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RESEARCH PAPER

Targeted Delivery to Neuroblastoma of Novel siRNA-anti-GD2-liposomes Prepared by Dual Asymmetric Centrifugation and Sterol-Based Post-Insertion Method

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ABSTRACT

Purpose To optimise and simplify preparation of targeted liposomes for efficient siRNA delivery to neuroblastoma, the most common solid tumour in early childhood.

Methods Liposomes containing siRNA were prepared by combining the novel dual asymmetric centrifugation (DAC) method and the recently optimised sterol-based post-insertion technique (SPIT) to couple anti-GD2 antibody for selective interaction with neuroblastoma cells. Cultured human neuroblastoma cell lines were used to evaluate the efficiency of siRNA delivery.

Results The size of liposomes prepared by DAC ranged from 190 to 240 nm; siRNA encapsulation efficiency was up to 50%. An average of 70 and 100 molecules of anti-GD2 antibody per particle were coupled. A significant association of liposomes with neuroblastoma cells as well as effective siRNA delivery was observed only when anti-GD2 antibody was coupled. Preliminary data suggest delivery of siRNA using anti-GD2-liposomes occurs via GD2-mediated endocytosis. Vascular endothelial growth factor A (VEGF-A) was down-regulated using siRNA delivered by anti-GD2-liposomes.

Conclusions DAC and SPIT allow for the straightforward preparation of liposomes for the targeted delivery of siRNA. Anti-GD2-liposomes thus produced can serve as versatile carriers of siRNA to neuroblastoma cells.

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KEY WORDS dual asymmetric centrifugation · GD2 antibody · neuroblastoma · post-insertion technique · siRNA delivery

ABBREVIATIONS

Ab	antibody
Chol	cholesterol
cryo-TEM	cryo-transmission electron microscope
DAC	dual asymmetric centrifugation
DDAB	dimethyldioctadecylammonium (bromide salt)
DSPE-PEG_{2000}	2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]
FCS	fetal calf serum
GD2	disialoganglioside
HN buffer	HEPES/NaCl buffer
HSPC	hydrogenated soybean phosphatidylcholine
L	liposomes
PBS	phosphate buffered saline
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
Rhd-PE	rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt
siRNA	short interfering RNA
SPIT	sterol-based post-insertion technique
TL	total lipid
VEGF-A	vascular endothelial growth factor A

INTRODUCTION

Since the discovery of siRNA and its ability to inhibit expression of specific genes (1), these molecules have become potentially the most powerful and promising therapeutics to be used for the regulation of gene

expression. The major challenge in applying siRNA therapeutics is the targeting and delivery of siRNA to specific cells (2). Several different carriers, such as polymers, antibodies, or cationic lipid complexes, have been designed in order to efficiently deliver siRNA to cells (3). Liposomes have been shown to be the most successful carriers for siRNA (4–6). In contrary to other carriers, liposomes are non-toxic, can be easily targeted by attaching specific ligands to their surface, and have favourable pharmacokinetic properties *in vivo* (7). However, liposomes prepared by conventional methods often result in particles with low siRNA encapsulation efficiency. Recently, a new method of liposome preparation using dual asymmetric centrifugation (DAC) has been shown to yield liposomes with a high efficiency of siRNA encapsulation (8). In the current study, we have applied this method to prepare liposomes for the delivery of siRNA to neuroblastoma cells.

Accounting for 7–10% of all childhood cancers, neuroblastoma is the most common solid tumour in children (9). In spite of aggressive treatment, such as chemotherapy, surgical intervention, and radiotherapy, the majority of patients are at high risk of developing incurable tumours. In contrast to this group, there are neuroblastomas which present with a good prognosis and even spontaneously regress (10). This heterogeneity is caused by specific genetic features (e.g. ploidy status, allelic loss, N−myc oncogene amplification) and the expression of certain proteins (e.g. neurotrophin receptor, or angiogenesis factors such as vascular endothelial growth factor (VEGF)) which define the progression of the disease and clinical behaviour. In view of the current knowledge about neuroblastoma biology, it seems that novel therapies that target specific genes and proteins, e.g. by siRNA, would be more effective in the treatment of this tumour than conventional approaches (11).

