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1. Chemical approaches to sphingolipid research

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Abstract. Sphingolipids are an important group of biomolecules that play important roles in the regulation of many cell functions. Many efforts have been made in recent years to design analogs suitable for a better understanding of the biological and biophysical roles of sphingolipids. In this review, some of the most relevant contributions in the field from our group are collected. In particular, this review deals with the development of new sphingolipid analogs as acid ceramidase inhibitors, and the design of fluorogenic probes to screen enzyme activities and to the study of biophysical properties.

Introduction

Sphingolipids (SLs) represent an important group of natural products that play crucial roles in cell survival and regulation [1]. Chemically, SLs in

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mammals contain a lipophilic 2-amino-1,3-diol backbone of eighteen carbon atoms, as found in sphingosine (So). Acylation of the 2-amino group with fatty acids affords ceramides (Cer), responsible for grow inhibition and apoptotis. The so-called complex SLs arise from functionalization at the primary hydroxyl group of Cer. In this case, glucosylation leads to glucosyl ceramide (GlcCer), the precursor of higher glycosphingolipids (GSLs), which play important roles in cell-cell recognition events at the outer membrane [2]. Esterification with phosphorylcholine leads to sphingomyelin (SM), while both Cer and Sph can be also phosphorylated in cells to the corresponding phosphate esters, ceramide-1-phosphate (CerP) [3] and sphingosine-1-phosphate (S1P) [4], which are important as second messengers and also in cell regulation as proliferative agents (Fig. 1).

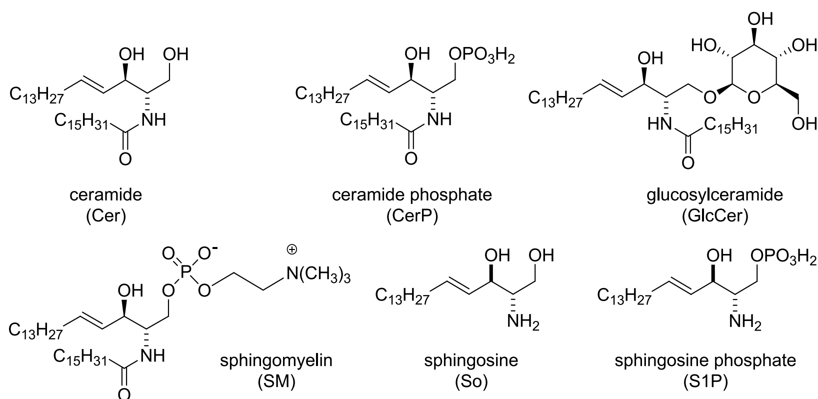

Figure 1. Some of the most representative sphingolipids in mammals.

The effective control of the cellular functions requires a delicate balance of SL levels, which is regulated by finely tuned complex metabolic pathways with the help of specific enzymes However, the enzymatic processes by themselves are not enough to understand this intricate scenario, whose operability depends on the cellular compartmentalization of the different pathways involved (Fig. 2) [1]. This cellular organization is especially relevant for signaling events mediated by SL that are often spatially separated in particular organelle. Since the subcellular distribution of lipids is not uniform [5], local changes in lipid concentrations can be responsible for diverse downstream effects.

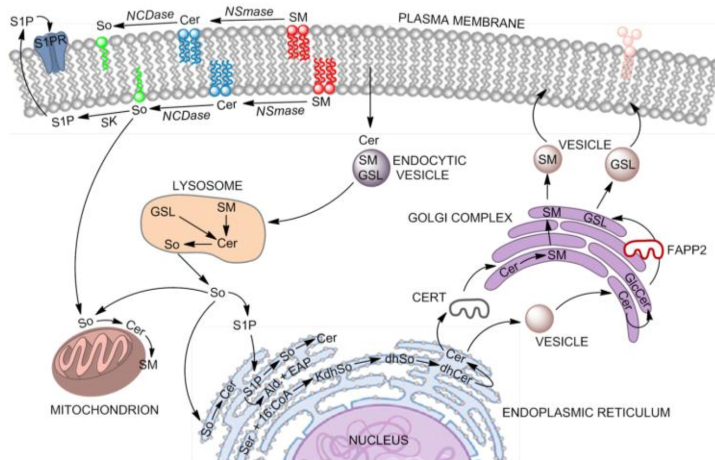

Figure 2. Metabolic pathways and compartmentalization in sphingolipid biosynthesis. (CERT: ceramide transporter protein; dhCer: dihydroceramide; dhKSo: ketosphingosine; dhSo: dihydrosphingosine; GSL: glycosphingolipids; NCDase: neutral ceramidase; NSmase: neutral sphingomyelinase; SK: sphingosine kinase; FAPP2: GlcCer transfer protein).

