



HAL
open science

Resistance to alkyl-lysophospholipid-induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin- and cholesterol-deficiency in lipid rafts

Arnold H. van Der Luit, Marianne Budde, Shuraila Zerp, Wendy Caan, Jeffrey B. Klarenbeek, Marcel Verheij, Wim J. van Blitterswijk

► To cite this version:

Arnold H. van Der Luit, Marianne Budde, Shuraila Zerp, Wendy Caan, Jeffrey B. Klarenbeek, et al.. Resistance to alkyl-lysophospholipid-induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin- and cholesterol-deficiency in lipid rafts. *Biochemical Journal*, 2006, 401 (2), pp.541-549. 10.1042/BJ20061178 . hal-00478643

HAL Id: hal-00478643

<https://hal.science/hal-00478643>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Resistance to alkyl-lysophospholipid-induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin and cholesterol deficiency in lipid rafts

Arnold H. VAN DER LUIT*¹, Marianne BUDDE*, Shuraila ZERP[†], Wendy CAAN*, Jeffrey B. KLARENBEK*, Marcel VERHEIJ[†] and Wim J. VAN BLITTERSWIJK*²

*Division of Cellular Biochemistry and [†]Department of Radiotherapy, The Netherlands Cancer Institute/ Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Short title: Apoptosis resistance due to lack of sphingomyelin synthesis

¹ Present address: IFR 100, Ath rome / Cancer, Facult  de M decine, 7 Bd Jeanne d'Arc, BP 87900, 21079 Dijon Cedex, France

² To whom correspondence should be addressed (Tel: +31-205121976; Fax: +31-205121989; email w.v.blitterswijk@nki.nl)

Abbreviations used: BSA, bovine serum albumin; SMS, sphingomyelin synthase; SM, sphingomyelin; exoSM, exogenous SM, C12:0-, C16:0-SM, lauroyl-, palmitoyl-SM; ALP, alkyl-lysophospholipid; FasL, Fas ligand; M β CD, methyl- β -cyclodextrin; PBS, phosphate-buffered saline; PC, phosphatidylcholine; LPC, lysoPC; PE, phosphatidylethanolamine; LacCer, lactosylceramide; GlcCer, glucosylceramide; So, sphingosine.

Synopsis

The alkyl-lysophospholipid (ALP) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine induces apoptosis in S49 mouse lymphoma cells. To this end, ALP is internalized by lipid raft-dependent endocytosis and inhibits phosphatidylcholine synthesis. A variant cell line, S49^{AR}, made resistant to ALP, was previously found incapable of ALP internalization via this lipid raft pathway. The reason for this uptake failure is not understood. Here, we report that S49^{AR} cells are unable to synthesize sphingomyelin due to downregulated sphingomyelin synthase (SMS1) expression. In parental S49 cells, resistance to ALP could be mimicked by small interfering RNA-induced SMS1 suppression, resulting in sphingomyelin deficiency and blockade of raft-dependent internalization of ALP and apoptosis induction. Similar results were obtained by treatment of the cells with myriocin/ISP-1, an inhibitor of general sphingolipid synthesis, or with U18666A, a cholesterol homeostasis disturbing agent. U18666A is known to inhibit Niemann-Pick C1 protein-dependent vesicular transport of cholesterol from endosomal compartments to the trans-Golgi network and the plasma membrane. U18666A reduced cholesterol partitioning in detergent-resistant lipid rafts and inhibited sphingomyelin synthesis in S49 cells, causing ALP resistance similar as in S49^{AR} cells. The data are explained by the strong physical interaction between (newly synthesized) sphingomyelin and available cholesterol at the Golgi, which facilitates lipid raft formation at the Golgi. We propose that ALP internalization by raft-dependent endocytosis represents the retrograde route of a constitutive SMS1- and raft-dependent membrane vesicular recycling process.

Key words: apoptosis resistance; edelfosine; lipid raft; sphingomyelin synthase; cholesterol; endocytosis

Introduction

The synthetic alkyl-lysophospholipid, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ALP; Et-18-OCH₃; Edelfosine) is the prototype of a group of new clinically used anti-cancer agents [1-4]. These unnatural phospholipids with one single alkyl chain readily insert into the plasma membrane lipid bilayer, accumulate because they are poorly degradable by lipases [5], and disturb lipid metabolism in the cell. For example, ALP inhibits the activity of CTP:phosphocholine cytidylyltransferase, the rate-determining enzyme for phosphatidylcholine (PC) biosynthesis [6]. We reported that this cytidylyltransferase inhibition is a direct trigger for apoptosis in S49 lymphoma and HeLa carcinoma cells [7,8]. Since cytidylyltransferase is active inside the cell (ER and nucleus [9]), ALP needs to be internalized to inhibit the enzyme. We found that ALP preferentially accumulates in a detergent-resistant sphingolipid- and cholesterol-rich membrane fraction, representative for microdomains known as lipid rafts [7]. ALP is then internalized by lipid raft- and dynamin-dependent, clathrin-independent endocytosis [7,8]. In an ALP-resistant cell variant, S49^{AR}, selected by continuous culturing of S49 cells in the presence of ALP, we found no raft-dependent ALP internalization and, consequently, no disturbance of the PC metabolism and no initiation of apoptosis [7]. The mechanism behind this uptake failure and consequent cellular resistance to ALP is unknown.

As a first approach to elucidate why lipid rafts in S49^{AR} cells are unable to mediate internalization of ALP, we questioned if the rafts in these resistant cells have a different lipid composition compared to the parental S49 cells. This question is the more relevant since we found that artificial breakdown of plasma membrane sphingomyelin (SM) by an exogenous (bacterial) sphingomyelinase, or extraction of cholesterol prevented ALP internalization and apoptosis [7]. We therefore focused on the analysis of the sphingolipids, as these are major raft constituents that, in association with cholesterol, determine the detergent insolubility [10]. It is well known that SM and cholesterol show tight mutual interaction [11,12] and that cellular SM levels therefore co-regulate cholesterol homeostasis [12,13]. Conversely, whether changes in membrane cholesterol may also affect the levels and/or synthesis of SM is less clear [13]. To disturb cholesterol

homeostasis in the present study, we used the amphiphilic drug U18666A, a well established inhibitor of Niemann-Pick C (NPC1) protein-mediated vesicular transport of cholesterol from endosomal compartments to the trans-Golgi network and the plasma membrane [14-17].

We report here that the ALP-resistant S49^{AR} cells are unable to synthesize SM through downregulated sphingomyelin synthase (SMS1) expression. This lack of SM is especially apparent in the detergent-resistant membrane fraction together with a decrease in cholesterol. Conversely, disturbance of cholesterol homeostasis, or siRNA-induced downregulation of SMS1 directly, prevented SM synthesis in parental S49 cells. We show in either cell variant that the lack of SM production is directly linked to abrogated ALP uptake and apoptosis resistance.

EXPERIMENTAL

Materials

ALP (Et-18-OCH₃; Edelfosine; 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) and U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) were purchased from BioMol (Plymouth Meeting, PA). [³H]Et-18-OCH₃ (³H-ALP; 58 Ci/mmol), was synthesized by Moravsek Biochemicals (Brea, CA). [*Methyl*-¹⁴C]choline chloride (58 mCi/mmol), L-[3-¹⁴C]serine (54 mCi/mmol) and [1α,2α(n)-³H]cholesterol (49 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). [1-³H]sphingosine was synthesized by Mr. Piet Weber at DSM (Delft, The Netherlands). N-Lauroyl-D-*erythro*-sphingosylphosphorylcholine (Lauroyl sphingomyelin; C12:0-SM) was from Avanti Polar Lipids, Inc. (Alabaster, AL). C6-NBD-ceramide (N-[6-[(7-nitro-benz-2-oxa-1,3-diazo-4-yl)amino]caproyl]-ceramide) was from Molecular Probes, Inc. (Invitrogen Life Technologies, Breda, The Netherlands). D609 (tricyclodecan-9-yl-xanthogenate) was from Calbiochem-Novabiochem GmbH (Schwalbach, Germany). Myriocin/ISP-1 was from Sigma. Soluble recombinant human Fas ligand (FasL; APO-1L) was from Alexis (Leiden, The Netherlands). Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 x 20 cm) were from Merck (Darmstadt, Germany).

Cells and culture conditions

Mouse S49.1 lymphoma cells (S49) were cultivated in Dulbecco's modified Eagle's medium (Gibco-Invitrogen), supplemented with 8% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin at 37°C and 5% CO₂. ALP-resistant S49^{AR} cells were obtained by two selection rounds of growth of S49 cells in the presence of 15 μM ALP (Et-18-OCH₃) for 72 hours, followed by plating in semi-solid medium and isolation of colonies of surviving cells [18]. The selected S49^{AR} clone could be grown continuously in 15 μM ALP with a doubling time of 12 h, similar to that of the parental S49 cells. S49^{AR} cells maintained resistance by treatment with ALP every two weeks. All experiments with S49^{AR} cells were performed with cells grown without the selection agent for at least three days.

Loading of S49^{AR} cells with exogenous C12:0-sphingomyelin was performed by injecting the ethanol-dissolved lipid into the complete culture medium (final concentration 20 μ M C12:0-SM; 0.02% ethanol) and culturing the cells in this medium for three days.

