Sulfatide-Rich Liposome Uptake by a Human-Derived Neuroblastoma Cell Line

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Abstract: Liposomes are bilayer membrane vesicles that can serve as vehicles for drug delivery. They are a good alternative to free drug administration that provides cell-targeted delivery into tumors, limiting the systemic toxicity of chemotherapeutic agents. Previous results from our group showed that an astrocytoma cell line exhibits selective uptake of sulfatide-rich (SCB) liposomes, mediated by the low-density lipoprotein receptor (LDL-R). The goal of this study was to assess the uptake of liposomes in a neuroblastoma cell line. For this purpose, we used two types of liposomes, one representing a regular cell membrane (DOPC) and another rich in myelin components (SCB). An astrocytoma cell line was used as a control. Characterization of liposome uptake and distribution was conducted by flow cytometry and fluorescence microscopy. Similar levels of LDL-R expression were found in both cell lines. The uptake of SCB liposomes was higher than that of DOPC liposomes. No alterations in cell viability were found. SCB liposomes were located near the cell membrane and did not colocalize within the acidic cellular compartments. Two endocytic pathway inhibitors did not affect the liposome uptake. Neuroblastoma cells exhibited a similar uptake of SCB liposomes as astrocytoma cells; however, the pathway involved appeared to be different than the hypothesized pathway of LDL-R clathrin-mediated endocytosis.

Keywords: neuroblastoma; drug delivery system; liposomes; sulfoglycosphingolipids; LDL receptor

1. Introduction

Liposomes are spherical vesicles composed of a phospholipid bilayer that contains an interior aqueous core, providing space to encapsulate hydrosoluble substances [1–3]. This property makes liposomes a drug delivery system that can boost drug efficiency in vivo [3,4]. These vesicles can comprise different lipids and other components that can modify their biological behavior [1,3]. One of the advantages of this drug delivery system is the possibility of targeting a particular cell or tissue to avoid unspecific accumulation [1,3]. This is of special interest in cancer treatment, in which the body is exposed to powerful systemic chemotherapeutic agents that could kill neoplastic and nonneoplastic cells. Liposomal formulation of some chemotherapeutic medications can decrease their systemic toxicity [4]. For example, compared with free doxorubicin, liposomal doxorubicin has fewer cardiotoxic effects and improves the overall response rate in women treated for breast cancer [5]. Our group has previously found that, compared with liposomes without sulfatides, sulfatide-rich liposomes are selectively taken up in an in vitro model of astrocytoma-derived cells [6,7]. Early studies suggest that this selectivity could be mediated by the low-density lipoprotein receptor (LDL-R) [6,7]. As some tumors overexpress LDL-R [8], this may be used as a target to treat cancers with a poor clinical prognosis. Neuroblastoma is one of the most common solid tumors outside of the brain in infancy [9].
It is derived from neural crest cells and has a variable prognosis ranging from disease that regresses on its own to disease with a high risk of metastasis and recurrence [10,11]. Children with recurrent neuroblastoma have a 5-year survival rate of 50% [12,13]. Treatment at this stage of the disease includes surgery, chemotherapy, and radiotherapy, but the latest treatment improvements have not shown a significant increase in life expectancy [12,13]. For this reason, finding new treatment approaches to increase these patients’ quality of life is paramount. Thus, the goal of this study is to assess the uptake of different liposome compositions, one representing a regular cell membrane (DOPC) and one rich in sulfatides (SCB), in a human neuroblastoma cell line. To our knowledge, this is the first study to assess the uptake of sulfatide-rich liposomes in a neuroblastoma cell line; previous works were conducted on glioblastoma models. The SCB liposome composition presented here was designed to resemble the myelin membrane. Our findings could be used in deriving a new possible selective drug delivery system for neuroblastoma.

2. Materials and Methods

2.1. Cell Lines and Cell Culture

Two commercial cell lines were used, the neuroblastoma cell line SH-SY5Y (CRL-2266) and the astrocytoma cell line CRL-1718 (both from American Type Culture Collection (ATCC), Manassas, VA, USA). Astrocytoma cells were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate (all from Gibco, Auckland, New Zealand), and 10% fetal bovine serum (Eurobio, Les Ulis, France). Neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1% streptomycin/penicillin (all from Gibco), and 5% fetal bovine serum (Eurobio). Cells were cultured in T25 or T75 flasks (Corning, NY, USA) at 37 °C in a humid atmosphere with 5% CO₂. All cell manipulation was performed in laminar flow IIB cabins (Labonco Corporation, Kansas City, MO, USA). When necessary, cells were detached using a trypsin-EDTA 0.25% solution (Gibco) and counted using a Neubauer chamber.