The fact that the expression of SL metabolizing enzymes is deregulated in many diseases has boosted the design of SL biosynthesis modulators as a rational approach to define new targets and new small molecule chemical entities with potential therapeutic applications [6].

In recent years, the interest on the biophysical properties of SLs, in particular So, Cer and their phosphorylated derivatives, has emerged as a major field of research. In this context, So is known to increase membrane permeability, while Cer increase lipid chain order, induce “flip-flop” motion of lipids and segregate laterally into rigid domains, among other effects [7]. Interestingly, it is the ability of these SL to aggregate into microdomains what accounts for the formation of high local concentrations of secondary messengers that are ultimately responsible for the triggering of some cellular effects.

1. Sphingolipid analogs as enzyme inhibitors

Abnormal SLs metabolism is known to occur in some diseases, such as certain sphingolipidoses [6], cancer [8], diabetes [9], and aterosclerosis [10]. The cellular contents of the various SLs species are controlled by enzymes

involved in their metabolic pathways. In this context, the search for potent and selective inhibitors of SL metabolizing enzymes offers new insights for the discovery of alternative therapeutic agents. Our interest in SL enzymes as potential targets led us to investigate on ceramidases, a type of amidohydrolases that catalyze the cleavage of Cer into So and fatty acids. According to their optimal pH, ceramidases fall into three groups, acidic (aCDase), neutral (NCDase) and alkaline ceramidases (alkCDase). While aCDase is ubiquitously expressed, NCDase is highly expressed in the small intestine along the brush border, where it is involved in the catabolism of dietary sphingolipids thus regulating the levels of bioactive sphingolipid metabolites in the intestinal tract. On the other side, alkaline ceramidases are expressed in the endoplasmic reticulum, where three different types have been identified, based on their localization and the encoding genes [11].

The role of CDases in human disease is well documented. In general, increased CDase activity leads to reduced levels of ceramides and increased amounts of S1P, which results in increased resistance to cytotoxic signals. This situation is often found in cancer progression and resistance to treatments. On the other hand, a decrease of ceramidase activity provokes cell death. A number of reports point to important roles of ceramidases, mainly aCDase, in the initiation and progression of cancer, and the response of tumours to therapy [12]. Overexpression of aCDase is found in several cancer cell lines and cancer tissues [13], which appears to contribute to decreasing the levels of Cer and increasing those of S1P, thereby resulting in resistance to cell death and enhancement of cell proliferation. In most cases, aCDase inhibition induces apoptosis. Multiple reports confirm the relationship between aCDase activity and radio- or chemotherapy resistance, as well as the interest of aCDase inhibitors as anticancer drugs, either alone or in combination with other therapies [11]. The research in this field has led to implicate an over-expression of acid ceramidase (aCDase) in metastatic prostate cancer [14]. Many tumor types express high levels of acid ceramidase (aCDase). Specifically, the expression levels of aCDase in prostate cancer have been reported to be elevated relative to normal prostate tissue [15]. With these considerations in mind, a rational design of an aCDase inhibitor was undertaken. Taking into account that aCDase is a cysteine hydrolase, a small family of Cer analogs modified at the amide linkage with thiol reactive functions was generated and tested. These compounds were inspired in reported cysteine protease inhibitors [16] and included two β-haloamides and several α,β–unsaturated amides as Michael acceptors, as shown in Fig. 3.

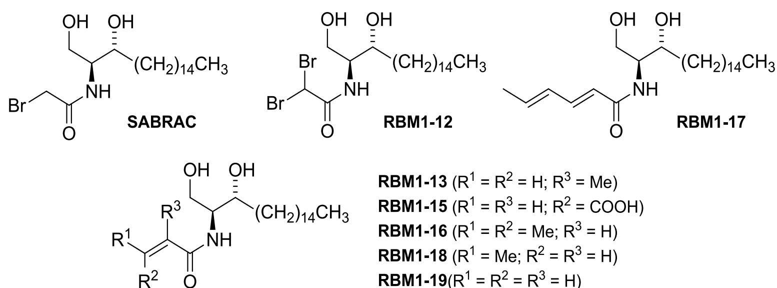

Figure 3. New acid ceramidase inhibitors.