PCR and retroviral transduction

In order to amplify SMS1 and SMS2 by reverse transcriptase PCR, the following primers were used: *forward* SMS1 primer CCGATGGCAGCTTCAGCATC; *reverse* SMS1 primer AGCCGCCGAGGAGAATACTC; *forward* SMS2 primer GTTGGCCATGGAATGGTACC; *reverse* SMS2 primer CTCACATCCTGTGCGGAGAC. As a control, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was amplified using *forward* primer TGCACCACCTGCTTAG and *reverse* primer GGATGCAGGGATGATGTTC. For real-time PCR, we used as *forward* SMS1 primer TGGCATGCATTTCAACTGTTC; and as *reverse* SMS1 primer AGCTTCATTA TTCTCCGCACTTG. The amplification was related to the hypoxanthine guanine phosphoribosyl transferase 1 (HRPT) gene, with as *forward* primer CTGGTAAAAGGACCTCTCG; and as *reverse* primer TGAAGTACTCATTATAGTCAAGGGCA. In order to silence the expression of SMS1, the following primers were used: *sense* GATCCCCGCATGGGAGTTGATTTAGATTCAAGAGATCTAAATCAACTCCCAT GCTTTTTGGAAA and *antisense* AGCTTTTCCAAAAAGCATGGGAGTTGATTTA GATCTCTTGAATCTAAATCAACTCCCATGCGGG.

For retroviral gene transduction, cDNAs were cloned into the retroviral vector pRETRO-SUPER [19]. Empty vector or vector containing the *siRNA* oligonucleotide directed to SMS1 were transfected into the 293T human embryonic kidney cell-derived packaging line Phoenix Ampho, using FuGene-6 transfection reagent, according to instructions of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Transfected cells were selected with 1 μ g/ml puromycin (Invitrogen Life Technologies (Breda, The Netherlands)). Virus-containing supernatants were harvested after 2-5 days and stored at -80 °C until further use. S49 cells were seeded on dishes coated with RetroNectin (Roche Molecular Biochemicals) and transduced with 1 ml of virus-

containing supernatant per 0.5×10^6 cells. Supernatants were removed after overnight incubation, and cells were cultured in fresh medium. Transduced cells were selected from 48 h after transduction for growth in the presence of 200 $\mu\text{g/ml}$ puromycin.

ALP uptake and apoptosis assay

Cells were grown to a density of $1 \times 10^6/\text{ml}$ and ALP (Et-18-OCH₃; Edelfosine) was added in the effective apoptotic concentration of 15 μM , supplemented with 0.2 μCi ³H-ALP/ml. At given time points, samples were taken, put on ice for 2 minutes and then washed three times with ice-cold PBS. Samples were lysed in 0.1 N NaOH prior to liquid scintillation counting.

Apoptosis was induced with ALP (15 μM) or FasL (500 ng/ml) for 6h. Cells were washed in PBS and lysed overnight at 4°C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 $\mu\text{g/ml}$ propidium iodide, according to Nicoletti et al. [20]. Fluorescence intensity of propidium iodide-stained nuclei was determined on a FACScan (Becton Dickinson), and data were analyzed using Lysis software.

Lipid analysis

Cells at 1×10^6 cells/ml were incubated with [*methyl*-¹⁴C]choline chloride (1 $\mu\text{Ci/ml}$), [³H]sphingosine (1 $\mu\text{Ci/ml}$), L-[3-¹⁴C]serine (0.4 $\mu\text{Ci/ml}$), or [³H]cholesterol (0.02 $\mu\text{Ci/ml}$) where indicated. At given time points, aliquots of cells were taken, washed and resuspended in 200 μl PBS. Lipids were extracted with chloroform/methanol (1:2, v/v) and phase separation was induced using 1 M NaCl. The organic phase was washed in a solution of methanol/H₂O/chloroform, 47:49:3 (v/v/v), and separated by one-dimensional silica TLC, using chloroform:methanol:acetic acid:water, 60:30:8:5 (v/v/v/v) or, in the case of sphingosine labeling, using chloroform:methanol:0.2% (w/v) CaCl₂, 60:40:9 (v/v/v). Two-dimensional TLC was performed using two times chloroform/methanol/NH₄OH (60:60:5, v/v/v), with intermittent drying, in the first dimension, followed by one time chloroform/methanol/acetic acid:water (50:30:8:4, v/v/v/v) in the second dimension. Radioactive lipids were visualized and quantified using a Fuji BAS 2000 TR Phosphor-Imager and identified using internal standards, which were visualized by iodine staining. Tritiated lipids were visualized after dipping the TLC

plate in 12.5% diphenyloxazole dissolved in diethylether, drying and subsequent autoradiography. Unlabeled lipids (e.g. C12:0-SM) were visualized by spraying the plate with sulfuric acid, followed by heating (charring) in an oven at 180 °C for 30 min.

Mass sphingolipid analyses were conducted by liquid chromatography, electrospray tandem mass spectrometry as described by Merrill et al. [21].

***In vitro* SM synthase assay**

SM synthesis *in vitro* was performed (1 hour, 37°C) on a crude membrane preparation from HeLa cells, using C6-NBD-ceramide (60 nM) as a substrate [22]. To this end, cells were homogenized by 5x passing through a 27½ G needle. Nuclei were spun down at 300 x *g*, 10 minutes, and from the supernatant, crude membranes were obtained by centrifugation at 100,000 x *g* for 1 hour. Lipids were extracted and separated by TLC (see above).

Isolation of lipid rafts

A lipid raft fraction was prepared by detergent extraction of cells and sucrose gradient centrifugation, essentially as described [23]. In brief, 2×10^8 cells were solubilized into 1 ml of an ice-cold buffer consisting of 25 mM MES, 150 mM NaCl and 1% Triton X-100, and homogenized using a Dounce homogenizer. The extract was centrifuged on a discontinuous sucrose gradient at 39,000 rpm in a SW41 rotor for 20 hours at 4°C, followed by 11×1.0 ml fractions manual collection from the top of the gradient.

Results

S49 lymphoma cells made resistant to ALP (S49^{AR} cells) show a defect in the synthesis of sphingomyelin

Previously, we reported that the synthetic ether-lipid Et-18-OCH₃ (Edelfosine; ALP) induces apoptosis in S49 lymphoma cells, in a dose- and time-dependent fashion. The onset of apoptosis in these cells was relatively fast, and already apparent after 3 hours [7]. An ALP-resistant variant cell line, S49^{AR}, generated by culturing S49 cells in the presence of ALP and continuous selection of surviving cells, did not undergo apoptosis by ALP. S49^{AR} cells were not derived from a single cell clone with a single genetic mutation, but is a population of cells in which the sensitivity to ALP is suppressed in a reversible manner: After 4-5 weeks of culturing in the absence of ALP, the cells gradually become resensitized to the lipid, and regain the phenotypic properties (to be described below) of the parental S49 cells (data not shown). We demonstrated that, in order to induce apoptosis, ALP needs to be internalized by endocytosis via lipid rafts [7,8]. This particular process was disrupted in the ALP-resistant S49^{AR} cells, although ALP still remained associated to the detergent-resistant raft fraction from these cells [7]. Since the uptake of ALP could be prevented by treatment of cells with cholesterol sequestering agents or the breakdown of SM [7], we addressed the question whether the difference in ALP sensitivity of the cells could be related to a different lipid composition of their lipid rafts, which may impede internalization. We focused on analyzing the sphingolipids, as being major raft constituents [10]. Importantly, we found that S49^{AR} cells were deficient in sphingomyelin (SM) (Figure 1; Table 1). Radiolabeling of the cells with the SM precursors [¹⁴C]choline or [¹⁴C]serine, followed by lipid analysis by 2D-TLC separation, revealed two typical SM spots in S49 cells, which were absent in S49^{AR} cells (Figure 1A). These two SM spots correspond respectively to a (major) pool with relatively short acyl chains (16 C-atoms) and a (minor) SM pool with long acyl chains (predominantly C24:1) and containing a C16-dihydro- (sphinganine) species (Table 1) [24]. No other conspicuous differences in lipid profile between the two cell lines were seen on these 2-D TLC plates.

The lack of SM synthesis in S49^{AR} cells was also reflected in SM mass levels in these cells, as determined by mass spectrometry (Table 1). S49 cells contain mainly the C16:0-SM species, which is reduced to about 20% in the S49^{AR} cells. Given the almost complete lack of SM synthesis in S49^{AR} cells (see also Figures 1B, 2 and 3), this residual mass SM has most likely been taken up by the cells directly from the fetal calf serum in the culture medium, as this serum is also rich in C16:0-SM (Table 1).

To visualize the synthesis of other sphingolipids in the cells, we radiolabeled the cells with a different metabolic precursor, [1-³H]-sphingosine. Figure 1B shows the time-dependent synthesis of sphingolipids in the two cell lines. It is clear that, during the 4 h time period, S49^{AR} cells were unable to synthesize SM from sphingosine, while S49 cells were able to do so (predominantly the SM pool with C16 acyl-length), evidently via the intermediate ceramide. No profound differences in the synthesis of glucosylceramide (GlcCer) or lactosylceramide (LacCer) were seen between the two cell lines. The spot of phosphatidylethanolamine (PE) represents a metabolite from sphingolipid degradation, via sphingosine-1-phosphate lyase-mediated phosphoethanolamine production, as shown and discussed before [25].

