# Lipidomic Analysis of *Toxoplasma gondii* Reveals Unusual Polar Lipids†

Ruth Welti,‡ Ernie Mui,§ Alexis Sparks,‡ Sarah Wernimont,§ Giorgis Isaac,‡ Michael Kirisits,§ Mary Roth,‡ Craig W. Roberts,|| Cyrille Botté,⊥ Eric Maréchal,⊥ and Rima McLeod*,§

*Kansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, Kansas 66506, Departments of Ophthalmology and Visual Sciences, Pediatrics (Infectious Diseases) Committees on Molecular Medicine, Immunology and Genetics and The College, University of Chicago, Chicago, Illinois 60637, Strathclyde Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, Glasgow, Scotland, UK, and Laboratoire de Physiologie Cellulaire Végétale, UMR 5168 CNRS-CEA-INRA-Université J. Fourier, Institut de Recherches en Technologies et Sciences pour le Vivant, CEA-Grenoble, Grenoble, France*

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ABSTRACT: Analysis of the polar lipids of *Toxoplasma gondii* by electrospray ionization tandem mass spectrometry provides a detailed picture of the lipid molecular species of this parasitic protozoan. Most notably, *T. gondii* contains a relatively high level, estimated to about 2% of the total polar lipid, of ceramide phosphoethanolamine. The ceramide phosphoethanolamine has a fatty amide profile with only 16- and 18-carbon species. Compared with the host fibroblasts in which it was grown, *T. gondii* also has higher levels of phosphatidylcholine but lower levels of sphingomyelin and phosphatidylserine. Analysis at the molecular species level indicated that *T. gondii* has greater amounts of shorter-chain fatty acid in its polar lipid molecular species than the host fibroblasts. Shorter-chain fatty acids with a combined total of 30 or fewer acyl carbons make up 21% of *Toxoplasma*'s, but only 3% of the host's, diacyl phosphatidylcholine. Furthermore, diacyl phosphatidylcholine with two saturated acyl chains with 12, 14, or 16 carbons make up over 11% of parasite phosphatidylcholine but less than 3% of the host phosphatidylcholine molecular species. The distinctive *T. gondii* tachyzoite lipid profile may be particularly suited to the function of parasitic membranes and the interaction of the parasite with the host cell and the host's immune system. Combined with *T. gondii* genomic data, these lipidomic data will assist in elucidation of metabolic pathways for lipid biosynthesis in this important human pathogen.

*Toxoplasma gondii* infects 2−3 billion people throughout the world and is known to cause diseases that impair neurologic function and sight. A *T. gondii* infection can be life-threatening to those who are immunologically immature or immunologically impaired by AIDS, cancer, organ transplantation, or their therapy and, in some instances, to persons without known immune compromise.

Potential differences in the lipid metabolism of *T. gondii* and mammalian hosts may provide promising targets for therapeutic drugs. Thus far, manipulation of fatty acid synthesis (*1*−*5*), phosphatidylcholine metabolism (*6*), and sphingolipid synthesis (*7*) have been shown to affect tachyzoite growth and survival. The origin of fatty acids for synthesis of acyl lipids has been intensely investigated by molecular and metabolic labeling approaches. The apicoplast harbors an active fatty acid synthase of type II (FASII) (*1*, *2*, *8*−*11*, *13*). *T. gondii* also contains a cytosolic fatty acid synthase of type I (FAS I) and fatty acyl-elongases (FAEs) (*1*, *5*, *12*). On the basis of metabolic labeling experiments, Bisanz et al. (*13*) showed that in free *T. gondii*, *de novo* fatty acid synthesis was one of the sources for the acyl moiety of glycerolipids. Since acyl lipid labeling is abolished by treatment with haloxyfop, a reported inhibitor of the apicoplast acetyl CoA carboxylase, Bisanz et al. (*13*) concluded that an active FAS II was critical for the bulk of the acyl lipid synthesis. Combining conditional mutant analyses and metabolic labeling, Mazumdar et al. (*5*) demonstrated that FAS II was critical for the biogenesis of the apicoplast itself, and subsequently for parasite survival, but was unlikely the source of the acyl moiety of the bulk of acyl lipids. Rather, most glycerolipids from free *T. gondii* cells appears to be produced using acyl chains generated by FAS I or FAEs, which are resistant to the FAS II inhibitor thiolactomycin but sensitive to the general FAS inhibitor cerulenin (*5*). Together, these analyses highlight the importance (1) of the parasite FAS for bulk acyl lipid syntheses in free stages and (2) of the FAS II activity for the apicoplast biogenesis. Bisanz

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* To whom correspondence should be addressed: Rima McLeod, M.D., The University of Chicago, AMBH S206, 5841 S. Maryland Ave., Chicago, IL 60637. Phone: 773-834-4130. Fax: 773-834-3577. E-mail: rmcleod@midway.uchicago.edu.

‡ Kansas State University.

§ University of Chicago.

|| University of Strathclyde.

⊥ Institut de Recherches en Technologies et Sciences pour le Vivant.

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et al. (*13*) surveyed lipid-synthesizing gene candidates in *T. gondii* and identified a glycerol-phosphate acyltransferase and a lysophosphatidate acyltransferase (phosphatidic acid synthase) that are predicted to be localized in the apicoplast, supporting the notion that fatty acids produced by FAS II are important for the organelle membrane expansion. Upon invasion, in spite of its autonomous capacity to synthesize its major membrane acyl lipids, *T. gondii* massively scavenges host cell lipid precursors for membrane biogenesis (*6*, *13*, *14*). Information about *T. gondii* lipids and their biosynthetic machinery and about the relationship between parasite and host cell lipids is still incomplete. Herein, analysis of the polar lipids of *T. gondii* tachyzoites (clonal type 1 RH) is described. Lipidomic analysis results in unprecedented compositional detail that provides new insight into the differences between host and parasite lipids. This information will facilitate metabolic reconstruction of lipid synthesis and metabolism in *T. gondii* following completion of genome sequence annotation.