The best inhibitors in intact cells were compounds **RBM1-12**, **RBM1-13**, **RBM1-18** and **SABRAC**, with percentages of inhibition ranging from 50 to 70%. Compounds **RBM1-12**, **RBM1-13**, and **SABRAC** were selected for further studies and were shown to be selective aCDase inhibitors in light of their lack of activity on NCDase, the enzyme that hydrolyses Cer in the cell membrane. *In vitro* dose-response determinations showed that **SABRAC** was the best inhibitor, with an IC_{50} value of 52 nM, followed by **RBM1-12** (IC_{50} = 0.53 μM) and **RBM1-13**, which exhibited the lowest potency (IC_{50} = 11.2 μM). Furthermore, in the presence of **SABRAC** and **RBM1-12**, the enzyme activity showed an exponential decay *versus* incubation time at two protein concentrations, this indicating an irreversible type of inhibition. The above observations confirmed aCDase as a therapeutic target in advanced and chemoresistant forms of prostate cancer and suggested that our new potent and specific inhibitors could act by counteracting critical growth properties of these highly aggressive tumor cells.

2. Sphingolipid analogs as fluorogenic probes

The perception that SL metabolism is composed of a highly intricate, interrelated system of enzymes, whose relative activities determine the intracellular concentration of SLs and, ultimately, the cell fate, has boosted the development of methods to monitor SL enzyme activity. In this context, the use of fluorogenic substrates (substrates that give rise to a fluorescent readout subsequent to a particular enzymatic reaction) represents a breakthrough in the design of probes suitable for determining enzyme activities. Guided by these interests, our group has been working actively in the development of new fluorogenic probes for the development of HTS methods for the screening of several SL metabolizing enzymes. With our focus on aCDase, the fluorogenic coumarinic substrates **RBM14** (Fig. 4)

were designed. After the enzymatic hydrolytic amide cleavage of the above substrates, oxidation of the resulting vicinal amino diol renders an intermediate aldehyde AL (Fig. 4), whose subsequent β-elimination under basic conditions liberates the fluorescent reporter (Fig. 4) [17,18]. Interestingly, the specificity of the substrates towards ceramidases could be modulated by choosing an appropriate acyl chain length. Thus, for aCDase, the highest rate of hydrolysis was observed for the probe with a dodecanoyl group (**RBM14-12**). The recombinant human neutral ceramidase preferred the hexadecanoyl derivative (**RBM14-16**), while the tetradecanoylamide (**RBM14-14**) was preferentially hydrolyzed by lysates of neutral ceramidase-null mouse embryonic fibroblasts at pH 8.5 in the presence of Ca^{+2}. It is worth mentioning that this fluorogenic method is currently used for the diagnosis of Farber disease, a rare disease characterized by the deficiency of aCDase [18].

The *in situ* generation of umbelliferone as a fluorescent reporter to monitor SL enzyme activity was also been applied for the development of a HTS protocol for sphingosine-1-phosphate lyase (SPL). This enzyme plays an important role in cellular functions linked to tumor progression and immunosuppression [19]. It catalyzes the retroaldol cleavage of long chain base phosphates into phosphoethanolamine and a fatty aldehyde (Fig. 5). Since both saturated and unsaturated, as well as truncated base phosphates are transformed by SPL and the reaction is highly stereoselective for the d-*erythro* isomer [20], we reasoned that compound **RBM13** contained the required structural features to behave as a suitable SPL substrate.

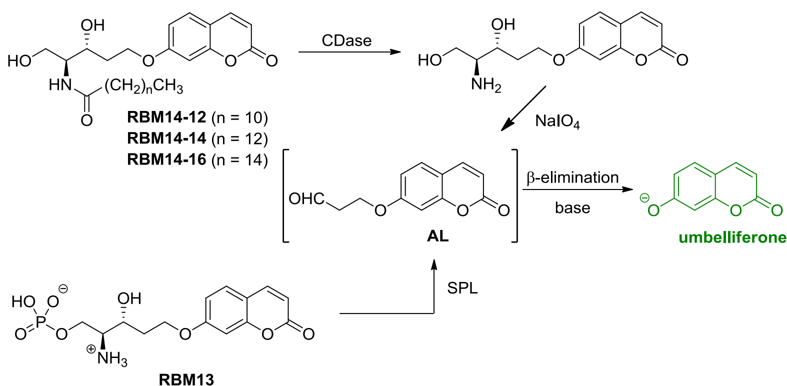

Figure 4. Fluorogenic coumarinic fluorogenic probes to determine CDase and SPL activities.