We next isolated the detergent-resistant lipid raft fractions from [³H]sphingosine-labeled cells, using sucrose density-gradient centrifugation. Figure 2 shows that, in S49 cells, SM is concentrated in the lipid raft fractions (3 to 5), as expected, whereas SM is lacking in the raft fractions from S49^{AR} cells. Again, no clear differences in the other lipids were seen between the two cell lines. GlcCer, LacCer and ceramide were normally present in the raft fractions of both cell lines. Taken together, the data indicate that ALP-resistant S49^{AR} cells show a specific defect in the synthesis of the lipid raft component SM, but not of other (sphingo)lipids. Furthermore, given the type of SM precursors used, and the unimpeded production of the precursor ceramide (Figures 1B and 2), it is apparent that the defect in SM production lies in the final biosynthetic step, catalyzed by SM synthase (SMS).

S49^{AR} cells do not express SMS, whereas S49 cells only express SMS1

In order to investigate the mechanism underlying the defective SM synthesis in S49^{AR} cells, we analyzed the expression of SMS1 and SMS2, the two recently identified

isozymes capable of executing the final enzymatic step of SM synthesis [26,27]. RT-PCR analysis revealed SMS1 expression in S49 but not in S49^{AR} cells (Figure 3A). SMS2 was not detectable at all in either cell type (transcripts were not visible after amplification up to 45 cycles of PCR). This result was verified by real-time PCR: Compared to S49 cells, SMS1 expression in S49^{AR} cells was about 50-fold reduced (Figure 3B). SMS2 was not expressed in either cell type (no detectable transcript after 50 PCR cycles, whereas it was readily detected in cDNA made from mouse embryo cells, as a positive control; data not shown). Thus, the lack of SMS expression in S49^{AR} cells directly explains the defect in SM synthesis in the S49^{AR} cells.

Downregulation of SMS1 expression by siRNA blocks SM synthesis and prevents ALP uptake and apoptosis induction in S49 cells

We previously demonstrated that S49^{AR} cells are resistant to ALP because, contrary to S49 cells, they are unable to internalize ALP via lipid rafts [7]. Since S49^{AR} cells lack SMS expression and SM synthesis, we first tried to introduce the SMS1 gene into S49^{AR} cells. This, however, resulted in loss of viability of SMS1-transfected cells (both in S49 and in S49^{AR} cells; data not shown). We next downregulated SMS1 expression in S49 cells by stable transduction of small interfering RNA (siRNA) specific for SMS1, yielding so-called S49^{siSMS1} cells, to see if reduced SM synthesis would lead to impaired ALP internalization, like in the S49^{AR} cells. SMS1 mRNA levels in S49^{siSMS1} cells were 6-fold reduced, as revealed by real-time PCR (Figure 3B). Figure 3C confirms that these SMS1-downregulated cells show significantly reduced SM synthesis, down to 10% of mock-transduced S49 control cells, whereas little or no changes were seen in the synthesis of the other sphingolipids. Thus, by inhibiting SMS1 gene expression specifically, we can block the synthesis of SM in S49 cells to a similar low level as in S49^{AR} cells.

We next tested if the inhibition of SM synthesis would lead to reduced ALP uptake. Figure 4A shows that, indeed, the time-dependent uptake of ³H-ALP by S49^{siSMS1} cells was decreased to a similar extent as observed in S49^{AR} cells, whereas S49 and S49^{mock} cells showed the same high uptake kinetics as previously reported [7]. The synthesis of SM is, therefore, essential for ALP internalization in these cells.

The production of SM can also be inhibited indirectly by preventing the synthesis of the SMS1 substrate ceramide, using myriocin/ISP-1, an inhibitor of serine-palmitoyltransferase [28], the initiating enzyme of the sphingolipid biosynthetic pathway. This inhibitor (at 5 μ M, 5 h) was found to decrease the incorporation of 14 C-serine precursor into 14 C-SM of S49 cells by 63%. Figure 4B shows that myriocin/ISP-1 inhibited the uptake of 3 H-ALP by S49 cells depending on the time of preincubation (1h or 16 h) with this inhibitor, to values that were intermediate between those of S49 and S49^{AR} cells.

We next tested if the reduced uptake of ALP in SMS1-downregulated S49^{siSMS1} cells would abrogate apoptosis induction in these cells, like in the S49^{AR} cells. Figure 4C shows that, indeed, the level of apoptosis induced by ALP in these SMS1-deficient cells dropped from 55% (S49 and S49^{mock} cells) to 15%. The sphingolipid synthesis inhibitor myriocin/ISP-1 (5 μ M, preincubated for 2h) inhibited ALP-induced apoptosis in S49 cells, measured at 6 h, by 31 \pm 6 % (n=4) (data not shown in figure). We note, however, that the inhibitor tended to be apoptotic by itself at longer preincubation times.

To answer the question if the reduced ALP uptake in the SMS1-deficient was due to the lack of SMS activity or the lack of SM as such in plasma membrane rafts, we loaded the S49^{AR} cells with exogenous SM. In our experience, the most efficient way to do this was to let the cells take up lauroyl-SM (C12:0-SM, which worked better than SM with longer acyl chains) via the serum lipoproteins in the culture medium. To this end, C12:0-SM was injected into the complete culture medium at 20 μ M final concentration (see 'Experimental'). After three days of culturing in this medium, cells were washed and their lipid rafts were isolated. Figure 5A shows that the exogenous C12:0-SM (exoSM) was incorporated in the S49^{AR} detergent-resistant raft (R) fractions to at least similar (or higher) proportions as the endogenous C16:0-SM in rafts from S49 cells. However, this exoSM loading of S49^{AR} cells did not result in more 3 H-ALP uptake by these cells (Figure 5B), nor did it affect the resistance of these cells to ALP-induced apoptosis (Figure 5C).

Collectively, these data strongly suggest that SM synthesis, rather than a mere SM accumulation in lipid rafts at the plasma membrane, is required for ALP internalization to induce apoptosis.

SM deficiency in lipid rafts is accompanied by reduced cholesterol content

Paradoxically, we demonstrated in previous work [7,8] that raft SM hydrolysis by exogenous (bacterial) sphingomyelinase prevented ALP internalization and apoptosis induction. The same happened when lipid rafts were disrupted by cholesterol sequestering agents such as methyl- β -cyclodextrin. Furthermore, it is well known that SM tightly associates with cholesterol [11,12], which favors lipid raft formation and causes detergent insolubility of these membrane microdomains [29]. We therefore questioned if the lack of SM synthesis in S49^{AR} cells would be accompanied by decreased cholesterol content in the lipid raft fraction. To answer this question, we incubated S49 and S49^{AR} cells with exogenous [³H]cholesterol overnight to label the various subcellular compartments to equilibrium, and then determined the distribution of [³H]cholesterol among the sucrose gradient fractions of Triton-solubilized cell membranes. Figure 6A shows that radiolabeled cholesterol is enriched in the detergent-insoluble raft fractions, and that cholesterol partitioning to these raft fractions is higher in the S49 cells than in the S49^{AR} cells.

Thus, detergent-insoluble lipid rafts from ALP-resistant S49^{AR} cells are not only deficient in SM but have also reduced cholesterol content. These results are consistent with the notion that the levels of SM and cholesterol in lipid rafts are mutually interdependent, conceivably due to the well known tight physical interaction between these two lipids [11,12].

SM synthesis and apoptosis sensitivity depend on cholesterol homeostasis

SMS1 is located in the Golgi apparatus [26] where its two enzymatic products, SM and diacylglycerol, play an important role in, respectively, new lipid raft formation and secretory vesicle formation at the trans-Golgi network for transport to the plasma membrane (see Discussion). SMS catalyses the production of SM and diacylglycerol from ceramide and PC, but can also catalyse the reverse reaction [30], resulting in an equilibrium. Given the strong physical association of SM with cholesterol, one would expect that the availability of cholesterol at the site of SM synthesis influences this enzymatic equilibrium and stabilizes SM formation through its sequestration in a

(transient) SM-cholesterol complex, thus facilitating/driving SM production and new lipid raft formation. We reasoned that, when cholesterol is deficient at this SMS1 location, SM and diacylglycerol can be more easily converted back into PC and ceramide, the net result being that SM production is inhibited. To test this hypothesis, we used U18666A to disturb NPC1-mediated vesicular transport of cholesterol out of the endosomal compartments [14,15] to the trans-Golgi network and lipid rafts [17], and we determined SM production and cholesterol partitioning in the lipid raft fractions. So, S49 cells were allowed to take up and process exogenous [³H]cholesterol in the presence or absence of U18666A. As shown in Figure 6B, cells that received U18666A showed less [³H]cholesterol partitioning in the raft fractions than control cells. In fact, the altered cholesterol profile from the U18666A-treated cells looked very similar to that from the S49^{AR} cells (compare the graphs of Figures 6A and 6B).

We next tested how this disturbance of cholesterol homeostasis affected SM synthesis. Figure 7A shows that the time-dependent synthesis of SM in S49 cells is inhibited by about 80% in the presence of U18666A. This inhibition of SM synthesis was indirect, via cholesterol, as confirmed by a control experiment shown in Figure 7B. In this experiment, we incubated crude cellular membranes with fluorescently NBD-labeled ceramide as a substrate for endogenous SMS. In this *in vitro* assay, U18666A did not inhibit SM synthesis, whereas D609, a known SMS inhibitor [31,32], indeed blocked SM production (Figure 7B). Collectively, the data suggest that SM synthesis is blocked indirectly through U18666A-mediated disturbance of intracellular cholesterol homeostasis.