## MATERIALS AND METHODS

*Toxoplasma Cell Pellet Preparation.* The RH strain of T. *gondii* was maintained by serial passage in human foreskin fibroblast (HFF) (*15*) that were grown to a confluent monolayer in glass Petri dishes (150 mm diameter). Cells were cultured in IMDM supplemented with 10% fetal calf serum (FCS) inactivated at 56 °C for 1 h, 1 IU of penicillin/mL, 100 $\mu$g/mL of streptomycin, 0.25 $\mu$g/mL of amphotericin B, and 10 mM L-glutamine at 37 °C with 5% $CO_2$. On day 4 of infection, tachyzoites were harvested from HFF cells by scraping the monolayer. The cell suspension was passed through a 25-gauge needle twice and a 27-gauge needle once. The cell suspension was then passed through a 3.0 $\mu$m filter (removing any HFF cells), before centrifuging at 1500 rpm (500*g*) for 15 min. The supernatant was removed, and the parasite pellet was frozen and stored at −70 °C.

*Fibroblast Preparation.* Human foreskin fibroblasts (HFF) that were not infected were analyzed for comparison. They were grown to a confluent monolayer in glass Petri dishes (150 mm diameter) in IMDM supplemented with 10% FCS inactivated at 56 °C for 1 h, 1 IU of penicillin/mL, 100 $\mu$g/mL of streptomycin, 0.25 $\mu$g/mL of amphotericin B, and 10 mM L-glutamine at 37 °C with 5% $CO_2$. The cells were obtained from the Petri dish by scraping the monolayer and centrifuging at 1500 rpm (500*g*) for 15 min. The supernatant was removed, and the cell pellet was frozen and stored at −70 °C.

*Lipid Extraction.* To 0.8 mL of cells, 1 mL of chloroform and 2 mL of methanol were added. The sample was shaken, and 1 mL of chloroform and 1 mL of water were added. The samples were again shaken and centrifuged at approximately 1500*g* for 5 min. The lower phase was removed. One part chloroform was added, the mixture was shaken again and centrifuged, and again the lower phase was removed. This was repeated, and the combined lower phases were washed once with 0.4 mL of 1 M KCl and once with 0.4 mL of water. The solvent was evaporated, and the sample was dissolved in 1 mL of chloroform.

*Lipidomics.* An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (*16*, *17*) with some modifications. An aliquot of extract (0.040 mL) was taken for mass spectrometry analysis. Internal standards were added, the solvent was evaporated, and the lipid was redissolved in chloroform/methanol/300 mM ammonium acetate in water (300/665/35) with a final volume of 1 mL. Internal standards, obtained and quantified as previously described (*16*), were 0.063 nmol di14:0 PC[1], 0.054 nmol di24:1 PC, 0.066 nmol 13:0 lyso PC, 0.066 nmol 19:0 lyso PC, 0.038 nmol di14:0 PE, 0.031 nmol di24:1 PE, 0.039 nmol 14:0 lyso PE, 0.034 nmol 18:0 lyso PE, 0.030 nmol di14:0 PA, 0.032 nmol di20:0(phytanoyl)-PA, 0.023 nmol di14:0 PS, 0.023 nmol di20:0(phytanoyl) PS, 0.015 nmol 16:0−18:0 PI, and 0.007 nmol di18:0 PI, 0.196 nmol d18:1/14:0 Cer, 0.155 nmol d18:1/12:0 Hex-cer and 0.124 nmol d18:1/12:0 Dihex-cer.

Unfractionated lipid extracts were introduced by continuous infusion into an Applied Biosystems 4000 Q-TRAP (MDS Sciex, Ontario, Canada). Samples were introduced using an automated nanospray chip ion source Advion TriVersa NanoMate (Advion BioSciences, Ithaca, NY) at a flow rate of 0.11 $\mu$L/min. Ionization voltage was set to 1.8 kV or −1.8 and gas pressure to 0.1 psi, and the source was controlled by ChipSoft 7.1.1 software. The collision gas pressure was set at 2 (arbitrary units) for phospholipids, 1 for glycosyldiacylglycerols, and 5 for Cer, Hex-cer, and Dihex-cer. The collision energies, with nitrogen in the collision cell, were 28 V for PE, 40 V for PC and SM, 47 V for Cer, Hex-cer, and Dihex-cer, −58 V for PI, −57 V for PA, and −34 V for PS. Declustering potentials were 100 V for PE, SM, PC, Cer, Hex-cer, and Dihex-cer and −100 V for PA, PI, and PS. Entrance potentials were 15 V for PE, 14 V for PC and SM, 10 V for Cer, Hex-cer, and Dihex-cer, and −10 V for PI and PA, and PS. Exit potentials were 11 V for PE, 14 V for PC, 15V for Cer, Hex-cer, and Dihex-cer, −15 V for PI, −14 V for PA, and −13 V for PS. The mass analyzers were adjusted to a resolution of 0.7 amu full width at half-height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 40 °C, the curtain gas was set at 10 (arbitrary units), and the two ion source gases were turned off.

Lipid species were detected, using the scans previously described, including neutral loss of 87 in the negative mode for PS, and additionally using precursor of 264.2 (sphingosine) in the positive mode for Cer, Hex-cer, and Dihex-cer (*16*, *18*, *19*). Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common fragment. The specificity of precursor scan of 264.2 was confirmed using a neutral loss scan of 162.2 (mono-hexose) and 342.2 (di-hexose) for Hex-cer and Dihex-cer respectively; only peaks observed in both sphingosine and sugar scans were used for quantification. Two internal standards were used for quantification, except for Cer, Hex-cer, and

[1] Abbreviations: Cer, ceramide; PE-cer, ceramide phosphoethanolamine; Dihex-cer, dihexosylceramide; ESI, electrospray ionization; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; ePC, (alkyl or alkenyl)/acyl glycerophosphocholine; PE, phosphatidylethanolamine; ePE, (alkyl or alkenyl)/acyl glycerophosphoethanolamine; PI, phosphatidylinositol; ePS, (alkyl or alkenyl)/acyl glycerophosphoserine; Hex-cer, hexosyl ceramide; PS, phosphatidylserine; SM, sphingomyelin; MS/MS, tandem mass spectrometry.