2.2. Liposomes

Six different lipids were used for vesicle generation. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), porcine brain sphingomyelin (SM), N-octanoyl-D-erythro-sphingosine (C8 ceramide, CER), porcine brain sulfatides (SCB), and cholesterol (CH) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Two different mixtures were prepared, one rich in sulfatides and one without sulfatides. The specific lipid compositions are shown in Table 1. Both lipid compositions were labeled with an additional 1 mol % N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE), a fluorescent lipid used to detect liposomes for analysis, purchased from Invitrogen (Carlsbad, CA, USA).

| Liposome | Lipid Composition (mol %) |
|----------|---------------------------|
| DOPC     | DOPC/SM/CH (46/28/25)     |
| SCB      | DOPC/SM/SCB/CER/CH (27/17/20/15/20) |

DOPC, 1,2-dioleoyl-sn-glycerol-3-phosphocholine; SM, porcine brain sphingomyelin; CER, N-octanoyl-D-erythro-sphingosine; SCB, porcine brain sulfatides; CH, cholesterol.

Each lipid was dissolved in chloroform and then mixed in the stated percentage. Afterwards, the solution was dried using a stream of nitrogen gas, lyophilized (Labonco Corporation) for at least 8 h, and then stored at −20°C. To form the vesicles, we hydrated the sample using 1 mL of vesicle buffer (100 mM NaCl, 10 mM HEPES, 1 mM EDTA, and 1 mM CaCl₂ at pH 7.4), mixed by vortex for 1 min, and heated to 55 °C for 10 min; this was repeated 5 times. Finally, the suspension was sonicated
for 30 min using the Sonics VCX 130 tip sonicator (Newtown, CT, USA) in pulses of 10 s on/off at 20% amplitude. For all experiments, we used a liposome concentration of 0.2 mM. This represents the total lipid concentration for each composition.

2.3. Liposome Characteristics

Characteristics of the liposomes such as size, z-potential, and polydispersity index (PDI) were determined using a Zetasizer Nano ZS90 analyzer (Malvern Instruments, Malvern, UK). Measurements were performed using diluted liposomes in vesicle buffer at room temperature.

2.4. LDL-R Expression

For both cell lines, we evaluated the expression of LDL-R using flow cytometry and indirect immunofluorescence. In the flow cytometry assay, $1 \times 10^5$ cells were placed in cytometry tubes. Experimental tubes were incubated for 30 min with 50 µL of a primary IgG anti-LDL-R antibody AB30532 (Abcam, Cambridge, UK) at a 1:1000 dilution. After washing the excess antibody, we added 500 µL of a 1:500 dilution of the secondary antibody IgG goat anti-rabbit Alexa Fluor 488 AB150077 (Invitrogen, Eugene, OR, USA). The cells were incubated with the secondary antibody for 25 min. The control tubes followed a similar process, but instead of antibodies, the same volume of PBS was added. Finally, 5 µL of propidium iodide (PI) (BD Biosciences, San José, CA, USA) was added to the experimental and control tubes. After 5 min, the samples were read in a FACSCanto II cytometer (BD Biosciences), and data were gathered using FACSDiva 6.1 software (BD Biosciences). The cells were characterized by their relative size and granularity (forward scatter and side scatter, respectively), and viability was assessed according to the mean fluorescence of PI.

For the evaluation of LDL-R using immunofluorescence, we cultured $2 \times 10^5$ cells in standard conditions in immunofluorescence slides. After 24 h, the cells were fixed using a 4% paraformaldehyde solution (Sigma-Aldrich) for 10 min and then permeabilized with a 1× PBS plus 0.1% Tween 20 solution for 10 min. Then, an antibody against LDL-R rabbit IgG AB30532 (Abcam) was used. A total of 100 µL at a 1:100 dilution of the antibody was added to each experimental well, and 1× PBS was added to the control wells. The cells were incubated for 1 h at room temperature in the dark and then carefully washed four times with 1× PBS. Then, the secondary antibody IgG goat anti-rabbit Alexa Fluor 488 AB150077 (Invitrogen) was diluted in 1× PBS at a 1:1000 dilution, and 100 µL of this solution was added to each well. The slides were washed with 1× PBS and left to air dry in the dark. Finally, Gelvatol (Sigma-Aldrich) was used as the mounting medium before the cells were observed under a Nikon Eclipse T1 microscope (Nikon Instruments, Melville, NY, USA). Pictures were taken using the Hamamatsu Orca 2 camera (Hamamatsu Photonics, Bridgewater, NJ, USA), and image analysis was performed using Fiji, an open source image analyzer [14].