Since U18666A not only disturbs cholesterol homeostasis, but abrogates SM synthesis concomitantly, we next tested if this inhibitor would prevent ALP uptake and apoptosis induction, like in the SMS1-deficient cells. Figure 8A shows the time-dependent uptake of [³H]ALP by S49 and S49^{AR} cells. In the presence of U18666A, the uptake by S49 cells decreased to the level of S49^{AR} cells. Furthermore, U18666A treatment dose-dependently blocked ALP-induced apoptosis in S49 cells, whereas the drug did not induce apoptosis by itself (Figure 8B). As an additional control, U18666A had no effect on the induction of apoptosis by an entirely different inducer, Fas ligand

(FasL; Figure 8B), indicating that the drug specifically blocked the ALP uptake, but exerted no aspecific effect on apoptotic signaling steps.

We previously reported that ALP induces apoptosis primarily by raft-mediated endocytic uptake and subsequent inhibition of PC synthesis inside the cell [7,8]. Since U18666A appeared to compromise 'normal' lipid raft formation (both SM synthesis and cholesterol incorporation in the detergent-resistant raft fraction were inhibited), and inhibits ALP internalization, one would expect that U18666A also prevents PC inhibition. Figure 8C shows that indeed ALP-induced PC inhibition is alleviated by U18666A. Thus, it appears that U18666A, through disturbing cholesterol homeostasis and decreased SM synthesis, which disables cholesterol-SM association in lipid rafts, prevents ALP from reaching its intracellular target, cytidylyltransferase in the ER.

Discussion

In this paper, we have shown that S49 lymphoma cells express only one of the two known mammalian sphingomyelin synthase isotypes, SMS1, and that the activity of this enzyme is required for ALP internalization and ALP-induced apoptosis in these cells. To initiate apoptotic signaling, ALP has to be internalized by endocytosis via lipid rafts to inhibit the rate-determining enzyme, CTP:phosphocholine cytidyltransferase, of PC synthesis in the ER [7,8]. In cells that were made resistant to ALP, S49^{AR} cells, this raft-dependent process is blocked [7]. We have demonstrated by using three different radiolabeled precursors, [¹⁴C]choline, [¹⁴C]serine and [³H]sphingosine, that these ALP-resistant S49^{AR} cells do not synthesize SM. The lack of SM in these cells, confirmed by mass spectrometry, was due to absence of SMS expression as determined by PCR. Furthermore, siRNA-induced downregulation of SMS1 expression in the parental S49 cells prevented SM production, ALP uptake and apoptosis induction, like in the S49^{AR} cells. Similar results were obtained when the S49 cells were treated with U18666A, which disturbs cholesterol homeostasis and in this way inhibits SM synthesis indirectly. That this inhibition by U18666A is indeed indirect, was verified by the fact that the compound did not affect SMS activity in a cell-free system (isolated membranes), whereas D609, a known SMS inhibitor [31,32], blocked this *in vitro* activity. D609 (at 30 µg/ml) also blocked the *in vivo* synthesis of SM (but notably inhibited the biosynthesis of other lipids to some moderate extent as well) and prevented ALP-induced apoptosis in S49 cells (data not shown). Indirect inhibition of SM production by myriocin/ISP-1, an inhibitor of serine palmitoyltransferase, an early enzymatic step in sphingolipid synthesis, likewise inhibited ALP uptake and apoptosis induction in these cells. The results obtained by gene silencing of SMS1 could thus be mimicked by pharmacological inhibition of the enzyme directly, or of the entire biosynthetic pathway of sphingolipids.

How precisely S49 cells become resistant to ALP upon continuous culturing in the presence of this ether-lipid remains unknown. Our results suggest a mechanism that includes transcriptional downregulation of SMS1. Possible involvement of stress mediators such as heat shock proteins or lipid (ALP?)-sensitive transcription factors may be considered in future studies. To escape ALP toxicity, the cells apparently shut off their

raft-mediated endocytic uptake, by downregulating the synthesis of the raft key-component SM (but not of other sphingolipids; Figures 1B and 2). This SM synthesis occurs in the Golgi, the location of SMS1 [26], and must somehow be linked to endocytic uptake of ALP at a spatially distant site, the plasma membrane. An appealing physico-chemical linker of these two spatio-temporal processes is the dynamic, and at any time transient (SM containing) lipid raft that is newly assembled in the trans-Golgi network, is subject to vesicular trafficking to the plasma membrane [10,33] and from there mediates ALP internalization through endosomes [7,8,33]. In this concept, ALP internalization by raft-dependent endocytosis may thus be viewed as part of a constitutive SMS1-dependent membrane recycling process.

In the Golgi apparatus, SMS1 and its two enzymatic products, SM and diacylglycerol, play a major role in microdomain (raft) formation and vesicle biogenesis at the trans-Golgi network [13, 34-36], respectively. Diacylglycerol represents a major cue for protein kinase D (PKD1) recruitment to trans-Golgi membranes [37]. This PKD1 regulates the fission of transport vesicles on their way to the plasma membrane [34]. Inhibition of SMS1-mediated diacylglycerol production was shown to block this process [37]. In the Golgi, newly synthesized SM is assumed to seek association with cholesterol in nascent lipid rafts [10,38]. Cholesterol partitioning in new rafts was shown to occur before it reaches the plasma membrane [39] and to be required for the formation of secretory vesicles from the trans-Golgi network [33,40]. Availability of cholesterol at this site has been shown to be regulated by the NPC1 protein [17]. In NPC1-knockout cells, cholesterol transport from endosomal compartments to the trans-Golgi network is blocked, and this defect can, in normal cells, be mimicked by the compound U18666A [14,15]. Treatment of S49 cells with this inhibitor of cholesterol homeostasis indeed reduced cholesterol distribution in lipid rafts and inhibited SM production indirectly. The latter may be tentatively explained by insufficient stabilization of newly synthesized SM through lack of cholesterol at this site (see also reasoning in Results section). In turn, we speculate that this deficient SM synthesis with consequent lack of SM-cholesterol assembly in new rafts might be a causal link to inhibition of ALP uptake and apoptosis induction, like in the ALP-resistant S49^{AR} cells.

Blockade of constitutive SM synthesis (and of concomitant production of diacylglycerol), as we effectuated in various ways (siRNA of SMS1, myriocin/ISP-1, D609) is likely to inhibit anterograde vesicular trafficking, as noted [34,36]. The mere accumulation of SM in plasma membrane rafts, by supplying SM-deficient S49^{AR} cells with exogenous SM, failed to facilitate ALP internalization and apoptosis induction (Figure 5). Likewise, loading of the plasma membrane with exogenous (cyclodextrin-formulated) cholesterol did not facilitate ALP uptake and apoptosis (see ref. [18]; and data not shown). Our results are therefore consistent with the possibility that constitutive biosynthesis of SM, in concert with recruited (endogenous) cholesterol, directs the assembly of SM-containing lipid rafts at the trans-Golgi network and further translocation to the plasma membrane, and that this dynamic and constitutive process is essential for the raft-dependent internalization of ALP. Collectively, these results support the idea that ALP internalization is carried by the retrograde route of constitutive raft-vesicular cycling, that may exist between the trans-Golgi, the plasma membrane and the endosomal compartments [10,33,41-44]. This hypothesis and the role of SMS1 in this particular recycling process and ALP sensitivity needs to be further verified in future studies.

Acknowledgements

We thank Alfred Merrill, Elaine Wang and Samuel Kelley for conducting the sphingomyelin analyses by mass spectrometry using the Sphingolipid Analysis Core funded by NIH GM069338 as part of the Lipid MAPS Consortium. We thank Jannie Borst, Nullin Divecha, Jon Halstead and Menno van Lummel for useful discussions. Cathelijne Stortelers is thanked for advice on real-time PCR. This study was funded by the Dutch Cancer Society, grant NKI 2005-3377.