In order to achieve specific uptake of siRNA by neuroblastoma cells, anti-GD2 antibody was coupled to the surface of liposomes containing siRNA using the sterol-based post-insertion technique (SPIT) (12). Disialoganglioside (GD2) is known to be highly expressed on the surface of some tumours, including neuroblastoma (13,14). In healthy humans GD2 is present only in the peripheral nervous system and the cerebellum. The function of GD2 in neuroblastoma is not fully understood; however, it has been shown to be involved in the process of tumour cell adhesion, invasion, and migration (15). Because of its specific occurrence in the tumour, GD2 has been used as a ligand in novel neuroblastoma targeted treatments. Several types of anti-GD2 antibodies have been developed and are under investigation in clinical trials for immunotherapy against neuroblastoma (16).

A combination of the DAC and SPIT methods was tested on two different liposome formulations. The first type of liposomes examined was characterised by having rather rigid membranes consisting of hydrogenated phosphatidylcholine and cholesterol. The second set consisted of more fluid, positively charged liposomes which were composed of palmitoyloleoylphosphocholine and dimethyldioctadecylammonium. These particles were extensively characterised with regard to size, ζ-potential, morphology, efficiency of siRNA encapsulation and antibody coupling. In addition, the association of anti-GD2-liposomes, the uptake of siRNA, and the effects of siRNA were studied in cultured human neuroblastoma cells.

In summary, we show in this study that targeted liposomes prepared by DAC and SPIT are stable particles with a high siRNA content and good antibody coupling efficiency, which can specifically and effectively deliver siRNA to neuroblastoma cells. To our knowledge, this is the first study which combines these two methods for the preparation of targeted liposomes loaded with siRNA.

MATERIALS AND METHODS

Materials

Hydrogenated soybean phosphatidylcholine (HSPC) and 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}) were kindly provided by Lipoid (Ludwigshafen, Germany). Dimethyldioctadecylammonium (bromide salt) (DDAB) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster AL, USA). Cholesterol, chlorpromazine, genestein and methyl-β-cyclodextrine were from Sigma-Aldrich®, and rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Rhd-PE) was from Molecular Probes (Karlsruhe, Germany). Fluorescently labeled AllStars Negative siRNA Alexa488 was purchased from Qiagen (Hilden, Germany). The VEGF-A and scrambled siRNA with sense strand sequence 5'—AUGUGAAUGCAGACCAAAGAA—TT and 5'—GAUAGCAAUGACGAAUGCGUA—TT, respectively (17), were synthesised by Eurofins MWG Operon (Ebersberg, Germany). Anti-GD2 antibody (hu 14.18) was a generous gift from Rupert Handgretinger (University Children's Hospital Tübingen, Tübingen, Germany). Culture medium RPMI 1640, fetal calf serum (FCS), PBS buffer and trypsin/EDTA 0.05%/0.25% (w/v) were from Biochrom AG (Berlin, Germany).

Preparation of Liposomes

Liposomes were prepared using the dual asymmetric centrifugation (DAC) method described by Massing *et al.*

(8) with small modifications. Briefly, 21 mg of lipids from stock solutions in absolute ethanol were mixed in the 2 ml Eppendorf cup in the following molar ratios: HSPC/Chol/DSPE-PEG_{2000} (12.1/8.2/1.14) or POPC/DDAB/DSPE-PEG (94/5/1). When indicated, 0.4 mol% rhodamine-PE was added. The lipids were dried in a Speed Vac (Eppendorf, Germany) until the ethanol was evaporated. The lipid mixture (35% of the total batch weight) was hydrated with 40 µl (65% of the batch) of either HN buffer pH 7.4 (10 mM HEPES, 140 mM NaCl) or, when appropriate, with siRNA solution (0.75 nmol siRNA/1 µmol of total lipids) for 10 min. Next, glass beads of 1 mm diameter were added, and the sample was centrifuged in a dual asymmetric centrifuge (DAC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany) at 3,540 rpm for 30 min. In order to disperse the liposomes, 80 µl of HN buffer pH 7.4 was added, and the sample was centrifuged at 3,540 rpm for 3 min. Subsequently, the volume of the liposome dispersion was adjusted to 1 ml with HN buffer at pH 7.4 and stored at 4°C until further use.