Dihex-cer, for which one internal standard was used. Sphingomyelin was determined from the same mass spectrum as PC (precursors of *m*/*z* 184 in positive mode) (*18*−*20*) and by comparison with PC internal standards using a molar response factor for sphingomyelin (in comparison to PC) determined experimentally to be 0.36. PC 28:0 was determined in the absence of added internal standards by comparison with another naturally occurring PC species, which was determined in comparison to internal standards. Ceramide phosphoethanolamine was determined in a similar fashion to sphingomyelin, from the same mass spectrum as PE (neutral loss of *m*/*z* 141) and by comparison with PE internal standards. Because no authentic compound was available, no response factor for PE-cer was determined nor employed. The background of each spectrum was subtracted, the data were smoothed, and peak areas were integrated using a custom script and Applied Biosystems Analyst software. Isotopic overlap corrections were applied, and the lipids in each class were quantified in comparison to the two internal standards of that class using corrected curves determined for the API 4000 mass spectrometer.

*Acyl Group Identification and Determination of Ceramide Phosphoethanolamine Structure.* The acyl groups of phospholipid species in the *Toxoplasma* lipid extract (without standards) were identified as acyl anions from the appropriate negative ion precursors. The collision energies were 20−55 V. The solvent was chloroform/methanol/300 mM ammonium acetate in water (300/665/35). PI and PE were analyzed as $[M - H]^-$ ions, and PC was analyzed as $[M + OAc]^-$. The relative abundance of the fatty acyl ions was used to designate the position of the acyl chain, as the acyl group in the 2-position generally produces the more abundant ion, although this designation is somewhat equivocal (*21*). The data for determination of PE-cer structure were obtained on an API 4000 without addition of internal standard. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV was applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), the two ion source gases were set at 45 (arbitrary units), the collision gas pressure was 2, and the collision energy was 55 V.

## RESULTS

*Lipid Classes.* Quantitative analysis of polar lipids with masses less than 1000, including diacyl, ether-linked (ePC; i.e. alk(en)yl/acyl PC), and lyso PC, diacyl, ether-linked (ePE), and lyso PE, diacyl and ether-linked (ePS) PS, diacyl PI, diacyl PA, SM, PE-cer, MHexDG (monohexosyldiacylglycerol identified based on MGDG standards), DHexDG (dihexosyldiacylglycerol identified based on DGDG standards), Cer, Hex-cer, and Dihex-cer, of *T. gondii* and its host fibroblasts was performed, using electrospray ionization tandem mass spectrometry (ESI-MS/MS). As shown in Figure 1, when the lipid classes were considered as mol % of the total of these lipids from each source, *T. gondii* was found to be enriched in PC (57.4% vs 43.4%), PE-cer (2.4% vs 0.01%), and PA (0.7% vs 0.5%) relative to its host fibroblasts, while the host fibroblasts had (as mol %) relatively more ePC, ePE, lysoPE, diacyl PS, SM, and Hex-cer.

*Glycerolipid Molecular Species.* An advantage of ESI-MS/MS over traditional methodologies for lipid analysis is

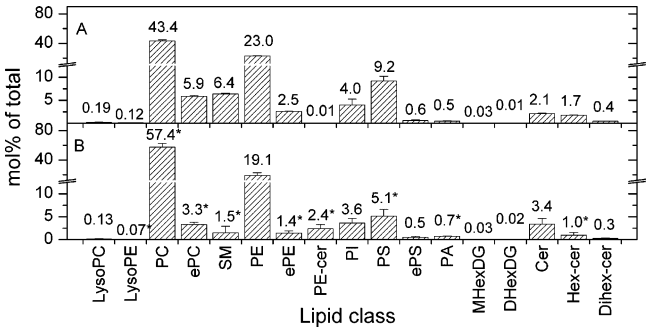


FIGURE 1: Composition of lipid classes in host fibroblasts (A) and *Toxoplasma gondii* (B). Lipids are determined from the total of those lipid classes shown. Numbers above each column indicate the mol % value for that species. The detection of MHexDG and DHexDG was equivocal with trace amounts of several species just at the limit of detection and of uncertain significance. Error bars are standard deviation ($n = 4$). The asterisks indicate lipid classes that differed significantly ($p < 0.05$) in mol % in *Toxoplasma gondii* as compared to the host fibroblast.

that individual lipid molecular species can be identified. In Figure 2, PC molecular species from *T. gondii* and host fibroblasts are depicted. From these data, it is obvious that there are significant differences between the molecular species profile of the parasite and its host. Most strikingly, there are high levels of shorter-chain (28, 30, and 32 carbons in the combined two acyl chains) species in the parasite lipids. PCs with a combined count of 28 and 30 carbons make up about 21% of the *T. gondii* PC molecular species, while these species make up only 3% of the host molecular species. The most prominent of these 28- and 30-carbon species were identified by product ion analysis as 1−16:0,2−14:0 PC, 1−16:1,2−14:0 PC, di14:0 PC, and 1−16:0,2−12:0 PC (Table 1), indicating that saturated 14-carbon and even 12-carbon acyl species are used in phospholipids considerably more frequently by this parasite than by its host. While there are some qualitative differences, the amounts of 34-, 36- and 38- carbon (combined diacyl count) PC molecular species relative to the total polar lipids are roughly similar between the parasite and host. In the longer chain species, the parasite has more PC 40:7, which was shown to be 1−18:1,2−22:6 PC (Table 1). Overall, the PC of *T. gondii* appears to contain PC similar to the host with additional 28-, 30-, and 32-carbon species. Thus, the shorter-chain and mostly saturated species are fairly specific to the parasite.

PE (Figure 3) of *T. gondii* is quite similar to its host in molecular species composition, but as in PC there are more 28−30- and 32-carbon PE species in the parasite than in the host. Twenty-eight- and 30-carbon species make up about 1.6% of the parasite PE, but only 0.1% of the host PE. PE 30:0 from *T. gondii* was identified as 1−16:0,2−14:0 PE (Table 1).

Whereas PI makes up a similar fraction of the polar lipids of *T. gondii* and host fibroblasts (Figure 1), the molecular species of PI (Figure 4A) of *T. gondii* are less dominated by the PI 38:4 molecular species, which makes up 48% of host PI and 10% of parasite PI. PI 34:1 makes up a greater percentage of the *T. gondii* PI (50%) than of the host PI (2%). This species was determined by product ion analysis (Table 1) to be PI containing 16:0 and 18:1 fatty acyl species.