2.5. Effect of Liposomes on Cell Viability

To verify the effect of liposomes on cell viability, we cultured $1 \times 10^5$ neuroblastoma cells in 48-well plates. They were cultured in standard conditions for 24 h, and then 0.2 mM SCB or DOPC liposomes were added to the experimental wells. The same volume of vesicle buffer was added to a control well. Cells were cocultured with the liposomes for another 24 h. Afterwards, the cells were detached using trypsin-EDTA, washed with 1× PBS, and centrifuged. Then, 5 µL of PI (BD Biosciences) was added to each tube. After 5 min of incubation at room temperature, the cells were assessed in a FACSCanto II cytometer (BD Biosciences), and cell viability data were analyzed using FACSDiva 6.1 software (BD Biosciences).

2.6. Liposome Uptake

At least $5 \times 10^5$ astrocytoma and neuroblastoma cells were placed in small glass Petri dishes; 3 different conditions were set for each cell line: one with cells only, another with cells plus 0.2
mM DOPC liposomes, and one with cells and 0.2 mM SCB liposomes. They were cultured at room temperature in slow continuous agitation protected from light. We collected samples at 4 different times: at the beginning of the experiment and after 30, 60, and 120 min of coculture. The excess liposomes and media were washed away with 1× PBS, and the cells were resuspended in 200 μL of FACS flow (BD Biosciences). Then, at least $1 \times 10^4$ cells were assessed with the FACSCanto II cytometer (BD Biosciences). The cells were characterized by their relative size and granularity (forward scatter and side scatter, respectively), and the mean liposomal NBD-PE fluorescence intensity was determined in the green detector (510/20 nM).

Furthermore, we evaluated liposome uptake by fluorescence microscopy. For this, we cultured neuroblastoma cells in 35-mm glass dishes with 10-mm microwells for 24 h in standard culture conditions. They were incubated for 6 and 24 h with 0.2 mM SCB liposomes. Vesicle buffer was used as a control. Then, 1 μL of lysotracker blue (Invitrogen) was added and incubated at 37 °C for 30 min. Finally, cells were observed under a Nikon eclipse T1 microscope (Nikon Instruments), and pictures were taken using the Hamamatsu Orca 2 camera (Hamamatsu Photonics) at 40× magnification. Image analysis was performed using Fiji, an open source image analyzer [14].

2.7. Endocytic Pathway Inhibition Assays

To study the potential pathways of SCB liposome uptake, we used two different active transport inhibitors: cytochalasin D (CYD) (Sigma-Aldrich), which inhibits macropinocytosis, and chlorpromazine (CPZ) (Sigma-Aldrich), which inhibits clathrin-mediated endocytosis. First, a concentration assay of the inhibitors was performed. Cells were incubated with different drug concentrations at 37 °C in continuous agitation for 2.5 h. Then, 7AAD (Invitrogen) was added to assess cell viability. After 10 min of incubation with 7AAD, $1 \times 10^4$ cells were acquired by the FACS canto II cytometer (BD Biosciences).

For the inhibition assays, neuroblastoma cells were cultured for 30 min with each inhibitor at two different concentrations. Then, 0.2 mM of SCB liposomes were added and incubated at 37 °C in continuous agitation for an additional 2 h. Liposome uptake was determined in viable cells (7AAD negative). The results were reported as the percentage of uptake relative to the one observed in control cells incubated with the solvent used to prepare the inhibitors.

3. Results

3.1. Liposome Characteristics

Characteristics of the SCB and DOPC liposomes such as size, z-potential, and PDI were determined. The diameter for SCB liposomes was 76.93 ± 0.63 nm on average, while the mean diameter of the DOPC liposomes was 96.08 ± 1.09 nm. The z-potential of SCB liposomes was $-14.3 \pm 2.9$ mV and for DOPC liposomes was $-2.0 \pm 1.18$ mV. Both liposomes had a PDI below 0.160, indicating a monodispersed suspension. The PDIs of the SCB and DOPC liposomes were 0.131 ± 0.024 and 0.156 ± 0.01, respectively.

3.2. Similar LDL-R Expression in Both Cell Lines

LDL-R is expressed in various cells, including cells of the central nervous system. Expression of this receptor in both cell lines is shown in Figure 1. Data are displayed as fluorescence values (arbitrary units, AU) obtained by flow cytometry. The delta (Δ) mean fluorescence intensity (MFI) was calculated as the MFI of experimental cells (with LDL-R antibody) minus the MFI of control cells (without LDL-R antibody). For the neuroblastoma cell line, the median Δ MFI was 170 AU, and for astrocytoma cells it was 132 AU. No significant difference was found between the Δ MFI of the two cell lines (Mann–Whitney test p-value 0.34), but the neuroblastoma cells tended to have higher values.
Low-density lipoprotein receptor (LDL-R) expression was measured by flow cytometry. Delta (Δ) in mean fluorescence intensity (MFI) from cells incubated with LDL-R anti-antibodies minus the MFI of cells without LDL-R anti-antibodies. Median and interquartile ranges of 75–25 are shown (Mann–Whitney test p-value 0.34) (n = 6).