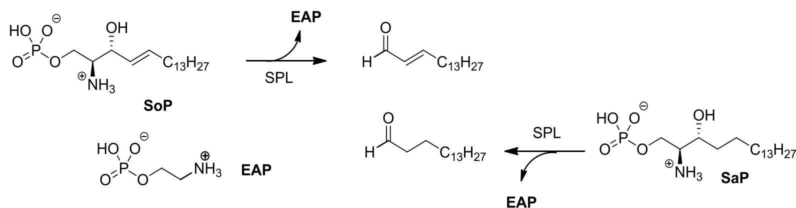

Figure 5. Retroaldol reaction catalyzed by SPL.

Upon enzymatic cleavage, aldehyde AL (Fig. 4) is first produced to render the fluorescent umbelliferone reporter after β-elimination under alkaline conditions [21]. In our optimized protocol, the assay can be performed in microtiter wells, and can be easily adapted to HTS formats.

The above substrates were inspired in the pioneering works by Reymond and co-workers for the development of a fluorogenic assay for hydrolytic enzymes [22].

The synthesis of the above probes can be carried out starting from Garner's aldehyde, following the approach indicated in Fig. 6. The common intermediate **B**, obtained from acidic hydrolysis of **A**, arising, in turn, from Garner's aldehyde in five synthetic steps [17], was selectively phosphorylated at the primary hydroxyl group, to give **C**, and further deprotected in a one-pot two-step process to the required amino phosphate **RBM13**. Alternatively, Boc removal from intermediate B, followed by standard *N*-acylation afforded the required **RBM14** probes.

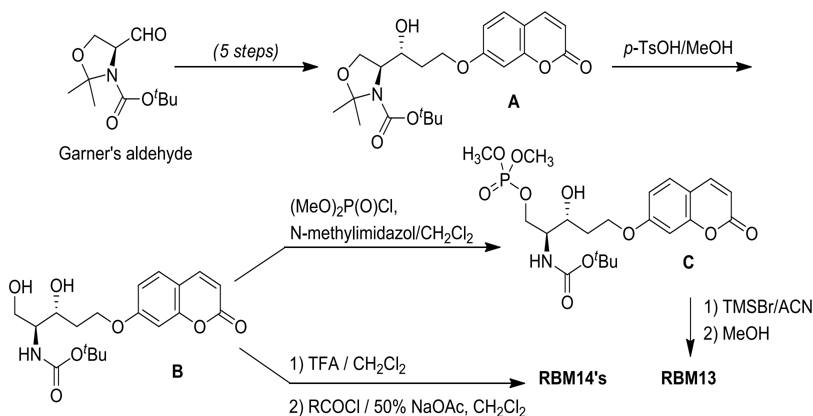

Figure 6. Synthesis of the fluorogenic coumarinic probes **RBM13** and **RBM14**.

3. Azidosphingolipids as probes to study membrane organization

The use of synthetic lipid probes for biophysical applications is a well-recognized strategy in lipid research [23]. In particular, the use of fluorescent tags is useful for the visualization of the membrane architecture and the study of its dynamic properties. The suitability of the substrate is determined by its ability to afford a fast and sensitive detection and also to behave similarly as its untagged counterpart. These two requirements are somehow contradictory when large aromatic fluorescent moieties are used. Because natural membrane lipids do not have such bulky fluorescent tags, dramatic effects on the properties of the resulting probes can be expected, especially as far as trafficking, sorting and/or domain formation is concerned [24]. Ideally, a suitable probe should be structurally similar to its natural counterpart and allow an efficient *in situ* chemoselective functionalization with a suitable fluorescent reagent in a natural environment. These requirements can be envisaged by a judicious use of bioorthogonal chemical reporter strategies, a technique that has become common place for the labelling of biomolecules [25,26]. Based on these premises, and aiming to widen the scope of our research, we undertook the synthesis of the α- and ω-azido probes **RBM2-79** and **RBM 2-77** shown in Fig. 7. As sphingolipid analogs, these probes are amenable to incorporation into natural or artificial membranes. In addition, due to the presence of the terminal azido group, the possibility of a bioorthogonal alkyne-azide cycloaddition "click" reaction with the fluorogenic tag **D** [27] was considered.