PS molecular species are shown in Figure 5. In general, *T. gondii* and host PS are similar, but host PS 36:1 species

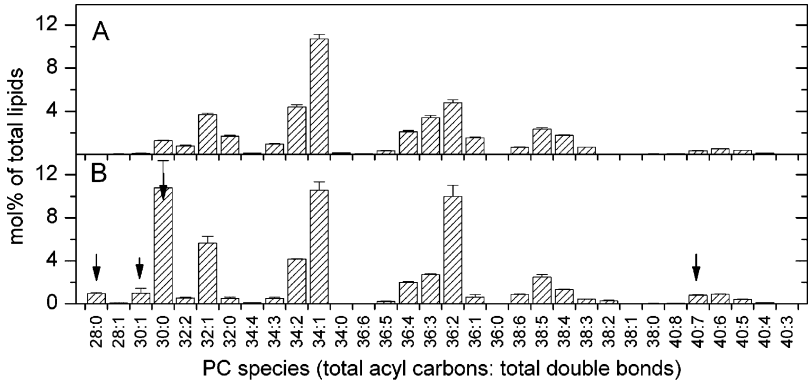


FIGURE 2: Diacyl phosphatidylcholine molecular species of host fibroblasts and *Toxoplasma gondii*. Species shown in A and B are indicated by the total number of acyl carbons: the total number of double bonds. Species are indicated as mol % of total lipids in classes shown in Figure 1. Error bars are standard deviation ($n = 4$). (A) Diacyl phosphatidylcholine molecular species of host fibroblasts. (B) Diacyl phosphatidylcholine molecular species of *Toxoplasma gondii*. Species marked by the arrows, chosen because they are higher in *Toxoplasma* than in the host fibroblasts, were subjected to product ion analysis (Table 1).

Table 1: Identification of Lipid Molecular Species in *Toxoplasma gondii* Tachyzoites. Selected Lipid Molecular Species That Represented a Larger Fraction of *Toxoplasma gondii*'s Lipids than of Fibroblast Lipids Were Subjected to Product Ion Analysis in the Negative Ion Mode in Order To Identify the Individual Fatty Acyl Components[a]

| identification in head group scan | acyl composition |
|---|---|
| PC 28:0 | 14:0−14:0 PC > 16:0−12:0 PC |
| PC 30:1 | 16:1−14:0 PC |
| PC 30:0 | 16:0−14:0 PC |
| PC 40:7 | 18:1−22:6 PC |
| PE 30:0 | 16:0−14:0 PE |
| PI 34:1 | 16:0/18:1 PI[b] |

[a] The fatty acyl group presumed to be on the 1-position, because of lower signal abundance as compared to its paired acyl species, is listed first; this method is only suggestive with regard to acyl position (*21*). [b] Cannot determine acyl position. Intensities for 16:0 and 18:1 anions are similar.

is common, making up about 24% of the total PS. *T. gondii* has only about half the relative amount of PS found in the host and has a more equitable distribution of PS among its molecular species, with the highest species (PS 36:2) making up 15% of the PS species.

*Sphingolipid Composition.* Besides the PC species composition, the most notable difference between the polar lipid composition of *T. gondii* and that of its host fibroblasts is in the sphingolipids containing phosphate. Figure 6 shows a scan of the sphingosine-containing compounds (i.e., precursors of *m*/*z* 264 in positive ion mode) in host fibroblasts (Figure 6A) and *T. gondii* (Figure 6B). Both parasite and host contain Cer, Hex-cer, and Dihex-cer, each with a predominance of 16- and 24-carbon fatty-amide species. Indeed, the sphingolipid spectra were qualitatively quite similar except for signals for SM (e.g., SM 16:0 at *m*/*z* 703) which was higher in the host cells than in *T. gondii*, and the presence of a large peak at *m*/*z* 661 and a smaller one at *m*/*z* 689, which were present in the parasite, but undetectable or at the limit of detection in the host cells. The SM 16:0 peak and other SM peaks were also detectable in a positive ion scan for precursors of phosphocholine (Pre 184), and peaks at *m*/*z* 661 and *m*/*z* 689 were also present in the positive ion scan for the neutral loss of phosphoethanolamine (NL 141) (data not shown). Peaks at *m*/*z* 661 and *m*/*z* 689 were identified by product ion analysis as PE-cer 16:0 and PE-cer 18:0; product ion spectra in the positive ion mode are shown in Figure 6C and 6D. The structures of the SM 16:0, the predominant phosphosphingolipid of host fibroblasts, and PE-cer 16:0, the predominant phosphosphingolipid of *T. gondii*, are shown in Figure 7. Sphingomyelin and PE-cer are related lipids with an ethanolamine moiety in PE-cer in contrast to a choline moiety in SM.

PE-cer and SM molecular species were quantified (Figure 8A and 8B). Because an authentic compound for PE-cer is not available, PE was quantified in relation to diacyl PE internal standards. Our lack of knowledge of a "response factor" for PE-cer only allows us to estimate the PE-cer content at about 2% of the polar lipids. Because the same method was used to analyze PE-cer in *T. gondii* and in the host cells, we were able to ascertain that *T. gondii* has a much higher, on the order of 100-fold higher, concentration of PE-cer than its host.

*Correction for Host Cell Membrane Contamination.* In separate studies (data not shown), uninfected host cells were passed through a needle and a 3-$\mu$m filter in precisely the same manner as the infected cells. The lipidomic data obtained were subtracted from those obtained with the infected cells (i.e., those from the isolated parasites). Because the amount of filtered fibroblast lipids was less than 4% of that of the filtered *T. gondii* lipid species, employing this correction did not significantly change the lipid composition determined for the isolated parasites.

