 24 h [Fig. 4]. This then illustrates the re-acylation of the 17Sph by the various Cer synthases.[10]

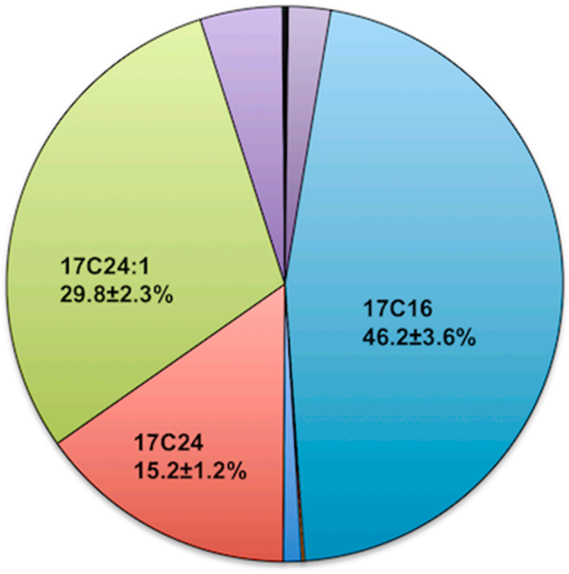

Fig. 4. Major metabolites (>10% of total 17Cn-Cer) of 5uM 17C6-Cer in MCF7 cells at 24 h. Cells were treated with 5uM 17C6-Cer for 24 h before cell pellets were isolated, lipids were extracted and cellular level of 17Cn-Cer were measured by UHPLC-MS/MS approach. Results are expressed as pmol/nmol lipid phosphate, and are presented as % total 17Cn-Cer. All results are presented as means ± st dev. of duplicates.

We therefore examined further the metabolism of the full set of B13 isomers using UHPLC-MS/MS. The results showed that after 24 h incubation, compared to the standards, mainly C16 homologs and a very small amount of the C24:1 homologs were observed for all four isomers [Suppl Fig 1 and Table 1]. UHPLC-MS/MS conditions were the same as previously reported.[11]

To confirm our findings, we also quantified each metabolite (free base, B13-homologs with C16 and C24:1 fatty acyl chain length) [Fig. 5]. Unexpectedly, the results indicated that all isomers could be hydrolyzed and more or less metabolized to form homologs with C16 and C24:1 fatty acyl chain length, but C16- were 10 times more prevalent than C24:1 homologs [Fig. 5**B-C**]. Surprisingly, it was not (1R, 2S) but (1S, 2S) showing relatively high level of these two metabolites. Moreover, we only detected trace amount of the free base hydrolyzed from the parent B13 isomers, and there were no statistical differences among the isomers [Fig. 5**A**], suggesting rapid re-acylation, possibly through the action of Cer synthases.

Since de-acylation/re-acylation appeared to act on all 4 isomers, we searched for additional metabolites, especially for (1R, 2S) that could explain its declining levels in cells, which suggest metabolism to some other compounds. When measuring the phosphorylation of the aromatic SLs, we found that phosphorylated free-base was specific for (1R, 2S) isomer, resulting in the highest phosphorylation among the full set of B13 isomers [Fig. 5**D**]. The data also suggest that free base that released from (1R, 2S) isomer can be a substrate of kinases.

2.4. Effects of the full set of B13 isomers on endogenous SL profile in MCF7 cells

Last, by using the UHPLC-MS/MS, we also measured the key SL profile of the full set of B13 isomers. First, we compared the Cn-Cer profile of different isomers after 10uM treatment [Fig. 6**A-B**].