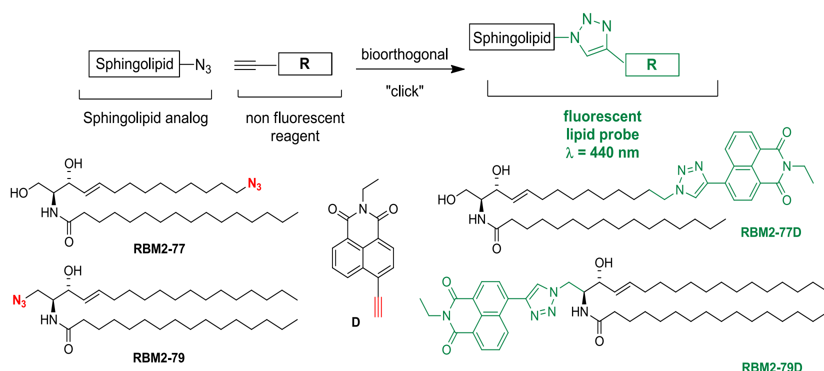

Figure 7. Design of bioorthogonal "click" reactions with azido sphingolipid probes **RBM2-77** and **RBM2-79**. Doble enlace en triazol

In this context, the recent advances in the development of bioorthogonal reactions have boosted their applications in chemical biology [28]. In particular, those involving the Cu(I)-catalyzed Huisgen [3+2] cycloadditions of terminal alkynes with azides [29] (CuAAC, the paradigm of "click chemistry" [30]) have become attractive to researchers due to their simplicity and high reactivity. In order to avoid the potential toxicity of Cu(I) salts, several modifications have been developed to reduce Cu(I) concentration, as the use of water-soluble Cu(I) ligands [31,32] or Cu(I)-chelating azides [33].

In our case, the probes shown in Fig. 7 have been designed to mimic the behaviour of natural ceramides in artificial membranes [34]. Membrane ceramides are important metabolic signals [35,36] that are known to separate laterally to give rise to gel-like ceramide-enriched domains [37–39]. Because of their structural similarity, our probes **RBM2-77** and **RBM2-79** were able to orient in lipid bilayers in parallel with the phospholipids, and eventually to give rise to domains similarly to natural ceramides. Gratifyingly, our *in situ* synthetic method allowed the observation of ceramide domains in living cells.

Click reactions required the use of an *in situ* generated Cu^{+} catalyst by ascorbate promoted reduction of a Cu^{2+} salt. The photoactivation of the probes was checked by microscopy experiments using giant unilamellar vesicles (GUVs) of ePC:**1** and ePC:**2** (10 mol% of clickable probe in both cases). When GUVs were treated with the labeling solution, a clear fluorescence intensity was collected in both cases between 450-500 nm, which was attributed to the formation of the corresponding fluorescent click cycloadducts shown in Fig. 7. This fluorescence was not observed when GUVs were incubated under control conditions (in the absence of the Cu^{2+} salt catalyst precursor) after 3h incubation. These results constitute a proof of principle that fluorescent ceramide derivatives may be formed within lipid membranes starting from minimally modified non-fluorescent azido sphingolipids. This technique can be extended to the study of ceramide-enriched domains by fluorescent confocal microscopy and also to the study of the so-called ceramide platforms [40]. Finally, despite Cu^{2+} may be toxic to cells, localization of ceramide-rich domains in cell membranes can be performed on fixed cell preparations. In any, case, the use of Cu-free click chemistry protocols [41] is also considered as a natural evolution of this technique.

The above probes were synthesized following standard protocols, as exemplified for **RBM2-77** in Fig. 8. Thus, the cross methathesis [42,43] of 11-bromo-1-undecene with vinyl alcohol **E**, obtained from Garner's aldehyde following a reported protocol [44], afforded bromide **F** in moderate

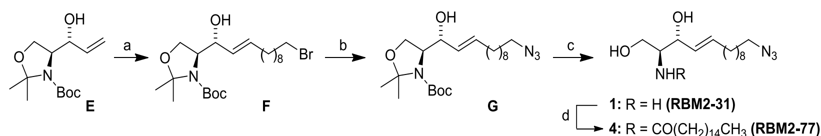

Figure 8. Synthesis of probe **RBM2-77**; a: 11-bromo-1-undecene, Grubb's 2nd generation, CH_2Cl_2, 45 ºC, 59%; b: NaN_3, DMF, 80 ºC (93%); c: $ClCOCH_3$, MeOH, rt, 1h (84%); d: $C_{15}H_{31}COOH$, EDC. HOBt, Et_3N, CH_2Cl_2, 65%.