## DISCUSSION

Defining the composition and abundance of *T. gondii* lipids provides not only information about the nature of plasma and organelle membranes but also insight into nutrient requirements for parasite metabolism and host parasite interactions (*5*, *23*). Collectively, this information might inform design of new antimicrobials. Herein, a broad-based analysis of composition and relative abundance of T. *gondii* RH strain intracellular tachyzoite polar lipids is reported. Overall the relative abundance of the lipid classes is in reasonable agreement with a previous study (*6*), but, in addition to information about the relative abundance of the various lipid classes, the current study provides information about lipid molecular species and, in particular, about diacyl, ether-linked (i.e., alk(en)yl/acyl) and ceramide-based polar lipids. In addition, this study identifies a class of sphingolip-

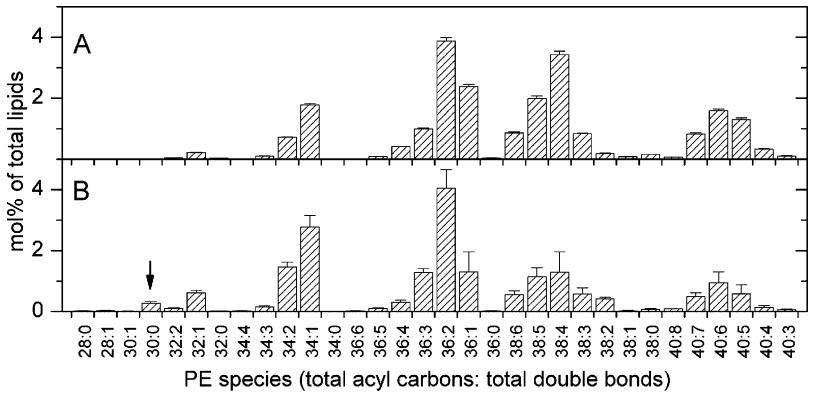


FIGURE 3: Diacyl phosphatidylethanolamine molecular species of host fibroblasts and *Toxoplasma gondii*. Species shown in A and B are indicated by the total number of acyl carbons: the total number of double bonds. Species are indicated as mol % of total lipids in classes shown in Figure 1. Error bars are standard deviation ($n = 4$). (A) Diacyl phosphatidylethanolamine molecular species of host fibroblasts. (B) Diacyl phosphatidylethanolamine molecular species of *Toxoplasma gondii*. The species marked by an arrow, chosen because it is higher in *Toxoplasma* than in the host fibroblasts, was subjected to product ion analysis (Table 1).

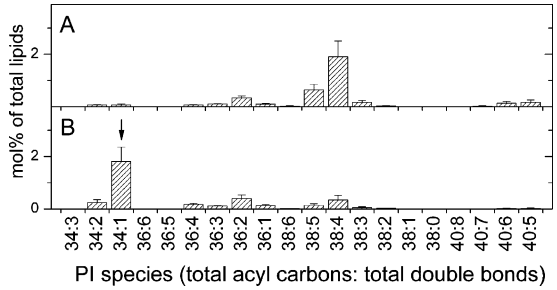


FIGURE 4: Diacyl phosphatidylinositol molecular species of host fibroblasts and *Toxoplasma gondii*. Species shown in A and B are indicated by the total number of acyl carbons: the total number of double bonds. Species are indicated as mol % of total lipids in classes shown in Figure 1. Error bars are standard deviation ($n = 4$). (A) Diacyl phosphatidylinositol molecular species of host fibroblasts. (B) Diacyl phosphatidylinositol molecular species of *Toxoplasma gondii*. The species marked by an arrow, chosen because it is higher in *Toxoplasma* than in the host fibroblasts, was subjected to product ion analysis (Table 1).

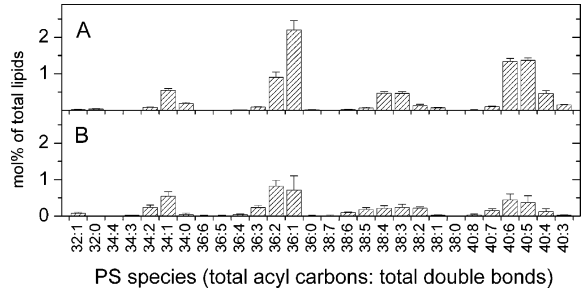


FIGURE 5: Diacyl phosphatidylserine molecular species of host fibroblasts and *Toxoplasma gondii*. Species are indicated by the total number of acyl carbons: the total number of double bonds. Species are indicated as mol % of total lipids in classes shown in Figure 1. Error bars are standard deviation ($n = 4$). (A) Diacyl phosphatidylserine molecular species of host fibroblasts. (B) Diacyl phosphatidylserine molecular species of *Toxoplasma gondii*.

ids, PE-cer, which is common in the parasite but is found at very low levels in host cells.

*Phosphatidylcholines.* Higher levels of PC were found in the parasite as compared with the host cells. Gupta et al. (*6*) noted the high PC levels in *T. gondii* and found that replacing PC with phosphatidyldimethylethanolamine drastically inhibited parasite growth. The difference in PC quantity between host and parasites is largely due to the presence of parasite PC species with relatively short acyl chains, i.e., containing only a combined 28 or 30 carbons in the two acyl species. The 28:0, 30:1, and 30:0 lipids are composed of 12-, 14-, and 16-carbon fatty acids. Shorter-chained PC species potentially could be derived by scavenging from the host or by either type I or type II fatty acid synthesis (FAS), since *T. gondii* is capable of acquiring lipids by all three routes (*24*−*26*). Shorter-chained fatty acids or shorter-chain PC species are more water-soluble than longer-chained species and so could be transferred from the host more easily than longer-chain molecular species. Recent studies on *Typanosoma brucei* indicate that this organism synthesizes 14-carbon fatty acid by an unexpected pathway, involving two of three modular elongases (*27*). The three elongases yield 10-, 14- and 18-carbon fatty acids sequentially. Trypanosomes regulate this pathway to produce varied length fatty acids under differing environmental conditions. It remains under speculation that *Toxoplasma* might also regulate the length of its fatty acyl chains under differing environmental conditions or in different stages of its life cycle.