References

1. Mollinedo, F., Fernandez-Luna, J.L., Gajate, C., Martin-Martin, B., Benito, A., Martinez-Dalmau, R. and Modolell, M. (1997) Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH₃ (Edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-X(L). *Cancer Res.* **57**, 1320-1328
2. Ruiter, G.A., Zerp, S.F., Bartelink, H., van Blitterswijk, W.J. and Verheij, M. (1999) Alkyl-lysophospholipids activate the SAPK/JNK pathway and enhance radiation-induced apoptosis. *Cancer Res.* **59**, 2457-2463
3. Ruiter, G.A., Verheij, M., Zerp, S.F. and van Blitterswijk, W.J. (2001) Alkyl-lysophospholipids as anticancer agents and enhancers of radiation-induced apoptosis. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 415-419
4. Ruiter, G.A., Zerp, S.F., Bartelink, H., van Blitterswijk, W.J. and Verheij, M. (2003) Anti-cancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase-Akt/PKB survival pathway. *Anticancer Drugs* **2**, 167-173
5. Van Blitterswijk, W.J., Hilkmann, H. and Storme, G.A. (1987) Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* **22**, 820-823
6. Boggs, K.P., Rock, C.O. and Jackowsky, S. (1995) Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidyltransferase step. *J. Biol. Chem.* **270**, 7757-7764
7. Van der Luit, A.H., Budde, M., Ruurs, P., Verheij, M. and van Blitterswijk, W.J. (2002) Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. *J. Biol. Chem.* **277**, 39541-39547
8. Van der Luit, A.H., Budde, M., Verheij, M. and van Blitterswijk, W.J. (2003) Different modes of internalization of apoptotic alkyl-lysophospholipid and cell-rescuing lysophosphatidylcholine. *Biochem. J.* **374**, 747-753
9. Kent, C. (1997) CTP:phosphocholine cytidyltransferase. *Biochim. Biophys. Acta* **1348**, 79-90

10. Van Meer, G. and Lisman, Q. (2002). Sphingolipid transport: rafts and translocators. *J. Biol. Chem.* **277**, 25855-25858
11. Van Blitterswijk, W.J., van der Meer, B.W. and Hilkmann, H. (1987). Quantitative contributions of cholesterol and the individual classes of phospholipids and their degree of fatty acid (un)saturation to membrane fluidity measured by fluorescence polarization. *Biochemistry* **26**, 1746-1756
12. Slotte, J.P. (1999). Sphingomyelin-cholesterol interactions in biological and model membranes. *Chem. Phys. Lipids* **102**, 13-27
13. Ridgway, N.D. (2000). Interaction between metabolism and intracellular distribution of cholesterol and sphingomyelin. *Biochim. Biophys. Acta* **1484**, 129-141
14. Liscum, L. and Faust, J.R. (1989). The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one. *J. Biol. Chem.* **264**, 11796-11806
15. Lange, Y., Ye, J., Rigney, M. and Steck, T. (2000) Cholesterol movement in Niemann-Pick type C cells and in cells treated with amphiphiles. *J. Biol. Chem.* **275**, 17468-17475
16. Te Vruchte, D., Lloyd-Evans, E., Veldman, R.J., Neville, D.C., Dwek, R.A., Platt, F.M., van Blitterswijk, W.J. and Sillence, D. (2004). Accumulation of glycosphingolipids in Niemann-Pick C disease disrupts endosomal transport. *J. Biol. Chem.* **279**, 26167-26175
17. Garver, W.S., Krishnan, K., Gallagos, J.R., Michikawa, M., Francis, G.A. and Heinenreich, R.A. (2002). Niemann-Pick C1 protein regulates cholesterol transport to the trans-Golgi network and plasma membrane caveolae. *J. Lipid Res.* **43**, 579-589
18. Smets, L.A., van Rooij, H. and Salomons, G.S. (1999) Signaling steps in apoptosis by ether lipids. *Apoptosis* **4**, 419-427
19. Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **3**, 243-247

20. Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**, 271-279
21. Merrill, A.H. Jr., Sullards, M.C., Allegood, J.C., Kelly, S. and Wang, E. (2005) Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods*. 36(2):207-224.
22. Nikolova-Karakashian, M. (1999) Assays for the biosynthesis of sphingomyelin and ceramide phosphoethanolamine. *Methods Enzymol.* **311**, 31-42
23. Lisanti, M.P., Tang, Z., Scherer, P.E. and Sargiacomo, M. (1995) Caveolae purification and glycosylphosphatidylinositol-linked protein sorting in polarized epithelia. *Methods Enzymol.* **250**, 655-668
24. Ramstedt, B., Leppimäki, P., Axberg, M. and Slotte, J.P. (1999) Analysis of natural and synthetic sphingomyelins using high-performance thin-layer chromatography. *Eur. J. Biochem.* **266**, 997-1002
25. Hanada, K., Nishijima, M., Kiso, M., Hasegawa, A., Fujita, S., Ogawa, T. and Akamatsu, Y. (1992) Sphingolipids are essential for the growth of Chinese hamster ovary cells. *J. Biol. Chem.* **267**, 23527-23533
26. Huitema, K., van den Dikkenberg, J., Brouwers, J.F.H.M. and Holthuis, J.C.M. (2004) Identification of a family of animal sphingomyelin synthases. *EMBO J.* **23**, 33-44
27. Yamaoka, S., Miyaji, M., Kitano, T., Umehara, H. and Okazaki, T. (2004) Expression cloning of a human cDNA restoring sphingomyelin synthesis and cell growth in sphingomyelin synthase defective lymphoid cells. *J. Biol. Chem.* **279**, 18688-18693
28. Miyake, Y., Kozutsumi, Y., Nakamura, S., Fujita, T. and Kawasaki, T. (1995) Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. *Biochem. Biophys. Res. Commun.* **211**, 396-403

29. Brown, D.A. and London E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275**, 17221-17224
30. Van Helvoort, A., van 't Hof, W., Ritsema, T., Sandra, A., and van Meer, G. (1994). Conversion of diacylglycerol to phosphatidylcholine on the basolateral surface of epithelial (Madin-Darby canine kidney) cells. Evidence for the reverse action of a sphingomyelin synthase. *J. Biol. Chem.* **269**, 1763-1769
31. Luberto, C. and Hannun, Y.A. (1998). Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation. Does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase C? *J. Biol. Chem.* **273**, 14550-14559
32. Meng, A., Luberto, C., Meier, P., Bai, A., Yang, X., Hannun, Y.A. and Zhou, D. (2004) Sphingomyelin synthase as a potential target for D609-induced apoptosis in U937 human monocytic leukemia cells. *Exp. Cell Res.* **292**, 385-392
33. Ikonen, E. (2001). Roles of lipid rafts in membrane transport. *Curr. Opin. Cell Biol.* **13**, 470-477
34. Bankaitis, V.A. and Morris, A.J. (2003) Lipids and the exocytotic machinery of eukariotic cells. *Curr. Op. Cell Biol.* **15**, 389-395
35. Van Lint, J., Rykx, A., Maeda, Y., Vantus, T., Sturany, S., Malhotra, V., Vandenheede, J.R. and Seufferlein, T. (2002) Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol.* **12**, 193-200
36. Lev, S. (2006) Lipid homeostasis and Golgi secretory function. *Biochem. Soc. Trans.* **34**, 363-366
37. Baron, C.L. and Malhotra, V. (2002) Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. *Science* **295**, 325-328
38. Simons, K. and Ikonen, E. (2000) How cells handle cholesterol. *Science* **290**, 1721-1726
39. Heino, S., Lusa, S., Somerharju, P., Ehnholm, C., Olkkonen, V.M. and Ikonen, E. (2000). Dissecting the role of the golgi complex and lipid rafts in

- biosynthetic transport of cholesterol to the cell surface. *Proc. Natl. Acad. Sci. USA* **97**, 8375-8380
40. Wang, Y., Thiele C. and Huttner, W.B. (2000) Cholesterol is required for the formation of regulated and constitutive secretory vesicles from the trans-Golgi network. *Traffic* **1**, 952-962
41. Hoekstra, D. and Van Ijzendoorn, S.C.D. (2000) Lipid trafficking and sorting: how cholesterol is filling gaps. *Curr. Opin. Cell Biol.* **12**, 496-502
42. Van Blitterswijk, W.J., van der Luit, A.H., Veldman, R.J., Verheij, M. and Borst, J. (2003). Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem. J.* **369**, 199-211
43. Nabi, I.R. and Le, P.U. (2003). Caveolae/raft dependent endocytosis. *J. Cell Biol.* **161**, 673-677
44. Sannerud, R., Saraste, J. and Goud, B. (2003) Retrograde traffic in the biosynthetic-secretory route: pathways and machinery. *Curr. Opin. Cell Biol.* **15**, 438-445

Table 1 Sphingomyelin mass levels in S49 and S49^{AR} cells and in the fetal calf serum used for cell culturing

Sphingomyelins in lipid extracts were assayed by liquid chromatography, electrospray tandem mass spectrometry [21]. Data are means of triplicates \pm S.D.

SM type	S49 cells	S49 ^{AR} cells	Fetal calf serum
	(pmol/ cell x 10 ⁶)		(pmol/mg protein)
C16:0	101.6 \pm 8.4	20.3 \pm 1.4	651.9 \pm 128.4
C16:0-DH ^a	6.9 \pm 0.1	1.8 \pm 0.4	77.8 \pm 17.4
C18:0	2.3 \pm 0.2	1.8 \pm 0.1	115.1 \pm 24.0
C24:1	6.8 \pm 0.6	1.7 \pm 0.3	146.5 \pm 32.1
Sum others ^b	3.7 \pm 0.2	2.1 \pm 0.1	162.9 \pm 16.0
Total	121.3	27.7	1154.1

^a C16:0-DH, sphingomyelin containing an N-palmitoyl-dihydrosphingosine moiety.

^b C20:0, C22:0, C24:0, C26:0 and C26:1 in amounts less than 1.9 and 0.8 pmol/cell x 10⁶ for S49 and S49^{AR} cells, respectively, and less than 60.1 pmol/mg protein for fetal calf serum.

LEGENDS TO FIGURES

Figure 1 ALP-resistant S49^{AR} cells are devoid of sphingomyelin synthesis

(A) ALP-sensitive S49 and ALP-resistant S49^{AR} cells were labeled overnight with the precursors [*methyl*-¹⁴C]choline or L-[3-¹⁴C]serine. Lipids were extracted and separated by two-dimensional TLC (see “Experimental”). The location of SM (typically two spots; see text) is marked by a white dotted box. (B) S49 and S49^{AR} cells were labeled with [³H-]sphingosine for the times indicated. (Sphingo)lipids were extracted and separated by one-dimensional TLC (see “Experimental”). The location of SM and other sphingolipids, ceramide (Cer), glucosylceramide (GlcCer) and lactosylceramide (LacCer) is indicated. Phosphatidylethanolamine (PE, indicated) is a catabolic end-product of sphingosine degradation.