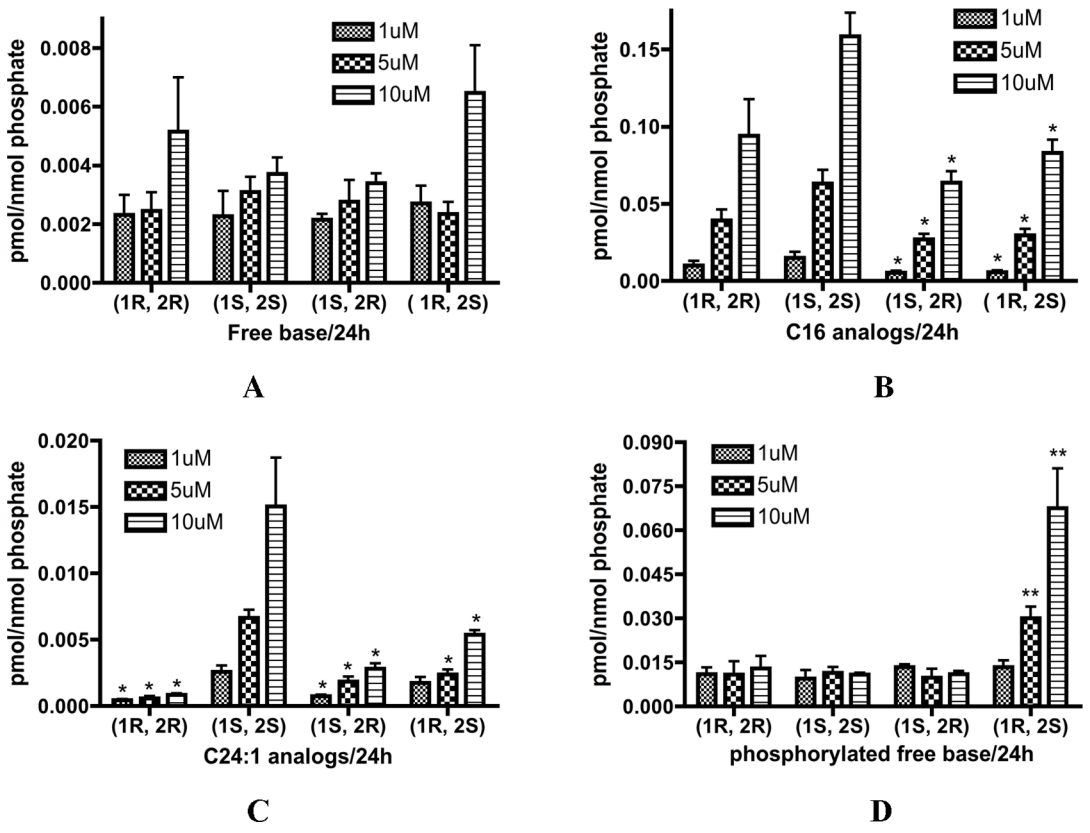

Fig. 5. Intracellular levels of the full set of B13 isomers' key metabolites in MCF7 cells (24 h). Cells were treated with each of 10uM B13 isomer for 24 h before cell pellets were isolated, lipids were extracted, and cellular levels of key metabolites (free base, C16 and C24:1 analogs) were measured by UHPLC-MS/MS approach. Results are expressed as pmol/nmol lipid phosphate, and are presented as means ± st dev. of triplicates. **A**. Free base; **B**. C16 analogs; **C**. C24:1 analogs; **D**. Phosphorylated free base. (* $p < 0.01$ (*vs* (1S, 2S)); ** $p < 0.05$ (*vs* (1S, 2S)).

Interestingly, at 1 h, the elevation of Cn-Cer was only observed for (1R, 2S) B13. Remarkably, the ACDase inhibitor, (1R, 2R) B13, at 10uM which is significantly below its IC50 in MCF7 cells growth, did show specific increases in C14 and 16-Cer, although the decrease was also significant in C24, C26:1 and C26-Cer very long chain Cn-Cer. When we prolonged the incubation time, at 24 h, (1R, 2R) exhibited a similar Cn-Cer profile as (1R, 2S), which clearly elevated long chain Cn-Cer (≤C18), however (1S, 2S) was the only isomer that kept decreasing almost all Cer species, and no specific changes were found with (1S, 2R) isomer. Our results also imply that Cn-Cer showed a response prior to cell killing.