Anti-GD2 antibody was coupled to the liposomes using SPIT, as has been recently reported (12). Briefly, in the first step, 2.25 mol% of succinimide-activated sterol-PEG_{1300} (sterol-PEG_{1300}-NHS) anchor to the total lipid concentration was incubated with anti-GD2 antibody (ratio antibody to anchor 1 to 50 mol%) for 15 min at 17°C and 750 rpm speed in the Termomixer (Eppendorf). In order to stop the coupling reaction of the anchor to the antibody, a 50-fold molar excess of L-histidine (Fluka) was added, followed by a 10 min incubation at 17°C and 750 rpm mixing. In the second step, liposomes were added to the sterol-PEG_{1300}-NHS-anti-GD2 antibody, allowing insertion of these constructs into the outer membrane of the liposomes for 100 min at 17°C and 750 rpm. Subsequently, uncoupled antibodies, anchors, and, when appropriate, non-encapsulated siRNA were removed from liposomes using Centrisart I columns (Satorius Stedim Biotech GmbH, Göttingen, Germany) with a 300.000 mwco membrane. Liposomes were washed with HN pH 7.4 buffer and centrifuged in the Centrisart I columns for 20 min at 2,000 g for a total of three times. Control liposomes containing siRNA were purified from non-encapsulated siRNA following the same procedure but using Centrisart I columns with a 100.000 mwco membrane.

Characterisation of Liposomes

The lipid concentration of each preparation were measured by a phosphorous assay according to Bartlett (18), and the total lipid concentration was corrected to the amount of lipids that did not contain phosphate groups.

The size and size distribution of liposomes were determined by photon correlation spectroscopy (PCS) (Zeta Plus, Brookhaven Instruments, NY, USA) directly after preparation was completed. Size stability studies of liposomes during storage were performed by weekly measurement of the sample stored at 4°C.

For ζ-potential measurements, in order to reduce the ionic strength of the HN buffer, pH 7.4, 20 µl of liposome preparation was diluted in 1 ml of sterile filtered 5 mM phosphate buffer (3 mM Na_2HPO_4*$2H_2O$, 2 mM KH_2PO_4) at pH 7.0. The ζ-potential was determined using the Zetasizer Nano Series (Malvern Instruments, UK). The final measurement of each sample is a mean of 10 runs performed by the instrument.

The morphology of liposomes was studied using cryo-TEM microscopy as described previously (19).

For the measurement of the antibody concentration coupled to the liposomes, particles were destroyed by 0.3% of deoxycholate, and the proteins were precipitated through the use of 70% TCA. The concentration of antibody was then analyzed using the BioRad DC Protein Assay (BioRad) according to the manufacturer's protocol. The number of antibody molecules coupled to the surface of the liposomes was calculated from the formula described previously (20), assuming the molecular mass of anti-GD2 antibody to be 156 kDa.

The efficiency of siRNA encapsulation into liposomes was determined with the Quanti-iT™ RiboGreen® assay (Invitrogen) according to the protocol of the manufacturer. The measurements of each sample were done in both the absence (intact particles) and presence (destroyed particles) of 0.1% Triton x-100 (Sigma). The final efficiency of siRNA encapsulation into liposomes was calculated by subtracting the amount of siRNA measured without Triton x-100 from the siRNA measured in the presence of the detergent.