yield and excellent *E*-selectivity. Reaction of **F** with excess NaN_3 in DMF at 80 ºC, followed by the simultaneous deprotection of the oxazolidine and *N*-Boc groups of intermediate azide **G** under acidic conditions, afforded **RBM2-31** in excellent yield. Acylation with palmitic acid, using EDC/HOBt as coupling system, afforded probe **RBM2-77**.

4. Conclusions

In this review we have tried to show the potential of the chemical modifications of sphingolipids by means of a selection of some of our recent results in this area. Thus, the biochemical functions of natural sphingolipids can be efficiently modulated by the judicious design of analogs addressed at interfering with specific enzymes of key sphingolipid metabolic pathways. In this account, this approach has been illustrated with the design of a new family of aCDase inhibitors, which have also allowed the identification of this enzyme as a therapeutic target in chemoresistant forms of prostate cancer. In a conceptually different approach, chemical modifications of sphingolipids have also been used to design chemical probes with specific applications in structural and cell biology. This is the case of the fluorogenic probes **RBM13** and **RBM14**, designed to develop HTS protocols to monitor the activity profiles of SPL and CDases, respectively. In a related context, the azido sphingolipids **RBM2-77** and **RBM2-79** have found applications in structural biology for their ability to visualize the membrane organization of natural ceramides after a biorthogonal click reaction with a suitable fluorogenic reporter.

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References

1. Hannun, Y. A. , Obeid, L. M. 2008, *Nat. Rev. Mol. Cell Biol.*, 9, 139.
2. Sillence, D. J. 2007, *Int. Rev. Cytol.*, 262, 151.
3. Gomez-Munoz, A. 2006, *Biochim. Biophys. Acta*, 1758, 2049.
4. Spiegel, S., Milstien, S. 2002, *J. Biol. Chem.*, 277, 25851.
5. Lev, S., 2010, *Nat. Rev. Mol. Cell Biol.*, 11, 739.
6. Kolter, T., 2011, *Chem. Phys. Lipids*, 164, 590.
7. Goñi, F. M., Sot, J., Alonso, A. 2014, *Biochem. Soc. Trans.*, 42, 1401.
8. Ryland, L. K. , Fox, T. E., Liu, X., Loughran, T. P., Kester, M. 2011, *Cancer Biol. Ther.*, 11, 138.
9. Hla, T. , Dannenberg, A. J. 2012, *Cell Metab.*, 16, 420.
10. Bismuth, J. , Lin, P., Yao, Q. , Chen, C. 2008, *Atherosclerosis*, 196, 497.
11. Fabrias, G., Bedia, C. , Casas, J., Abad, J. L., Delgado, A. 2011, *Anticancer Agents Med. Chem.*, 11, 830.
12. Liu, X., Elojeimy, S., Turner, L. S., Mahdy, A. E. M., Zeidan, Y. H., Bielawska, A., Bielawski, J., Dong, J.-Y., El-Zawahry, A. M., Guo, G. W., Hannun, Y. A., Holman, D. H., Rubinchik, S., Szulc, Z., Keane, T. E., Tavassoli, M., Norris, J. S. 2008, *Front. Biosci.*, 13, 2293.
13. Elojeimy, S. , Liu, X., McKillop, J. C., El-Zawahry, A. M., Holman, D. H., Cheng, J. Y., Meacham, W. D., Mahdy, A. E. M., Saad, A. F., Turner, L. S., Cheng, J., A Day, T., Dong, J. Y., Bielawska, A., Hannun, Y. A., Norris, J. S. 2007, *Mol. Ther.*, 15, 1259.
14. Camacho, L., Meca-Cortés, O., Abad, J. L., García, S., Rubio, N., Díaz, A., Celiá-Terrassa, T., Cingolani, F., Bermudo, R., Fernández, P. L., Blanco, J., Delgado, A., Casas, J., Fabriàs, G., Thomson, T. M. 2013, *J. Lipid Res.*,
15. 54, 1207.
16. Seelan, R. S., Qian, C., Yokomizo, A., Bostwick, D. G., Smith, D. I., Liu, W. 2000, *Genes Chromosom. Cancer*, 29, 137.
17. Otto, H. H., Schirmeister, T. 1997, *Chem. Rev.*, 97, 133.
18. Bedia, C., Casas, J., Garcia, V., Levade, T., Fabrias, G. 2007, *ChemBioChem*,
19. 8, 642.
20. Bedia, C., Camacho, L., Abad, J. L., Fabrias, G., Levade, T. 2010, *J. Lipid Res.*, 51, 3542.
21. Bourquin, F., Riezman, H., Capitani, G., Grutter, M. G. 2010, *Structure*,
22. 18, 1054.
23. van Veldhoven, P. P., Mannaerts, G. P. 1993, *Adv. Lipid Res.*, 26, 69.
24. Bedia, C., Camacho, L., Casas, J., Abad, J. L., Delgado, A., Van Veldhoven, P. R., Fabrias, G. 2009, *ChemBioChem*, 10, 820.
25. Wahler, D., Badalassi, F., Crotti, P., Reymond, J. L. 2001, *Angew. Chem. Int. Ed. Engl.*, 40, 4457.
26. Loura, L. M., Ramalho, J. P. 2011, *Molecules*, 16, 5437.
27. Maier, O., Oberle, V., Hoekstra, D. 2002, *Chem. Phys. Lipids*, 116, 3.
28. Neef, A. B., Schultz, C. 2009, *Angew. Chem. Int. Ed. Engl.*, 48, 1498.
29. Lim, R. K. V., Lin, Q. 2010, *Chem. Commun.*, 46, 1589.