As for the Cn-dhCer profile, after 1 h incubation, the only highly elevated Cn-dhCer was also from (1R, 2S) B13, in particular, the very long fatty acyl chain length were all enhanced [n ≥ 22, Fig. 6**C**]. Yet when we prolonged the incubation time (24 h), the response of Cn-dhCer went to the opposite way. Although both *threo-* isomers showed distinct increase of Cn-dhCer, in particular dhC14, dhC16, as well as all the very long chain Cn-dhCer (n ≥ 22), the responses from *erythro-* isomers were all moved to the control level, in particular (1R, 2S) [Fig. 6**D**]. Our results also suggest that rather than CDases, (1S, 2S) may have inhibitory effects on dihydroceramide desaturase (DES), due to its ability to increase of Cn-dhCer and decrease of Cn-Cer at 24 h.

Our previous study showed that 10uM (1R, 2R) B13 (ACDase inhibitor) increased Cer and somewhat decreased Sph at 24 h in the same cell line, which indicated its inhibitory effects on cellular ACDase.[12] Thus, by using the UHPLC-MS/MS, we also measured endogenous Sph and calculated the Cer/Sph ratio to briefly explore whether we could evaluate a similar or better inhibitory response among the full set of B13 isomers. The results [Fig. 7**A**] showed that except for 10uM (1R, 2S) treatment, all other isomers displayed a dose dependent decrease of endogenous Sph, in particular (1S, 2R) B13, which led to a significant decrease in the level of endogenous Sph at all doses that we selected (1, 5 and 10uM). However, when we calculated the Cer/Sph ratio [Fig. 7**B**], up to the 10uM concentration, only (1R, 2R) B13 showed a slightly elevated ratio that did not achieve statistical significance compared to the control, while other isomers decreased this ratio, thus, indicating much more complex effects on Cer and Sph metabolism, with Cer either being decreased or Sph being increased, or both.

3. Summary and conclusion

Aromatic analogs of SL have been designed and pre-clinically validated as inhibitors of enzymes of SL metabolism because of their structural similarity to natural SL. Moreover, aromatic analogs of SL are UV active, thus are easy to monitor.

To search for novel structures that can mimic the action of natural SL or inhibit the formation of SL, so as to further investigate SL metabolic pathways, our group has developed various sets of aromatic analogs of SL with a structural feature containing phenyl-amino-alcohol. Among them, B13 [N-((1R, 2R)-1,3-dihydroxy-1-(4-nitrophenyl)-propan-2-yl) tetra-decanamide] and D-e-MAPP [N-((1S, 2R)-1-hydroxy-1-phenylpropan-2-yl)tetra-decanamide] were both synthesized by A. Bielawska et al in 1992. Later, B13 was found to inhibit ACDase, while D-e-MAPP inhibits alkaline CDase[13]. Moreover, our group also discovered that L-e-MAPP (1R, 2S), the enantiomer of D-e-MAPP, which has the comparable stereo-structure to natural Cer, was a substrate of alkaline CDase, and it was metabolized by alkaline CDases to an extent similar to what occurred with C16-Cer[13].

In this study, we now report on the synthesis of *erythro-* B13 isomers and determine the action of the full set of B13 isomers on MCF7 breast carcinoma cells. Cell cytotoxicity showed that the *erythro-* pair was more effective than the *threo-* pair on MCF7 cells growth. Interestingly, each of the B13 isomers could be metabolized in the cells forming mainly their