Figure 2 Comparison of sphingolipid distribution of detergent-resistant lipid raft fractions isolated from S49 and S49^{AR} cells

Raft fractions (3 + 4) from ALP-resistant cells (S49^{AR}), but not from parental cells (S49) are devoid of SM, while the distribution of the other sphingolipids shows no profound differences among the two cell lines. Cells were labeled for 4 h with [³H-]sphingosine. Triton-X100-insoluble lipid rafts were isolated on sucrose density gradients, and lipids were extracted and separated by TLC (see “Experimental”). T, total lysate extract. Positions of lipids are indicated as in Figure 1B.

Figure 3 Expression of SMS1 and SMS2 in S49 and S49^{AR} cells

(A) RT-PCR for gene transcription of SMS1 and SMS2 on cDNA generated from S49 cells (indicated as S) and ALP-resistant S49^{AR} cells (indicated as R) cells. Primer sets were used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; serving as a control), SMS1, and SMS2 (indicated). (B) Real-time PCR analysis of SMS1 in S49,

S49^{AR}, S49^{mock}, and S49^{siSMS1} cells. Gene transcription is expressed as percentage of that in S49 cells, with the expression of the hypoxanthine guanine phosphoribosyl transferase 1 (HRPT) gene serving as internal standard. (C) S49^{siSMS1} cells are almost devoid of SM biosynthesis, while the synthesis of other (sphingo)lipids is unaffected. Sphingolipids were labeled with ³H-sphingosine for 4 h. Lipids were extracted and separated on TLC. Positions lipids are indicated as in Figure 1B. So, sphingosine.

Figure 4 Inhibition of SM synthesis, through siRNA-induced SMS1 silencing (S49^{siSMS1} cells) or myriocin/ISP-1 treatment (S49 cells) abrogates ALP uptake and apoptosis induction, similar to S49^{AR} cells

(A) Uptake of ³H-ALP in S49 (●), S49^{mock} (○), S49^{AR} (■) and S49^{siSMS1} cells (□) followed over time. (B) Time-dependent uptake of ³H-ALP in S49 that were preincubated with myriocin/ISP-1 (5 μM) for 1 h (Δ) or 16 h (▲), to inhibit sphingolipid biosynthesis. Other symbols are as in (A). Data are given in ³H-ALP radioactivity (dpm) bound per 10³ cells, and represent the averages of four experiments ± SD; (absence of error bars means that the SD value is within the size of the symbol). (C) Similar to S49^{AR} cells, S49^{siSMS1} cells show reduced ALP-induced apoptosis compared to parental S49 cells or mock-transduced S49 cells. Cells were stimulated with Et-18-OCH₃ (ALP; 15 μM) for 6 h, and apoptosis (nuclear fragmentation) was determined by FACScan analysis.

Figure 5 Exogenous SM incorporates in lipid rafts of S49^{AR} cells but does not facilitate ALP uptake and apoptosis induction in these cells

(A) S49^{AR} cells were cultured in the presence of exogenous C12:0-SM (exoSM, 20 μM, added to the culture medium, for 3 days). Cells were then washed and detergent-insoluble lipid raft fractions (R) were isolated and combined, separated from non-raft fractions (NR, combined). Lipids were extracted, separated by TLC and visualized by charring with sulfuric acid (see 'Experimental'). Positions of phospholipids are indicated. Note that C12:0-SM has become enriched in lipid rafts (R) from S49^{AR} cells, and migrates on TLC slightly lower than (more hydrophobic) endogenous (C16:0)SM (compare with S49

rafts). (B) Uptake of ^3H -ALP (at 2 h) by S49^{AR} cells cultured with (*grey bars*) or without exogenous C12:0-SM (exoSM) (*white bars*) in comparison with S49 cells (*black bars*). Experimental conditions were the same as in Figure 4. Data are means of triplicates \pm SD, and are representative of three separate experiments. (C) ALP-induced apoptosis in S49 cells and in S49^{AR} cells that were cultured with (*grey bars*) or without exogenous C12:0-SM (exoSM) (*white bars*). Cells were stimulated with Et-18-OCH₃ (ALP; 15 μM) for 6 h, and apoptosis (nuclear fragmentation) was determined by FACScan analysis. Data are means of triplicates and are representative of four separate experiments.

Figure 6 Cholesterol levels are decreased in lipid raft fractions from S49^{AR} cells and U18666A-treated S49 cells

(A) Cholesterol profiles of gradient fractions from S49 and S49^{AR} cells. Cells were incubated overnight with [^3H]cholesterol (0.02 $\mu\text{Ci}/\text{mL}$; trace amount dissolved in ethanol injected into the serum-containing culture medium), followed by Triton X-100 solubilization and sucrose gradient centrifugation. [^3H]cholesterol was quantified by liquid scintillation counting. (B) U18666A treatment of S49 cells causes redistribution of cholesterol out of the raft fractions, yielding a profile similar to S49^{AR} cells (compare with panel A). S49 cells were incubated with [^3H]cholesterol as above. Prior to solubilization, cells were treated with U18666A (2 $\mu\text{g}/\text{ml}$; open symbols) or without U18666A (closed symbols; control) for 4 h. [^3H]cholesterol in the gradient fractions was separated on TLC and was visualized by autoradiography (lower panel) or was quantified by liquid scintillation counting (upper panel), expressed as percent of total cholesterol on the gradient.

Figure 7 Disturbance of cholesterol homeostasis by U18666A indirectly inhibits SM biosynthesis

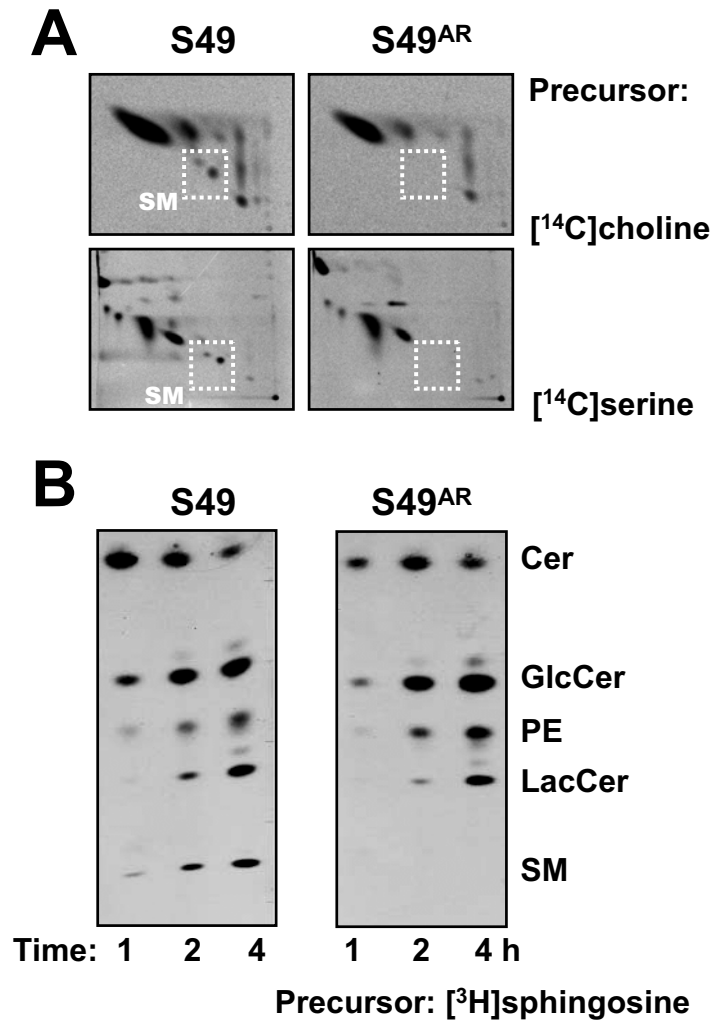
(A) S49 cells were labeled in time with [*methyl*- ^{14}C]choline, in the absence or presence of U18666A (2 $\mu\text{g}/\text{ml}$; preincubated for 4 h, as indicated). Lipids were extracted and separated by TLC. Positions of phospholipids are indicated. SM appears typically as two

spots (cf Fig.1A; see text). (B) D609 (50 $\mu\text{g/ml}$), but not U18666A (2 $\mu\text{g/ml}$), inhibits the synthesis of (NBD-conjugated) SM, when added to crude membranes with NBD-ceramide as a substrate (see “Experimental”).