In addition to the *T. gondii* PC species being shorter-chained than is typical for those of mammalian membranes, the PC species are also more saturated. PC 30:0, which was determined by product ion analysis to be 16:0/14:0 PC, makes up about 11% of *T. gondii* PC species but only about 1% of host PC species. While the relative intensity of the product ion spectral peaks suggests that 16:0 is in the predominant species in the 1-position of 16:0/14:0 PC, determination of position by peak intensity can provide ambiguous or incorrect positional assignments (*27*), and it is also possible that PC 30:0 may be a mixture of positional isomers. 1−16:0,2−14:0 PC has a gel-liquid crystalline phase transition temperature of 27.3 °C, and 1−14:0,2−16:0 has a transition temperature of 35.1 °C (*28*). It is speculated that this/these PC 30:0 species might contribute to the growth arrest caused by replacement of PC with phosphatidyldimethylethanolamine (*6*), since phosphatidyldimethylethanolamines have gel-to-liquid crystalline transition temperatures approximately 8 °C higher than PC species with the same acyl chains (*29*). Thus, if head group alteration occurred without significant changes in fatty acyl species in this group of lipids, phosphatidyldimethylethanolamine replacement for PC could potentially result in the formation of gel-phase lipid in the membranes of *T. gondii*. This might be related to the

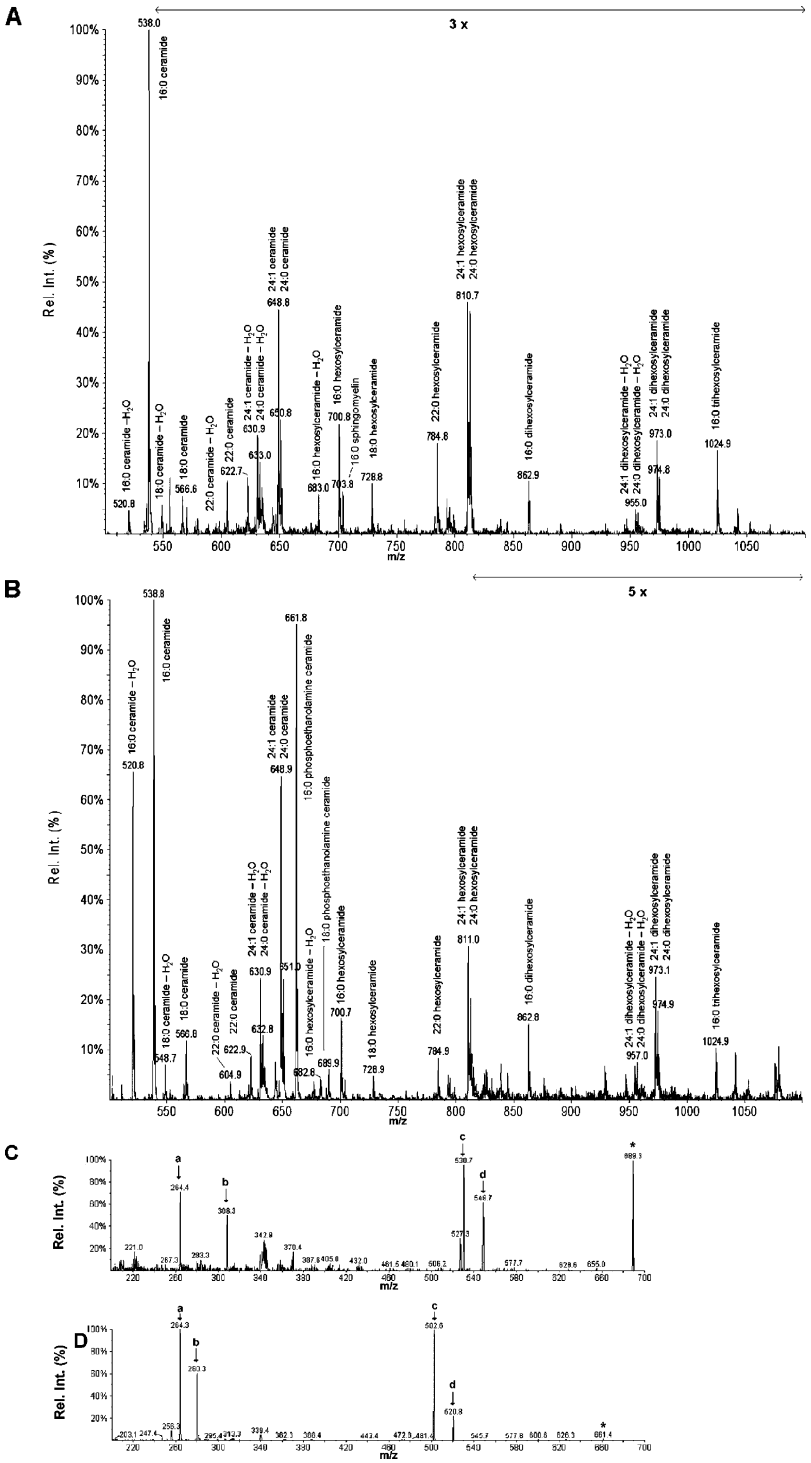


FIGURE 6: Sphingosine-containing molecular species of host fibroblasts and *Toxoplasma gondii as shown by ESI MS/MS in the positive mode*. (A and B) Precursors of *m/z* 264 (sphingosine) were identified as $[M + H]^+$ ions. Ceramides, hexosyl ceramides, di-hexosyl ceramides, and tri-hexosyl ceramides are identified in both host fibroblasts and *Toxoplasma gondii* as indicated with their amide-linked fatty acyl species. SM 16:0 is identified in host fibroblasts at *m/z* 703 but produced only a very small signal in *Toxoplasma*. It should be noted that SM produces *m/z* 264 only as a minor fragment, in contrast to other sphingolipids, which produce *m/z* 264 as a major fragment; thus, the signal for SM under-represents its amount in relation to the amounts of the other sphingolipids. (A) Sphingosine-containing molecular species of host fibroblasts. (B) Sphingosine-containing molecular species of *Toxoplasma gondii*. In the *Toxoplasma gondii* extract, PE-cer produced signals at *m/z* 689 for the 18:0 amide-linked species and at *m/z* 661 for the 16:0 amide-linked species. Product ion analysis of these species is shown in C and D. These species were absent from the precursors of *m/z* 264 scan of the host fibroblast extract (A). (C and D) Product ion analysis of the *Toxoplasma* phosphatidylethanolmine molecular species, "PE-cer 18:0" (*m/z* 689) (C) and "PE-cer 16:0" (*m/z* 661) (D). The species indicated by the arrow in B were subjected, as an $[M + H]^+$ ions (∗), to product ion analysis to confirm their identifications. Note that these product ion scans were performed on an unfractionated *Toxoplasma* extract, and so isobaric species, i.e., species with the same nominal mass as the molecular ion of interest may produce fragment ions in addition to those derived from the ion of interest. The *m/z* 264 ion, indicated as "a", is the characteristic ion for sphingosine (a dihydroxy 18-carbon sphingoid base). The ions indicated as "b", *m/z* 308 in A and *m/z* 280 in B, are characteristic of the fatty amide species, 18:0 and 16:0, respectively. Fragmentation of SM 16:0 produced the same *m/z* 280 ion, characteristic of the fatty amide (*22*). The ions labeled "c" and "d" are produced by a neutral loss of phosphoethanolamine (NL 141) ("d") and an additional water loss ("c").