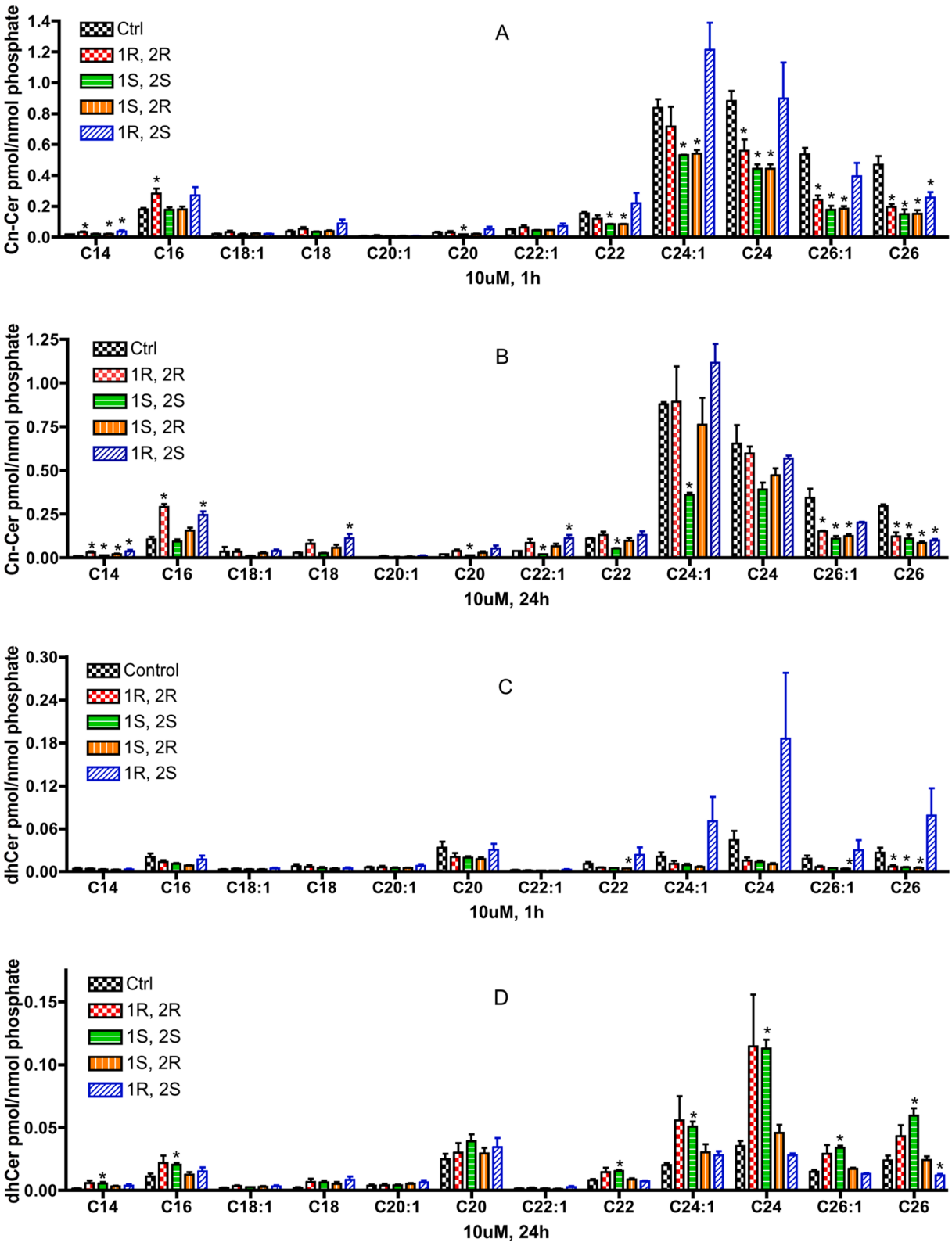

Fig. 6. Cn-Cer profile (**A**. 1 h; **B**. 24 h) and Cn-dhCer profile (**C**. 1 h; **D**. 24 h) of full set of B13 isomers at 10uM. Cells were treated with each of 10uM B13 isomer for the indicated time before cell pellets were isolated, lipids were extracted, and cellular level of Cn-Cer and Cn-dhCer were measured by UHPLC-MS/MS approach. Results are expressed as pmol/nmol lipid phosphate, and are presented as means ± st dev. of triplicates (*$p < 0.05$, *vs* Control).