LDL-R expression was confirmed by indirect immunofluorescence, as shown in Figure 2. A primary antibody for the intracellular portion of LDL-R was used along with a secondary antibody with fluorescein and DAPI for nuclear staining. Both cell lines had a granular pattern of LDL-R expression dispersed evenly throughout the cytoplasm. However, LDL-R expression appeared to be more prominent in neuroblastoma cells.

Figure 1. Low-density lipoprotein receptor (LDL-R) expression was measured by flow cytometry. Delta (Δ) in mean fluorescence intensity (MFI) from cells incubated with LDL-R anti-antibodies minus the MFI of cells without LDL-R anti-antibodies. Median and interquartile ranges of 75–25 are shown (Mann–Whitney test p-value 0.9004) (Figure S1).

Figure 2. Representative images of LDL-R expression by indirect immunofluorescence. Blue represents cell nucleus staining, and green shows the LDL-R distribution. (A) Astrocytoma cells; (B) neuroblastoma cells. Scale, 50 μm.

3.3. Liposomes Did Not Alter Neuroblastoma Cell Viability

To assess whether liposomes had a cytotoxic effect, we cocultured neuroblastoma cells with SCB liposomes for 24 and 48 h. We used 0.2 mM of total lipid concentration, which was the same liposome concentration used for the uptake and inhibition assays. Cell viability was measured by flow cytometry using PI. No difference was found between the cells under usual culture conditions and the cells with added SCB liposomes at 24 h (Mann–Whitney test p-value 0.1255) and at 48 h (Mann–Whitney test p-value 0.9004) (Figure S1).

3.4. SCB Liposome Uptake by Neuroblastoma

Figure 3 shows the curve of liposome uptake in both cell lines. The uptake of SCB liposomes was greater than that of DOPC liposomes in both cell lines at any point in time. At 2 h, a difference
between SCB and DOPC uptake was found in both the astrocytoma (Mann–Whitney test \( p \)-value 0.0022) and neuroblastoma (Mann–Whitney test \( p \)-value 0.0022) cell lines.

![Figure 3](image3.png)

**Figure 3.** Uptake of SCB and DOPC liposomes measured by flow cytometry. Liposomes had 1 mol % of \( N-(7\text{-nitrobenz}-2\text{-oxa-1,3-diazol}-4\text{-yl})\text{-}1,2\text{-dihexadecanoyl-sn-glycero-3-phosphoethanolamine} \) (NBD-PE), a fluorescent lipid that allows detection of the mean fluorescence intensity by flow cytometry. Measurements were made at 30, 60, and 120 min after coculture with the liposomes. Measurements of the average of six independent experiments are shown, and the data are shown as the mean and standard error \((n = 6)\). (A) Astrocytoma cell liposome uptake; (B) neuroblastoma cell liposome uptake.

Liposome uptake was also observed directly by fluorescence microscopy. Figure 4 shows pictures taken after the neuroblastoma cells were cocultured with SCB liposomes for 6 and 24 h. Lysotracker blue was used to evaluate whether the liposomes were directed to acidic compartments after internalization. In the neuroblastoma cells, the liposomes appeared to be concentrated at or near the cell membrane after 6 h. Meanwhile, at 24 h, the liposomes were more distributed throughout the cytoplasm. Additionally, no colocalization within acidic compartments was noted at 6 or 24 h.

![Figure 4](image4.png)

**Figure 4.** Representative images of SCB liposome uptake by neuroblastoma cells as seen by fluorescence microscopy. Blue represents acidic compartments, such as lysosomes, stained by Lysotracker blue. Green shows the SCB liposomes with 1 mol % of NBD-PE, a fluorescent lipid. (A) Photo after 6 h of coculture; (B) photo after 24 h of coculture. Scale, 50 \( \mu \text{m} \).
3.5. SCB Liposome Uptake Was Independent of Macropinocytosis or Clathrin-Mediated Endocytosis

CYD inhibits macropinocytosis. Concentrations of 1, 5, 20 and 40 μM were studied to assess cell viability. No decrease in neuroblastoma cell viability was observed at any CYD concentration compared with the control cells (data not shown). The effect of CYD on liposome uptake was then determined with 1 μM and 5 μM CYD concentrations (Figure 5A). No difference in SCB liposome uptake was found between the cells exposed to CYD and the control cells (Kruskal–Wallis test $p$-value 0.2034). Clathrin-mediated endocytosis is inhibited by CPZ. Neuroblastoma cell viability was examined with CPZ concentrations of 5, 10, 20, and 40 μM. No changes in cell viability were detected at any concentration (data not shown). The effect of 5 μM and 10 μM of CPZ on liposome uptake was then determined (Figure 5B). A slight but not significant change in liposome uptake was noted with a concentration of either 5 μM or 10 μM CPZ compared with the control cells (Mann–Whitney test $p$-value 0.1000 for both).