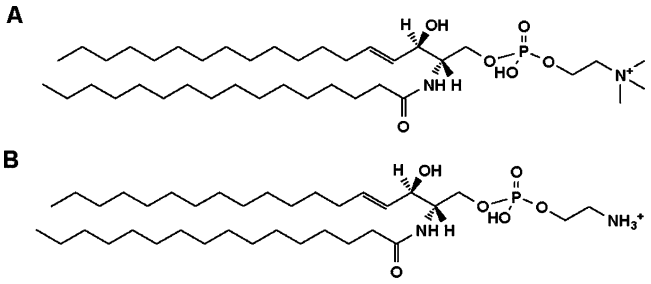


FIGURE 7: Structures of SM 16:0 and PE-cer 16:0 as $[M + H]^+$ ions. (A) SM 16:0, the predominant phosphosphingolipid of host fibroblasts. (B) PE-cer 16:0, the predominant phosphosphingolipid of *Toxoplasma gondii* tachyzoites.

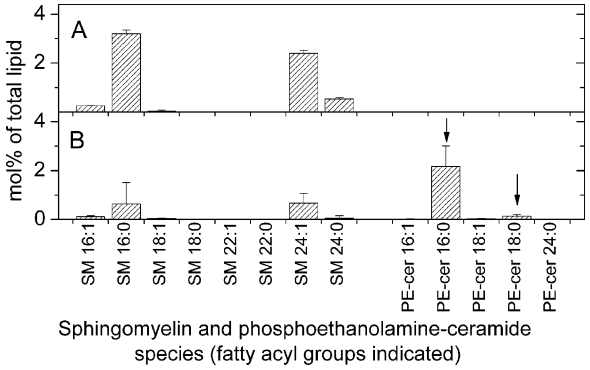


FIGURE 8: Quantification of sphingomyelin and phosphoethanolamine molecular species of host fibroblasts and *Toxoplasma gondii*. Species are indicated as mol % of total lipids in classes shown in Figure 1 and are indicated by the number of acyl carbons: the number of double bonds, assuming that the ceramide contains a dihydroxy18:1 base (sphingosine); this is demonstrated in Figure 6C and 6D for the species indicated. Error bars are standard deviation ($n = 5$). (A) Sphingomyelin and phosphoethanolamine molecular species of host fibroblasts. (B) Sphingomyelin and phosphoethanolamine molecular species of *Toxoplasma gondii*. The species marked by the arrows, chosen because they are higher in *Toxoplasma* than in the host fibroblasts, were subjected to product ion analysis.

observed intracellular accumulation of lipids and the negative effect of phosphatidyldimethylethanolamine on *T. gondii* multiplication (*6*). On the other hand, many organisms are able to alter fatty acid composition to compensate for altered phospholipid head group composition (e.g., (*30*)), but the partial dependence of *T. gondii* on host cell lipid biosynthesis may not permit the parasite to make compensatory changes in its lipid composition quickly.

*Phosphosphingolipids.* Gupta et al. (*6*) found that radiolabeled serine was incorporated into two unknown alkaline-stable materials and suggested that these unidentified materials might be sphingolipids. In the current work, a major phosphosphingolipid class was determined to be PE-cer. While the mol % of sphingomyelin in *T. gondii* is less than half that of the host fibroblasts, there is a large amount of PE-cer in *T. gondii* in comparison to host cells. PE-cer is generally found only in trace quantities in mammalian cells, but it has been previously detected in the rumen ciliate commensal *Entodinium caudatum* and oomycete plant pathogens (*31*, *32*). This might suggest that the production of PE-cer and the route to its biosynthesis is conserved or is an ancestral characteristic of the Chromoalveolates. PE-cer has also been reported in marine invertebrates (*33*), in insects (*34*, *35*), and in low amounts in mammalian cells (*36*, *37*). In the current studies the level of PE-cer in uninfected host fibroblasts was determined to be very low, about 0.01% of total polar lipids. Interestingly, the fatty amides associated with PE-cer included only 16- and 18-carbon species. Twenty four-carbon species, which are common (15−40%) in the other sphingolipid classes (Cer, Hex-cer, Dihex-cer, and SM), were not detectable in the PE-cer class.

PE-cer synthesis may occur through a transfer of a phosphoethanolamine group from PE to ceramide; this pathway was suggested to occur in rumen ciliate commensal *Entodinium caudatum* (*31*). This reaction might be catalyzed by a protozoan-encoded protein or could conceivably be due to an upregulation of the synthesis of PE-cer by the host as a result of the commensal interaction or parasite infection, as this lipid is known to occur at low levels in mammalian cells (*36*, *37*). Indeed, Apicomplexa genomes contain genes for putative sphingomyelin synthase (one candidate gene in *T. gondii*, i.e. 50.m03113 and two putative homologues in *P. falciparum*, i.e. the PFF1210w and PFF1215w contiguous genes), and we do not know if these enzymes might be capable of synthesizing ceramide phophoethanolamine as well or if a substantial amount of SM and ceramide phosphoethanolamine might be diverted from host cells.

Fouts and Boothroyd (*38*) and Kim et al. (*39*) recently analyzed the global transcriptomic responses of HFF cells after infection by *T. gondii*. A list of 46 "lipid biosynthesis" genes with expression induced or repressed by *T. gondii* infection was reported (*38*); of these, 10 are reported in the other study (*39*). Both analyses confirm a strong remodeling of host cell lipid metabolism upon infection.

In the study by Fouts and Boothroyd (*38*), a human gene encoding a phosphoserine aminotransferase 1 (AI015679) is shown to be repressed in tachyzoite infected HFF cells. Suppression of phosphoserine aminotransferase (if confirmed) would support the diversion of the phosphoserine metabolism toward serine production. Among different metabolic usages, serine can enter both glycerolipid and sphingolipid metabolism, since it is the substrate for both phosphatidylserine synthase (generating PS) and for serine palmitoyltransferase (entry point for the synthesis of sphinganine). In the study by Kim et al. (*39*), the phosphatidylserine synthase 1 (H28984) is consistently enhanced after infection by *T. gondii*.