C16-homologs and a small amount of C24:1-homologs thus acting similarly to 17C6-Cer cellular metabolism. Furthermore, (1R, 2S) B13, with the comparable stereo-structure to the natural Cer, could be hydrolyzed and phosphorylated, and was the most potent on MCF7 cell growth inhibition [Fig. 8]. At this point, we have not identified the kinase that acts on the (1R, 2S) B13. Based on these cumulative findings, we conclude that the (1R, 2S) isomer could be developed as a substitute of natural Cer for cell studies.

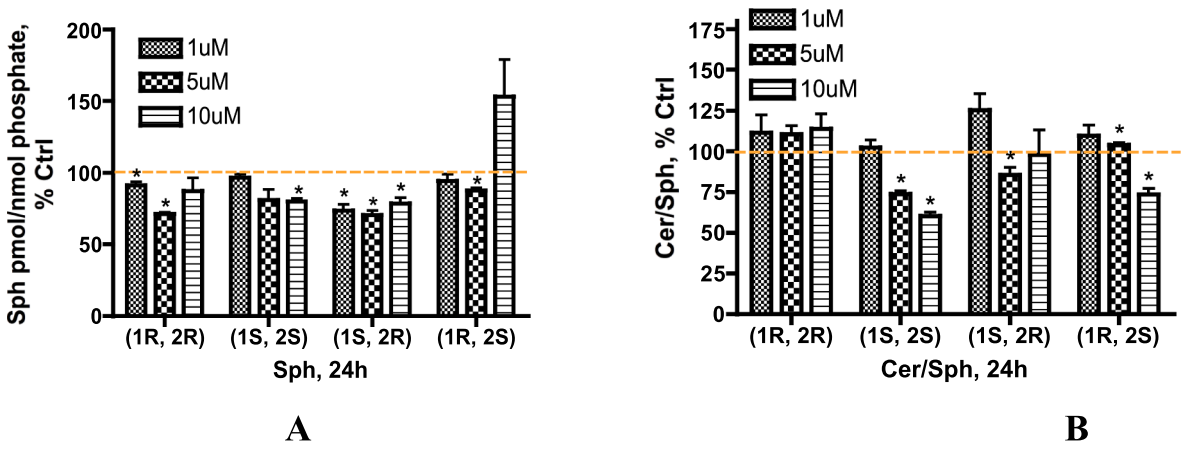

Fig. 7. Dose dependent response of full set of B13 isomers on Sph (**A**) and Cer/Sph ratio (**B**) at 24 h. Cells were treated with 1, 5 and 10uM of each B13 isomer for 24 h before cell pellets were isolated, lipids were extracted and cellular level of Sph was measured by UHPLC-MS/MS approach. Results are expressed as pmol/nmol lipid phosphate, and are presented as means ± st dev. of triplicates (*$p < 0.05$, *vs* Control).

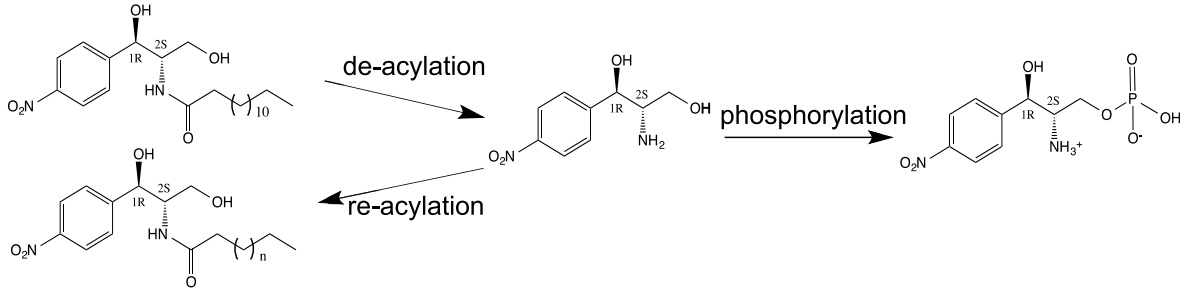

Fig. 8. (1R, 2S) B13′s possible metabolic pathway in MCF7 cells.