![Figure 5](image-url).

**Figure 5.** Effect of endocytic pathway inhibitors in the uptake of SCB liposomes as measured by flow cytometry. Cytochalasin D (CYD), a macropinocytosis inhibitor, and chlorpromazine (CPZ), a clathrin-mediated endocytosis inhibitor, were cultured with neuroblastoma cells for 30 min. Then, 0.2 mM of SCB liposomes were added and co-cultured for an additional 2 h, and finally the fluorescence of the liposomes was detected by flow cytometry. Bars indicate the mean and standard error. (A) Effect of CYD on SCB liposome uptake ($n = 6$); (B) effect of CPZ on SCB liposome uptake ($n = 3$).

4. Discussion

Liposomes are used as therapeutic vehicles to improve the in vivo efficiency of various medications [4]. Encapsulation of chemotherapeutic agents is of particular interest in cancer therapy, particularly in treatment-resistant tumors and in tumors located in areas where the bioavailability of chemotherapeutic agents is low [15,16]. Tumor cells can express specific receptors or molecules that could be used as targets for monoclonal antibodies or liposomes [4,16]. Additionally, the extracellular matrix (ECM) that supports tumor cell growth has specific ligands that could help increase the concentration of therapeutic agents. Thus, liposomes may serve as a medium for cell-targeted delivery of highly cytotoxic chemotherapeutic agents [15,16].

Previously, we showed that a human glioblastoma cell line can avidly uptake SCB liposomes [6]. The uptake did not affect the cell viability or immune-related function of the astrocytoma cells [6]. A glioma model in rats showed that doxorubicin encapsulated in liposomes containing sulfatides had less organ toxicity and reduced tumor size compared with free doxorubicin [17]. These effects could be associated with specific interactions between sulfatides and molecules overexpressed in tumor tissues, such as tenascin-C [18,19] or LDL-R [8,20–23]. The interaction between sulfates and tenascin-C, an ECM protein, ensures the accumulation of liposomes in the tumor region [24], whereas the interaction with LDL-R ensures its intracellular delivery to target cells [18,19,25]. In astrocytoma cells, LDL-R was
linked with the selective uptake of liposomes containing sulfatides [6,7]. Evidence shows that selective protein-mediated lipid internalization involves LDL-R in other tumors [26]; thus, the expression of this receptor by other tumors, such as neuroblastoma, was expected. We measured LDL-R expression in neuroblastoma cells. Previous research by our group showed that it was expressed in astrocytoma cells [6]. Here, neuroblastoma was also found to express LDL-R; the expression values were higher in neuroblastoma cells than in astrocytoma cells, but the difference was not significant.

Consequently, in the present study, the ability of a neuroblastoma cell line to uptake SCB and DOPC liposomes was assessed. DOPC liposomes represent the main components of a regular eukaryotic cell membrane, whereas SCB liposomes have a composition similar to the membrane of myelinated axons. This could provide more evidence of the role of SCB liposomes as targeted drug delivery vehicles for other types of cancer, such as neuroblastoma. One of the main differences of the SBC liposomes compared with DOPC liposomes was the z-potential. Due to the anionic characteristic of sulfatides, the expected decrease in z-potential was observed in SBC liposomes. This feature could be associated with the uptake of liposomes due to the charge. However, in a previous study, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG) liposomes with a z-potential similar to SCB liposomes did not have an increased uptake as did the SCB liposomes in an astrocytoma cell line [7].

Ceramides, which are precursors of sphingolipids, can induce apoptosis in neuroblastoma, and sulfatide accumulation produces neuron loss in diseases such as metachromatic leukodystrophy [27]. Hence, the impact of SCB liposomes on neuroblastoma cell viability was also evaluated. Our previous experience with astrocytoma cells showed no effect of SCB liposomes on cell viability [6,7]. Similar findings are presented here with neuroblastoma cells.