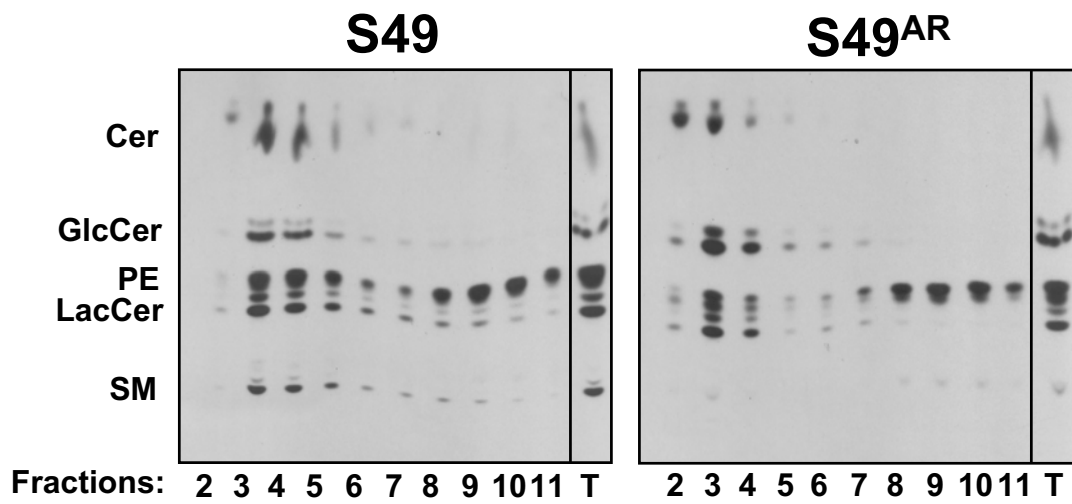
Figure 8 U18666A inhibits ALP uptake and ALP-induced apoptosis, and alleviates ALP-mediated inhibition of PC synthesis

(A) S49 cells (*circles*) and S49^{AR} cells (*squares*) were treated without (*closed symbols*) or with U18666A (2 $\mu\text{g/ml}$, for 4 hours) (*open symbols*) and then incubated with [³H]ALP for the times indicated. Uptake of [³H]ALP was measured as described in “Experimental”. (B) Induction of apoptosis in S49 cells by ALP (15 μM ; *solid circles*) or FasL (500ng/ml; *squares*) as a function of the concentration of U18666A. *Open circles* represent cells that received no ALP or FasL. Apoptosis was measured by nuclear fragmentation, using FACScan analysis, (A, B) No error bar means that its size is within the size of the symbol. (C) S49 cells were labeled with [*methyl*-¹⁴C]choline (1 $\mu\text{Ci/ml}$; 4 h) in the presence or absence of ALP (15 μM) and/or U18666A (2 $\mu\text{g/ml}$), as indicated. Radiolabeled PC was determined by TLC analysis (*upper panel*), and quantified by phosphoimaging (*lower panel*). Data are means of three experiments \pm S.D.

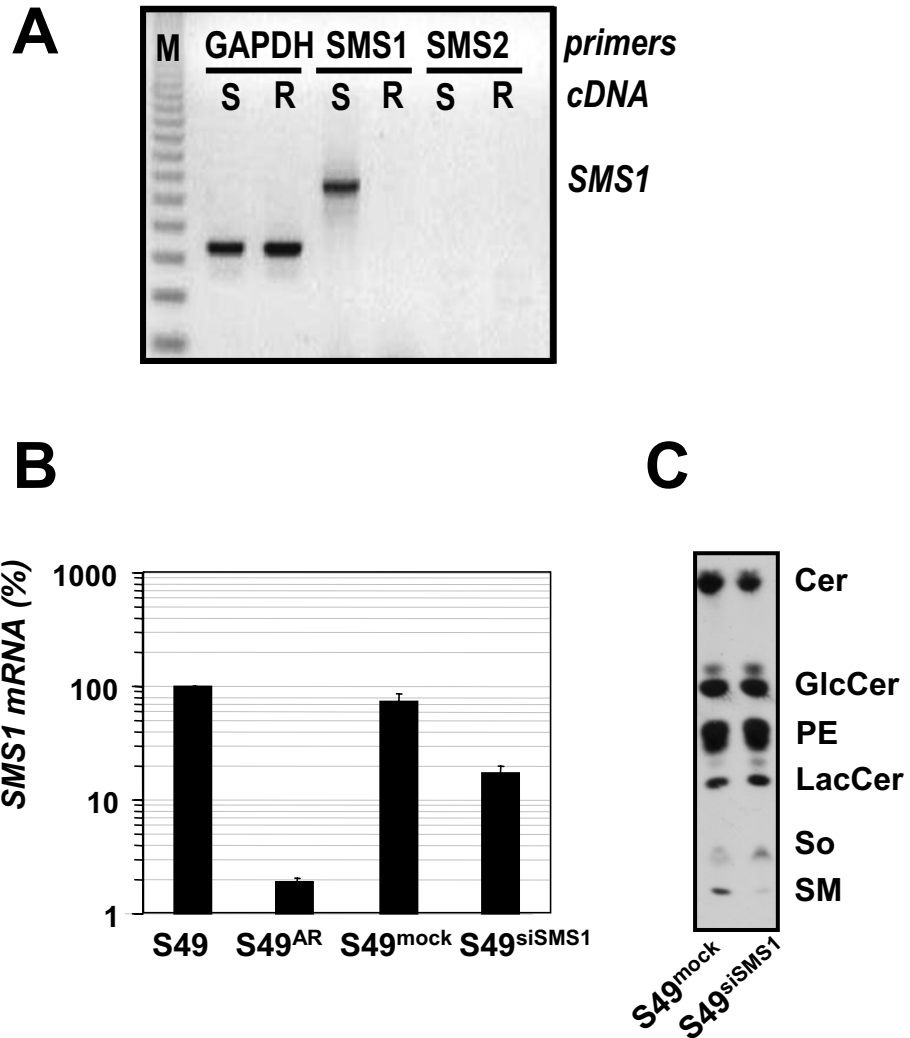
van der Luit et al., 2006 - Figure 1



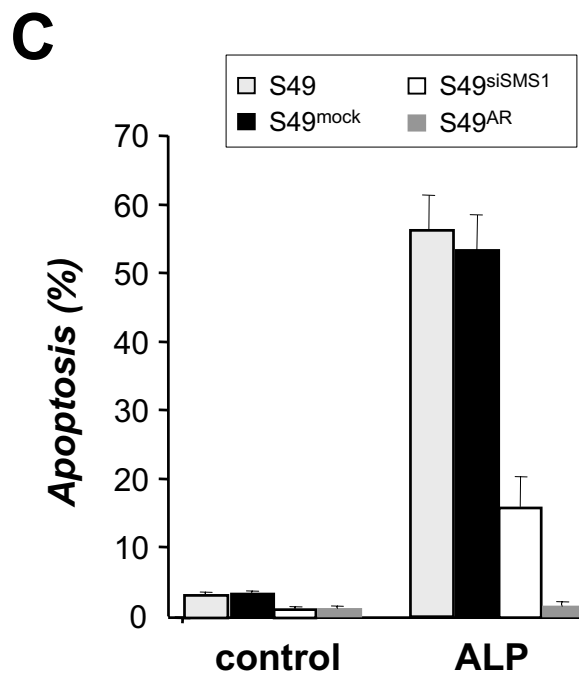
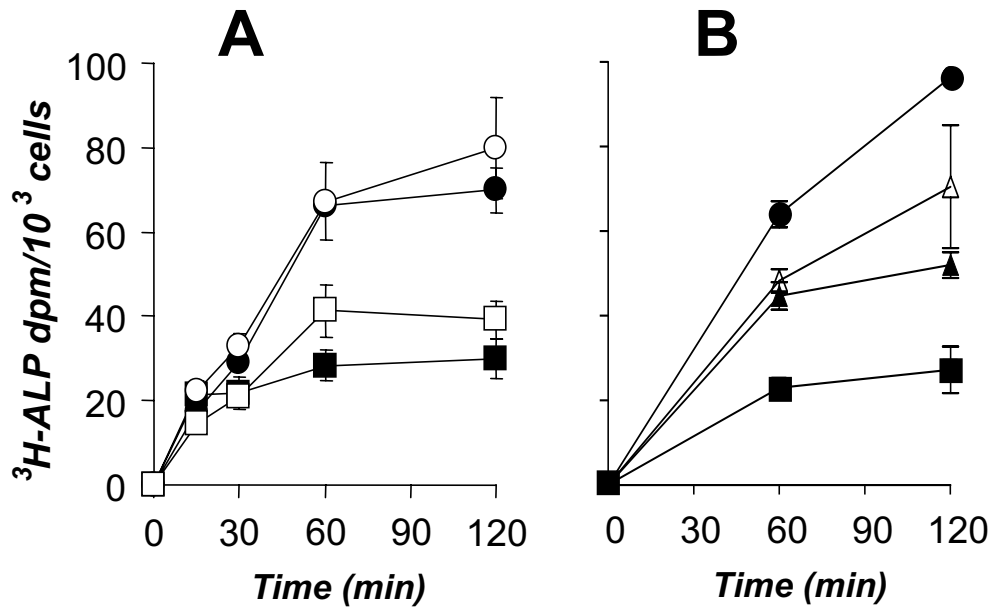
van der Luit et al., 2006 – Figure 2