4. Methods

4.1. Chemistry

All solvents and general reagents were purchased from Sigma-Aldrich and VWR. Synthetic outline for the preparation of *erythro* B13 was presented in scheme 1. Thin layer chromatography (TLC), ^{1}H NMR, optical rotation, and MS confirmed purity of the synthesized compounds. Detection was performed under ultraviolet light (254 nm). Flash chromatography was performed using EM Silica Gel 60 (230–400 mesh) with the indicated solvent systems. ^{1}H NMR spectra were recorded on Bruker AVANCE 400 MHz spectrometer equipped with Oxford Narrow Bore Magnet; chemical shifts were reported in ppm on the δ scale from the internal standard of residual chloroform (7.27 ppm). Optical rotation data were determined with a JASCO P-1010 polarimeter. Mass spectral data were reported in a positive ion electrospray ionization (ESI) mode on Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer. Samples were infused in methanol with an ESI voltage of 4.0 kV and capillary temperature of 200 °C. Specific structural features were established by fragmentation pattern upon electrospray (ESI/MS/MS) conditions, specific for each of the compound studied.

4.1.1. N-((1R, 2R)-1-hydroxy-1-(4-nitrophenyl)-3-(trityloxy)propan-2-yl) tetra-decanamide (2a) was prepared as previously described.[3]

4.1.2. N-((1S, 2S)-1-hydroxy-1-(4-nitrophenyl)-3-(trityloxy)propan-2-yl) tetra-decanamide (2b).

Crude compound 2b was purified by flush column chromatography at ethyl acetate-hexane, 1:2 (v/v) in yield of 81.8%. R_f 0.34; $[\alpha]_D^{22.3}$ + 27.0 (*C* 1.0, CH_3OH); ^{1}H NMR (400 MHz, $CDCl_3$) 8.109 (d, J = 8.8, 2H), 7.444 (d, J = 8.8, 2H), 7.245–7.394 (m, 15H), 5.965 (d, J = 8.4, 1H), 5.064(d, J = 3.2, 1H), 4.198 (m, 1H), 3.949 (d, J = 3.6, 1H), 3.435 (dd, J = 4.8, 4.8, 1H), 3.328 (dd, J = 3.6, 4.0, 1H), 2.063 (td, J = 7.2, 1.6, 2H), 1.560 (s, 2H), 1.460 (m, 2H), 1.127–1.3 (m, 18H), 0.864 (t, J = 7.2, 3H). ESI-MS (CH_3OH, relative intensity, %): *m/z* 687.13 ($[MNa]^+$, 100). Calcd. for C42H52N2O5 *m/z* 664.39 [M]; C42H52N2NaO5, *m/z* 687.38 [MNa].

4.1.3. (2R)-N-(1-(4-nitrophenyl)-1-oxo-3-(trityloxy)propan-2-yl)tetradecanamide (3a) was prepared from 2a, crude product 3a was purified by flush column chromatography at ethyl acetate-hexane, 1:2 (v/v) in yield of 58% as colorless oil. R_f 0.47; ^{1}H NMR (400 MHz, $CDCl_3$) 8.185 (d, J = 8.8, 2H), 7.917 (d, J = 9.2, 2H), 7.147 (m, 15H), 6.739 (d, J = 7.2, 1H), 5.599 (m, 1H), 3.572 (dd, J = 5.2, 3.6, 1H), 3.365 (dd, J = 4.0, 4.0, 1H), 2.296 (t, J = 7.6, 2H), 1.661 (m, 2H), 1.242 (m, 20H), 0.865 (t, J = 6.8, 3H). ESI-MS (CH_3OH, relative intensity, %): *m/z* 685.17 ($[MNa]^+$, 100). Calcd. for C42H50N2O5 *m/z* 662.37 [M]; C42H50N2NaO5, *m/z* 685.36 [MNa].