The liposome uptake capacity of the astrocytoma and neuroblastoma cell lines was compared. Both cell lines exhibited an increased uptake of SCB liposomes compared with DOPC liposomes, with astrocytoma cells showing a slightly higher SCB uptake capacity. Visualization under the microscope suggests that SCB liposomes are located near the inner leaflet of the neuroblastoma cell membrane at 6 h, especially as stringent washes were made to remove liposomes bound to the outer membrane. At 24 h, the changes in liposome distribution suggest that they are inside the neuroblastoma cells. Nevertheless, some liposomes may remain adhered to the external membrane. The observed pattern of SCB liposome distribution in neuroblastoma cells differed from the even cytoplasmic distribution described in astrocytoma cells [6,7]. Astrocytoma cell uptake of SCB liposomes is most likely mediated by LDL-R, with internalization via the clathrin pathway [6,7]. CPZ inhibition of this pathway produced a significant decrease in SCB lipid uptake in astrocytoma cells [7]. Physiologically, LDL and LDL-R are internalized via clathrin-coated pits; after internalization, LDL is released into endosomes while LDL-R is transferred again to the cell surface [28]. Within endosomes, the lipoprotein and its cargo start a degradation process that is finished in the lysosome [29]. This work presents indirect data, suggesting that the endocytic pathway involved in SCB lipid uptake by neuroblastoma cells is probably neither LDL-R-mediated internalization via clathrin pits nor macropinocytosis. Evidence to support this claim includes the difference in the distribution patterns of LDL-R and SCB liposomes shown in the immunofluorescence pictures. Additionally, colocalization of SCB liposomes does not appear in the acidic compartments of neuroblastoma cells. These observations differed from the ones in astrocytoma cells [6,7]. These results suggest that the uptake of SCB liposomes in this cell line relies on a different uptake mechanism. Drug delivery by liposomes can be achieved by several mechanisms depending on how the liposomes interact with the cell membrane. These features can influence drug delivery. The cargo delivery mechanisms of liposomes include (a) absorption into the cell membrane, (b) fusion with the cell membrane, and (c) internalization via endocytosis. In the first mechanism, the drug is released extracellularly and then diffused into the cell; in the second mechanism, the drug is released intracellularly. Finally, in the endocytic pathway the compound travels in endosomes [30]. In the endocytic pathway, the interaction between liposomes and lysosomes, which have a lower pH and contain enzymes, might affect the delivered drug mechanism and function.
For example, some specific liposomal compositions can release doxorubicin in a pH-dependent manner [31], and modified doxorubicin preparations with peptide sequences targeted by cathepsin B facilitate the release and induce antitumor activity [32].

Future work should clarify the role of LDL-R and tenascin-C as potential mediators of the uptake of sulfatides in neuroblastoma. Tenascin-C appears to be poorly expressed in the neuroblastoma-derived SHSY-5Y cells used in this work [33]. However, a retrospective analysis of RNA-sequencing data of 498 neuroblastoma tumors showed tenascin-C expression in both low-risk and high-risk tumors [34]. Additionally, as this is an in vitro study, the specificity of SCB liposomes needs to be tested in animal models to shed more light on this promising targeted cancer drug delivery vehicle.

5. Conclusions

Our research group designed SCB liposomes with a composition that resembles the membrane of myelinated axons. This similitude was intended to increase the uptake of liposomes by cells derived from the central nervous system with the aim of designing targeted drug delivery systems for neurogenically derived tumors. We found that neuroblastoma cells had an increased SCB liposome uptake compared with DOPC liposomes in vitro. No effect of SCB liposomes on cell viability was observed. The uptake did not appear to be associated with LDL-R. Additionally, inhibition of clathrin-dependent endocytosis or macropinocytosis did not result in a significant decrease in SCB liposome uptake. Further studies are needed to characterize the transport pathway involved and confirm the in vivo replicability of our findings.

Supplementary Materials: The following information is available online at http://www.mdpi.com/2227-9717/8/12/1615/s1. Figure S1: Neuroblastoma cell viability after SCB coculture.