In addition, Kim et al. (*39*) provide evidence for remodeling of host cell sphingolipid metabolism: in particular, expression of a sphingosine kinase (AI341901) is stimulated, supporting the synthesis of sphingosine 1-phosphate, which is the direct substrate for the sphinganine-1-phosphate aldolase that generates phosphoethanolamine. Three human genes encoding sphingomyelin synthases (transmembrane protein 23: AI261602, AA459293 and AA693488) are markedly repressed after infection. These proteins are bidirectional and capable of converting PC and ceramide to SM and diacylglycerol (DAG) and vice versa; direction is dependent on the relative concentrations of DAG and ceramide as phosphocholine acceptors. It is therefore difficult to deduce from the reduced expression of sphingomyelin synthase genes whether they are correlated to a decrease or an increase of HFF SM. It is also possible that some of these enzymes could be involved in ceramide phosphoethanolamine synthesis. If the enzyme involved in PE-cer synthesis belongs to the protozoan, its uncommon specificity for ceramides with 16C and 18C amides might suggest that the

enzyme has an unusual active site that might provide a parasite-specific target.

PE-cer and other ceramide derivatives have structural resemblance to lipopolysaccharide and represent alternative ligands for host toll receptors (*40*). If PE-cer does bind to toll receptors, PE-cer may be made by the parasite to modulate host cell function.

*Hexosylceramides.* Figure 6, a discovery scan for sphingolipids indicates that, besides the phosphosphingolipids, the major ceramide-containing species in the mass range between 500 and 1100 are ceramides, (mono)Hex-cers, and Dihex-cers. A peak at *m*/*z* 1024, consistent with trihexosyl ceramide 16:0, was also detected. The current data are consistent with the detection of mongalactosylceramide synthesis after metabolic labeling of *T. gondii* cells with radiolabled UDP-galactose (*41*). These data further confirm the synthesis of mono-, di-, and trihexosyl ceramides detected by Bisanz et al. (*13*) after acetate metabolic labeling.

*Ceramide.* The amount of free ceramide in *T. gondii* relative to the composition of the uninfected host cell is also of interest in that both ceramide and lipopolysaccharide bind to CD14 and CD36 which appear to coassociate with toll receptors 2 and 4 (*40*). These findings again are suggestive of a way in which the parasite lipids could modulate host cell functions.

*Glycosyldiacylglycerols.* The amounts of MHexDG and DHexDG in *T. gondii* tachyzoites are at or below the limit of detection. Maréchal et al. (*41*) could detect the synthesis of both MGDG and DGDG in *T. gondii* cells incubated with radiolabeled UDP-galactose and could detect a glycolipid reacting with an anti-DGDG antibody in *T. gondii* lipid extracts. Bisanz et al. (*13*) further detected a glycolipid comigrating with DGDG. Here, MHexDG and DHexDG with masses corresponding to diacyl galactolipids, similar to those found in plant chloroplasts, are, at most, very minor lipid species.

*Significance of PE-cer and Distinctive PC Species.* The work herein precisely characterizes lipid species in *T. gondii*, focusing initially on one clonal type and the tachyzoite life cycle stage. Bradyzoites have more lipid and more abundant lipid bodies than tachyzoites (*42*), and whether lipid species in tachyzoites and bradyzoites differ remains to be defined. It will also be of interest to compare lipid content in different isolates (clonal and atypical parasite types) and in *T. gondii* organelles (*43*). Mass spectrometry-based lipidomics is a robust methodology for comparing parasite and host cell lipid composition in detail. It identifies a novel sphingolipid, ceramide phosphoethanolamine, which is likely to play a role in the interaction between the parasite and the host. Ceramide phosphoethanolamine might also be an important component of *T. gondii* membrane rafts.

Occurrence of ceramide phosphoethanolamine suggests the existence of multiple, and possibly nonredundant, pathways for the synthesis of sphingolipids. SM can be generated either by conventional PC-ceramide phosphocholine transferases (one putative homologous gene in *T. gondii*, i.e. 50.m03113 and two putative homologous genes in *P. falciparum*, i.e. the PFF1210w and PFF1215w contiguous genes) or by a stepwise process involving PE-ceramide phosphoethanolamine transferases, generating ceramide phosphoethanolamine, and methyltransferases, generating SM. PE-ceramide phosphoethanolamine transferase activity might be harbored by PC-ceramide phosphocholine transferases with loose substrate specificity or by as yet unidentified enzymes. Subsequent methylation of the phosphoethanolamine polar head into phosphocholine might be catalyzed by a phosphoethanolamine methyltransferase (one gene characterized in *P. falciparum*, i.e. PfPMT or MAL13P1.214; no clear homologue in *T. gondii* genome). In *P. falciparum*, the PfPMT enzyme was shown to catalyze the *in vitro* conversion of phosphethanolamine into phosphocholine and is suspected to also catalyze the transmethylation of PE into PC *in vivo* (reviewed in ref *44*). Such an enzyme might therefore also catalyze the transmethylation of ceramide phosphoethanolamine into SM. PfPMT has attracted attention because it is inhibited by phosphocholine and synthetic analogues (miltefosine (*45*) and 1,12-bis-(*N*,*N*′-acetamidinyl)dodecane derivatives (*46*)), which represent some of the most promising future generations of antimalarial drugs. In *T. gondii*, the accumulation of ceramide phosphoethanolamine might be related to the apparent lack of a PfPMT homologue or a methyl transferase of different substrate affinity. In future studies, it would be of interest for comparisons of *T. gondii* and *P. falciparum* lipidomic profiles to focus on the ceramide phosphoethanolamine and SM contents, and on the enzymes that are involved in the transmethylations of phosphoethanolamine, ceramide phosphoethanolamine, and PE, in both *T. gondii* and *P. falciparum*.

The functional significance of the high-level of 12-, 14- and 16-carbon saturated *T. gondii* PCs and the effect of these lipids on the properties of the parasite's membranes remain to be determined. However, if their role is demonstrated to be essential, the enzymes involved in the synthesis of distinctive lipids may also provide unique molecular targets for chemotherapeutics (*7*, *47*, *48*).

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