4.1.4 (2S)-N-(1-(4-nitrophenyl)-1-oxo-3-(trityloxy)propan-2-yl)tetradecanamide (3b) was prepared from 2b, crude product 3b was purified by flush column chromatography at ethyl acetate-hexane, 1:3 (v/v) in yield of 45.4% as colorless oil. R_f 0.30; ^{1}H NMR (400 MHz, $CDCl_3$) 8.176 (d, J = 7.2, 2H), 7.893 (d, J = 7.2, 2H), 7.145 (m, 15H), 6.774 (d, J = 7.6, 1H), 5.591(m, 1H), 3.574 (dd, J = 3.6, 3.2, 1H), 3.355 (dd, J = 4.0, 3.6, 1H), 2.301 (t, J = 7.2, 2H), 1.665 (m, 2H), 1.243 (m, 20H), 0.865 (t, J = 6.8, 3H). ESI-MS (CH_3OH, relative intensity, %): *m/z* 685.03 ($[MNa]^+$, 100). Calcd. for C42H50N2O5 *m/z* 662.37 [M]; C42H50N2NaO5, *m/z* 685.36 [MNa].

4.1.5. N-((1S, 2R)-1-hydroxyl-1-(4-nitrophenyl)-3-(trityloxy) propan-2-yl)tetra-decanamide (4a) was prepared from 3a, crude product 4a was purified by flush column chromatography at ethyl acetate-hexane, 1:4 (v/v) in yield of 37% as waxy oil. R_f 0.16; $[\alpha]_D^{24.6}$ −9.5 (*C* 1, CH_3OH), ^{1}H NMR (400 MHz, $CDCl_3$) 8.02 (dd, J = 6.8, 2.4, 2H), 7.307 (m, 17H), 5.952 (d, J = 8.0, 1H), 5.038(dd, J = 3.2, 3.2, 1H), 4.456 (d, J = 7.6, 1H), 4.379 (m, 1H), 3.267 (dd, J = 4.8, 4.8, 1H), 3.097 (dd, J =

4.0, 3.2, 1H), 2.207 (t, J = 7.6, 2H), 1.622 (m, 2H), 1.242 (m, 20H), 0.863 (t, J = 6.8, 3H). ESI-MS (CH_3OH, relative intensity, %): *m/z* 687.20 ($[MNa]^+$, 100). Calcd. for C42H52N2O5 *m/z* 664.39, [M]; C42H52N2NaO5, *m/z* 687.38 [MNa].

4.1.6 N-((1R, 2S)-1-hydroxyl-1-(4-nitrophenyl)-3-(trityloxy)propan-2-yl)tetra-decanamide (4b) was prepared from 3b, crude product 4b was purified by flush column chromatography at ethyl acetate-hexane, 1:4 (v/v) in yield of 39.3% as waxy oil. R_f 0.16; $[\alpha]_D^{22.4}$+8.0 (*C* 1, CH_3OH); ^1H NMR (400 MHz, $CDCl_3$) 8.017 (dd, J = 7.2, 2.0, 2H), 7.332–7.237 (m, 17H), 5.964 (d, J = 8.0, 1H), 5.041(m, 1H), 4.46 (d, J = 7.6, 1H), 4.388 (m, 1H), 3.263 (dd, J = 5.2, 4.8, 1H), 3.101 (dd, J = 4.0, 4.0, 1H), 2.204 (t, J = 6.8, 2H), 1.63–1.242 (m, 22H), 0.863 (t, J = 6.8, 3H). ESI-MS (CH_3OH, relative intensity, %): *m/z* 687.13 ($[MNa]^+$, 100). Calcd. for C42H52N2O5 *m/z* 664.39 [M]; C42H52N2NaO5 *m/z* 687.38 [MNa].