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References

1. Allen, T.M.; Cullis, P.R. Liposomal drug delivery systems: From concept to clinical applications. Adv. Drug Deliv. Rev. 2013, 65, 36–48. [CrossRef] [PubMed]
2. Madni, A.; Sarfraz, M.; Rehman, M.; Ahmad, M.; Akhtar, N.; Ahmad, S.; Tahir, N.; Ijaz, S.; Al-Kassas, R.; Löbenberg, R. Liposomal drug delivery: A versatile platform for challenging clinical applications. J. Pharm. Pharm. Sci. 2014, 17, 401–426. [CrossRef] [PubMed]
3. Torchilin, V. Multifunctional nanocarriers. Adv. Drug Deliv. Rev. 2006, 58, 1532–1555. [CrossRef] [PubMed]
4. Li, M.; Du, C.; Guo, N.; Teng, Y.; Meng, X.; Sun, H.; Li, S.; Yu, P.; Galons, H. Composition design and medical application of liposomes. Eur. J. Med. Chem. 2019, 164, 640–653. [CrossRef] [PubMed]
5. Xing, M.; Yan, F.; Yu, S.; Shen, P. Efficacy and cardiotoxicity of liposomal doxorubicin-based chemotherapy in advanced breast cancer: A meta-analysis of ten randomized controlled trials. PLoS ONE 2015, 10, e0133569. [CrossRef]
6. Suesca, E.; Alejo, J.L.; Bolaños, N.I.; Ocampo, J.; Leidy, C.; González, J.M. Sulfocerebrosides upregulate liposome uptake in human astrocytes without inducing a proinflammatory response. Cytom. Part A 2015, 83A, 627–635. [CrossRef]
7. Suesca, E.; Bolaños, N.I.; Bustamante, N.C.; Badwaik, V.; Gonzalez, J.M.; Thompson, D.H.; Leidy, C.; Universidad de los Andes, Bogotá, DC, Colombia. Elucidating the uptake mechanism of liposomes containing sulfatides by astrocytoma cells. Unpublished work. 2020.
8. Menrad, A.; Anderer, F.A. Expression of LDL receptor on tumor cells induced by growth factors. *Anticancer Res.* 1991, 11, 385–390.

9. Ward, E.; DeSantis, C.; Robbins, A.; Kohler, B.; Jemal, A. Childhood and adolescent cancer statistics, 2014: Cancer in Children and Adolescents. *CA Cancer J. Clin.* 2014, 64, 83–103. [CrossRef] [PubMed]

10. Irwin, M.S.; Park, J.R. Neuroblastoma paradigm for precision medicine. *Pediatr. Clin. N. Am.* 2015, 62, 225–256. [CrossRef]

11. Matthay, K.K.; Maris, J.M.; Schiefermacher, G.; Nakagawara, A.; Mackall, C.L.; Diller, L.; Weiss, W.A. Neuroblastoma. *Nat. Rev. Dis. Primers* 2016, 2, 16078. [CrossRef]

12. Brodeur, G.M.; Hogarty, M.D.; Bagatell, R.; Mosse, Y.P.; Maris, J.M. Neuroblastoma. In *Principles and Practice of Pediatric Oncology*; Pizzo, P.A., Poplack, D.G., Adamson, P.C., Blaney, S.M., Blaney, S.M., Helman, L., Eds.; Wolters Kluwer Health: Philadelphia, PA, USA, 2016.

13. Ahmed, A.A.; Zhang, L.; Reddivilla, N.; Hetherington, M. Neuroblastoma in children: Update on clinicopathologic and genetic prognostic factors. *Pediatr. Hematol. Oncol.* 2017, 34, 165–185. [CrossRef]

14. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: An open-source platform for biological-image analysis. *Nat. Methods* 2012, 9, 676–682. [CrossRef]

15. Wakaskar, R.R. Promising effects of nanomedicine in cancer drug delivery. *J. Drug Target.* 2018, 26, 319–324. [CrossRef] [PubMed]

16. Kydd, J.; Jadja, R.; Velpurisiva, P.; Gad, A.; Paliwal, S.; Rai, P. Targeting Strategies for the Combination Treatment of Cancer Using Drug Delivery Systems. *Pharmaceutica* 2017, 9, 46. [CrossRef]

17. Lin, J.; Shidgar, S.; Fang, D.Z.; Xiang, D.; Wei, M.Q.; Danks, A.; Kong, L.; Li, L.; Qiao, L.; Duan, W. Improved efficacy and reduced toxicity of doxorubicin encapsulated in sulfatide-containing nanoliposome in a glioma model. *PLoS ONE* 2014, 9, e103736. [CrossRef] [PubMed]

18. Yoshida, T.; Akatsuka, T.; Imanaka-Yoshida, K. Tenascin-C and integrins in cancer. *Cell Adhes. Migr.* 2015, 9, 96–104. [CrossRef] [PubMed]

19. Herold-Mende, C.; Mueller, M.M.; Bonsanto, M.M.; Schmitt, H.P.; Kunze, S.; Steiner, H.-H. Clinical impact and functional aspects of tenascin-C expression during glioma progression. *Int. J. Cancer* 2002, 98, 362–369. [CrossRef] [PubMed]

20. Gal, D.; Macdonald, P.C.; Porter, J.C.; Simpson, E.R. Cholesterol metabolism in cancer cells in monolayer culture. III. Low-density lipoprotein metabolism. *Int. J. Cancer* 1981, 28, 315–319. [CrossRef]

21. Vitolis, S.; Peterson, C.; Larsson, O.; Holm, P.; Aberg, B. Elevated uptake of low density lipoproteins by human lung cancer tissue in vivo. *Cancer Res.* 1992, 52, 6244–6247.