30. Sawa, M., Hsu, T. L., Itoh, T., Sugiyama, M., Hanson, S. R., Vogt, P. K., Wong, C. H. 2006, *Proc. Natl. Acad. Sci. U. S. A.*, 103, 12371.
31. Lang, K., Chin, J. W. 2014, *ACS Chem. Biol.*, 9, 16.
32. Rostovtsev, V. V., Green, L. G., Fokin, V. V., Sharpless, K. B. 2002, *Angew. Chem. Int. Ed. Eng.*, 41, 2596.
33. Kolb, H. C., Finn, M. G., Sharpless, K. B. 2001, *Angew. Chem. Int. Ed. Engl.*, 40, 2004.
34. Hong, V., Steinmetz, N. F., Manchester, M., Finn, M. G. 2010, *Bioconjug. Chem.*, 21, 1912.
35. Besanceney-Webler, C., Jiang, H., Zheng, T., Feng, L., Soriano del Amo, D., Wang, W., Klivansky, L. M., Marlow, F. L., Liu, Y., Wu, P. 2011, *Angew. Chem. Int. Ed. Engl.*, 50, 8051.
36. Kuang, G.-C., Michaels, H. A., Simmons, J. T., Clark, R. J., Zhu, L. 2010, *J. Org. Chem.*, 75, 6540.
37. Garrido, M., Abad, J. L., Alonso, A., Goni, F. M., Delgado, A., Montes, L.-R. 2012, *J. Chem. Biol.*, 5, 119.
38. Stancevic, B., Kolesnick, R. 2010, *FEBS Lett.*, 584, 1728.
39. Gulbins, E., Kolesnick, R. 2003, *Oncogene*, 22, 7070.
40. Kooijman, E., Sot, J., Montes, R., Alonso, A., Gericke, A., De Kruijff, B., Kumar, S., Goñi, F. M. 2008, *Biophys. J.*, 94, 4320.
41. Goñi, F. M., Alonso, A. 2009, *Chem. Phys. Lipids*, 160, S2.
42. de Almeida, R. F., Loura, L. M., Prieto, M. 2009, *Chem. Phys. Lipids*, 157, 61.
43. Cremesti, A. E., Goñi, F. M., Kolesnick, R. 2002, *FEBS Lett.*, 531, 47.
44. Mizukami, S., Hori, Y., Kikuchi, K. 2014, *Acc. Chem. Res.*, 47, 247.
45. Chatterjee, A. K., Choi, T. L., Sanders, D. P., Grubbs, R. H. 2003, *J. Am. Chem. Soc.*, 125, 11360.
46. Peters, C., Billich, A., Ghobrial, M., Hogenauer, K., Ullrich, T., Nussbaumer, P. 2007, *J. Org. Chem.*, 72, 1842.
47. Herold, P. 1988, *Helv. Chim. Acta.*, 71, 354.