4.1.7. N-((1S, 2R)-1,3-dihydroxy-1-(4′-nitrophenyl)-propan-2-yl) tetradecanamide ((1S, 2R) B13; 5a) was prepared from 4a, crude product 5a was purified by flush column chromatography at chloroform–methanol, 4:1 (v/v) in yield of 61.4% as waxy solid. R_f 0.31; $[\alpha]_D^{22.7}$ −6.5 (*C* 0.25, CH_3OH); ^1H NMR (400 MHz, $CDCl_3$) 8.229 (d, J = 8.8, 2H), 7.603 (d, J = 8.8, 2H), 6.312 (d, J = 8.0, 1H), 5.161(d, J = 3.2, 1H), 4.105 (m, 1H), 3.790 (dd, J = 3.2, 3.2, 1H), 3.646 (dd, J = 3.6, 3.6, 1H), 2.241 (t, J = 7.2, 2H), 1.239 (m, 20H), 0.862 (t, J = 6.8, 3H). ESI-MS (CH_3OH, relative intensity, %), *m/z* 423.18 ($[MH]^+$, 100). Calcd. for C23H38N2O5, *m/z* 422.28 [M]; C, 65.38; H, 9.06; N, 6.63. Found: C, 63.72; H, 9.06; N, 6.43.

4.1.8. N-((1R, 2S)-1,3-dihydroxy-1-(4′-nitrophenyl)-propan-2-yl) tetradecanamide ((1R, 2S) B13; 5b) was prepared from 4b, crude product 5b was purified by flush column chromatography at chloroform–methanol, 4:1 (v/v) in yield of yield 84.2% as waxy solid. R_f 0.31; $[\alpha]_D^{22.6}$+5.1 (*C* 0.235, CH_3OH); ^1H NMR (400 MHz, $CDCl_3$) 8.222 (d, J = 8.4, 2H), 7.598 (d, J = 8.4, 2H), 6.337 (d, J = 7.6, 1H), 5.161(d, J = 4.0, 1H), 4.098 (m, 1H), 3.780 (dd, J = 3.2, 3.2, 1H), 3.638 (dd, J = 3.6, 3.6, 1H), 2.232 (t, J = 7.2, 2H), 1.238 (m, 20H), 0.861 (t, J = 6.8, 3H). ESI-MS (CH_3OH, relative intensity, %), *m/z* 423.11 ($[MH]^+$, 100). Calcd. for C23H38N2O5, *m/z* 422.28 [M]; C, 65.38; H, 9.06; N, 6.63. Found: C, 64.74; H, 9.23; N, 6.51.

4.2. Biology

4.2.1. Cell culture

The MCF7 human breast adenocarcinoma cell line, originally obtained from American Type Culture Collection (ATCC, Manassas, VA) and was cultured in complete medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 100 μg/ml Normocin (VWR)) and maintained under standard incubator condition (humidified atmosphere with 5% CO_2 at 37 °C). Cells in the exponential growth phase are harvested from the culture and used in all experiments.

4.2.2. Cell viability assay

The MTT assay was used to quantify viable cells according to the literature.[14] Results are expressed as % of control viable MCF7 cells, with means ± st dev. of 5x replicates.

4.2.3. Lipid extract preparation and UHPLC-MS/MS analysis for cellular SL and B13 isomers

Advanced analysis of Cer species, Sph and endogenous B13 isomers were performed on a Thermo Finnigan TSQ 7000, triple stage quadruple mass spectrometer operating in a multiple reaction monitoring (MRM) positive ionization mode as described.[15] Final results were presented as the level of SL or chemical (pmol) per phosphate (Pi); lipid phosphate was determined from the Bligh and Dyer lipid extraction[16] and expressed as total phospholipid phosphate (nmol).

4.2.4. Statistical analysis

Where indicated, data are represented as mean ± SD. Statistical analysis was performed using two-sided *t* test, with *p*-value < 0.05 considered statistically significant.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Financial support was provided in part by the National Cancer Institute [PO1-CA097132 (YAH)], and National Institutes of Health-National Center for Advancing Translational Sciences [UL1TR001450 Voucher Pilot Program (APB)]. Research was supported in part by the Lipidomics Shared Resource, Hollings Cancer Center, Medical University of South Carolina [P30 CA138313] and the Lipidomics and Pathobiology COBRE, Department Biochemistry, MUSC [P20 RR017677]. We especially acknowledge the National Center for Research Resources and the Office of the Director of the National Institutes of Health for the funding [C06 RR018823], which provided laboratory space for Lipidomics Facility in MUSC's Children's Research Institute.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116011.

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