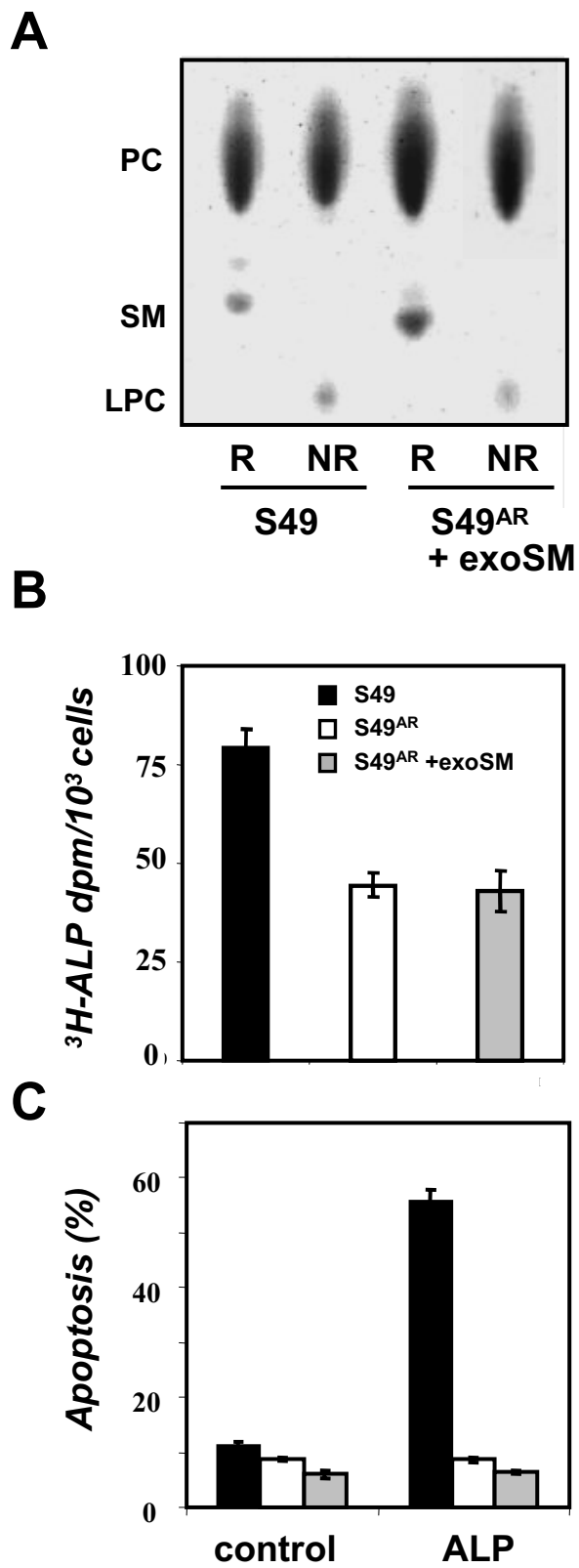
van der Luit et al., 2006 - Figure 3



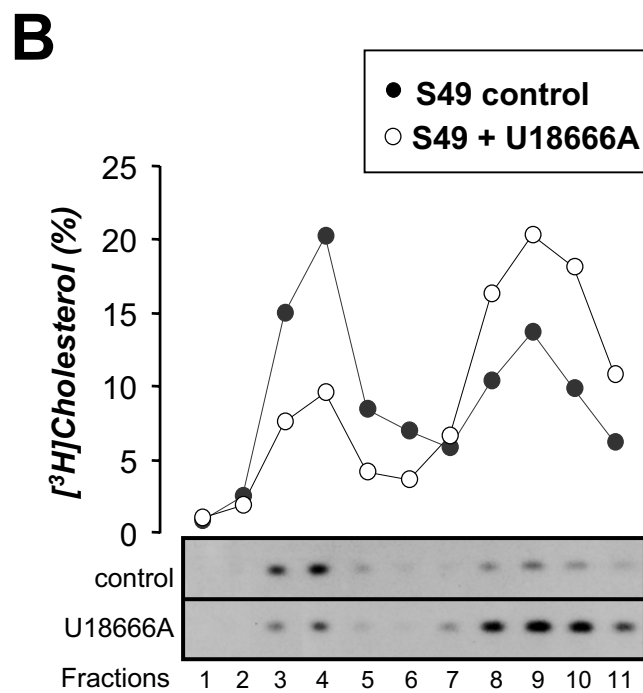
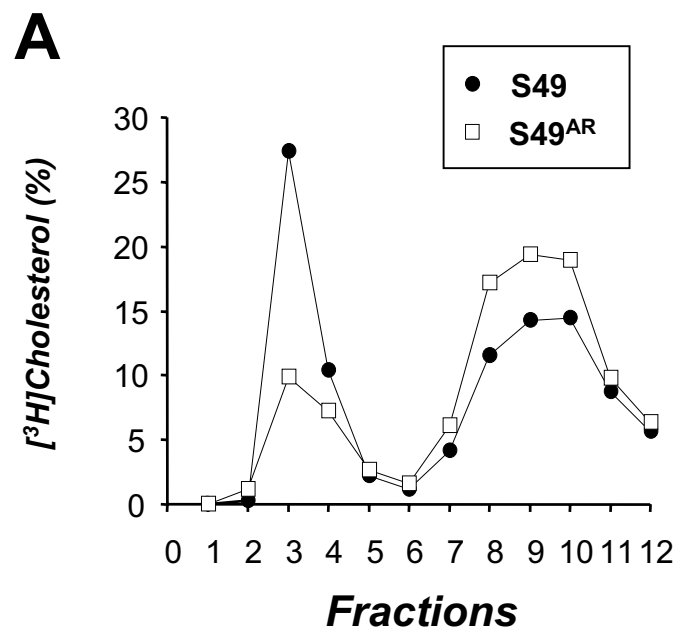
van der Luit et al., 2006 - Figure 4



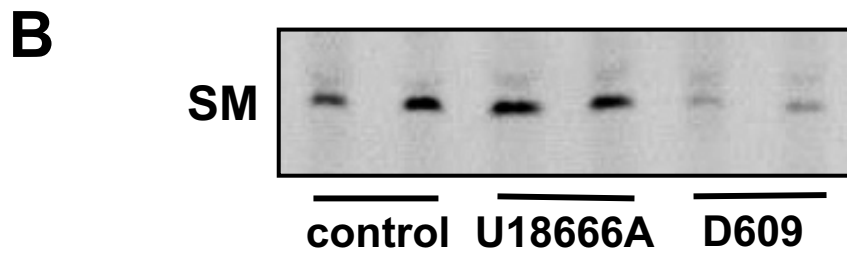
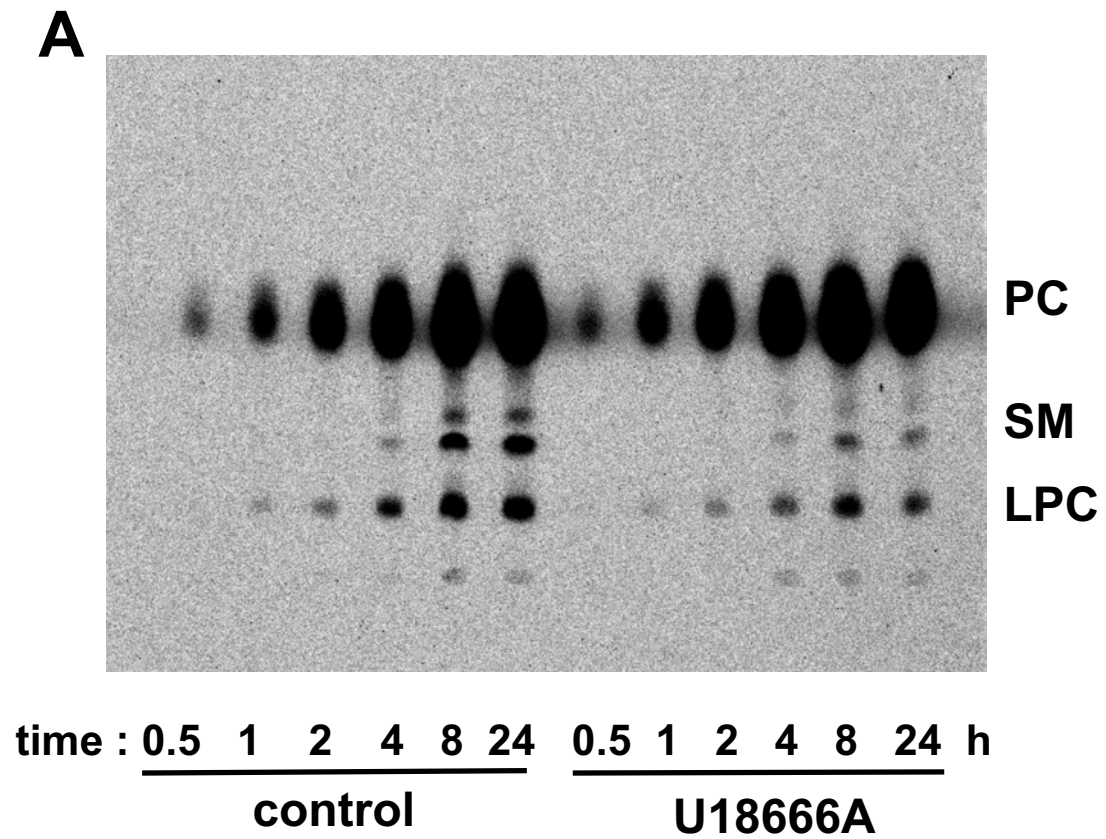
van der Luit et al., 2006 - Figure 5



Van der Luit et al., 2006-Figure 6



Van der Luit et al., 2006- Figure 7



Van der Luit et al., 2006-Figure 8