22. Pires, L.A.; Hegg, R.; Freitas, F.R.; Tavares, E.R.; Almeida, C.P.; Baracat, E.C.; Maranhão, R.C. Effect of neoadjuvant chemotherapy on low-density lipoprotein (LDL) receptor and LDL receptor-related protein 1 (LRP-1) receptor in locally advanced breast cancer. *Braz. J. Med. Biol. Res.* 2012, 45, 557–564. [CrossRef]

23. DeGonzalo-Calvo, D.; López-Vilaró, L.; Nasarre, L.; Perez-Olabarria, M.; Vázquez, T.; Escuin, D.; Badimon, L.; Barnadas, A.; Lerma, E.; Llorente-Cortés, V. Intratumor cholesteryl ester accumulation is associated with human breast cancer proliferation and aggressive potential: A molecular and clinicopathological study. *BMC Cancer* 2015, 15, 460. [CrossRef]

24. Shao, K.; Hou, Q.; Go, M.L.; Duan, W.; Cheung, N.S.; Feng, S.-S.; Wong, K.P.; Yoram, A.; Zhang, W.; Huang, Z.; et al. Sulfatide-tenascin interaction mediates binding to the extracellular matrix and endocytic uptake of liposomes in glioma cells. *Cell. Mol. Life Sci.* 2007, 64, 506–515. [CrossRef]

25. Wang, Z.; Duan, X.; Lv, Y.; Zhao, Y. Low density lipoprotein receptor (LDLR)-targeted lipid nanoparticles for the delivery of sorafenib and Dihydroartemisinin in liver cancers. *Life Sci.* 2019, 239, 117013. [CrossRef]

26. Toussignant, K.D.; Rockstroh, A.; Fard, A.T.; Lehman, M.L.; Wang, C.; McPherson, S.J.; Philip, L.K.; Bartonick, N.; Dinger, M.E.; Nelson, C.C.; et al. Lipid Uptake Is an Androgen-Enhanced Lipid Supply Pathway Associated with Prostate Cancer Disease Progression and Bone Metastasis. *Mol. Cancer Res.* 2019, 17, 1166–1179. [CrossRef] [PubMed]

27. Zeng, Y.; Cheng, H.; Jiang, X.; Han, X. Endosomes and lysosomes play distinct roles in sulfatide-induced neuroblastoma apoptosis: Potential mechanisms contributing to abnormal sulfatide metabolism in related neuronal diseases. *Biochem. J.* 2008, 410, 81–92. [CrossRef] [PubMed]

28. Zou, P.; Ting, A.Y. Imaging LDL receptor oligomerization during endocytosis using a co-internalization assay. *ACS Chem. Biol.* 2011, 6, 308–313. [CrossRef]
29. Boron, W.F.; Boulpaep, E.L. Medical Physiology; Elsevier Health Sciences: Philadelphia, PA, USA, 2016; ISBN 978-1-4557-3328-6.

30. Bozzuto, G.; Molinari, A. Liposomes as nanomedical devices. Int. J. Nanomed. 2015, 10, 975–999. [CrossRef]

31. Johnson, R.P.; Jeong, Y.-I.; Choi, E.; Chung, C.-W.; Kang, D.H.; Oh, S.-O.; Suh, H.; Kim, I. Biocompatible Poly(2-hydroxyethyl methacrylate)-b-poly(L-histidine) Hybrid Materials for pH-Sensitive Intracellular Anticancer Drug Delivery. Adv. Funct. Mater. 2012, 22, 1058–1068. [CrossRef]

32. Minko, T.; Kopečkova, P.; Pozharov, V.; Jensen, K.D.; Kopeček, J. The Influence of Cytotoxicity of Macromolecules and of VEGF Gene Modulated Vascular Permeability on the Enhanced Permeability and Retention Effect in Resistant Solid Tumors. Pharm. Res. 2000, 17, 505–514. [CrossRef] [PubMed]

33. Linnala, A.; Lehto, V.P.; Virtanen, I. Neuronal differentiation in SH-SY5Y human neuroblastoma cells induces synthesis and secretion of tenascin and upregulation of alpha(v) integrin receptors. J. Neurosci. Res. 1997, 49, 53–63. [CrossRef]

34. Otsuka, K.; Sasada, M.; Iyoda, T.; Nohara, Y.; Sakai, S.; Asayama, T.; Suenaga, Y.; Yokoi, S.; Higami, Y.; Kodama, H.; et al. Combining peptide TNIIA2 with all-trans retinoic acid accelerates N-Myc protein degradation and neuronal differentiation in MYCN-amplified neuroblastoma cells. Am. J. Cancer Res. 2019, 9, 434–448. [PubMed]

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