Cell Culturing

The human neuroblastoma cell lines Kelly, IMR-32 (both DSMZ, Braunschweig, Germany) and SK-N-AS (gift from Division of Paediatric Haematology and Oncology, University Hospital of Essen, Germany) were cultured in RPMI-1640 medium supplemented with 10% FCS at 37°C in a humidified incubator with 5% CO_2 atmosphere. The medium was replaced every 3 days, and the cells were seeded into a new culture dish when a confluency of 80% was reached using a trypsin/EDTA (0.05%/0.02% (w/v) in PBS) solution. For flow cytometry analysis, neuroblastoma cell lines were seeded onto 24-well plates 24 h prior to the experiment. For fluorescence and confocal microscopy experiments, cells were seeded 24 h prior to the experiment on sterile cover glasses added into the wells of a 24-well plate and coated with 0.2% gelatin in PBS overnight.

Expression of GD2 by Neuroblastoma Cells Lines

For flow cytometry analysis, the cells were washed with PBS, harvested using trypsin/EDTA solution and re-suspended in ice-cold FACS buffer (PBS containing 5% FCS). The cells were centrifuged for 4 min at 1,200 rpm and 4°C to spin them down. Subsequently, they were incubated either with anti-GD2 mouse anti-human IgG2a (BD Pharmingen) or an irrelevant IgG control, mouse anti-rat IgG2a (BD Pharmingen) for 1 h and 15 min at 4°C. Both antibodies were diluted 1:80 in FACS buffer. After washing with FACS buffer and centrifugation (4 min at 1,200 rpm and 4°C), the cells were incubated with the secondary antibody, goat anti-mouse IgG (H+L) MFP488 (MoBiTec, Göttingen, Germany) diluted 1:80 in PBS containing 5% FCS and 2% human serum for 45 min at 4°C. The cells were washed twice with FACS buffer and analysed using a FACS Calibur® (Becton-Dickinson, Heidelberg, Germany) in combination with Lysis II software analysis.

For fluorescence microscopy studies, after washing with PBS, cells were fixed with 4% paraformaldehyde solution for 30 min, washed with PBS, and incubated with 10% FCS in PBS for 30 min. Subsequently, incubation with anti-GD2 mouse anti-human IgG2a or an IgG control—mouse anti-rat IgG2a diluted 1:40 in PBS containing 10% FCS—was performed for 60 min at room temperature. The cells were washed twice with PBS for 5 min and stained with goat anti-mouse (H+L) MFP488 antibody diluted 1:25 in PBS containing 10% FCS and 2% human serum for 1 h at room temperature. After two steps of washing cells with PBS, the nuclei of the cells were stained with DAPI (10 nM) for 20 min and washed again with PBS. Cover glasses were mounted with MobiGLOW Mounting Medium (MoBiTec) and placed on the microscope slides. Microscopic analysis was done using a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging GmbH, Germany) equipped with AxioVision 4.7 software. Photographs were taken with an original magnification x 630.

Association of Liposomes with Neuroblastoma Cells

The neuroblastoma cell lines were incubated with Rhd-PE or Alexa488 siRNA-labelled liposomes diluted in culture medium at a concentration of 80 nmol/ml either for 1 h for flow cytometry analysis or for 3 h in the case of observation by fluorescence and confocal microscopy. When indicated, 5 min prior incubation with liposomes anti-GD2 antibody (hu 14.18) was added to the cells at a concentration of 0.1 mg/ml. When the effects of endocytosis inhibitors on the uptake of liposomes were to be studied, cells were incubated either for 1 h with 28 μM of chlorpromazine, 200 μM genistein, or for 15 min with 5 mM methyl-β-cyclodextrin prior to adding liposomes.

In order to halt the incubation with liposomes, the cells were washed twice with PBS. Samples for flow cytometry analysis were prepared as follows. The cells were harvested using trypsin/EDTA solution, transferred to ice-cold FACS buffer, and centrifuged for 4 min at 1,200 rpm and 4°C. Next, the cells were washed twice with FACS buffer and analysed as described above.

For analysis by fluorescence and confocal microscopy, the cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature and washed twice with PBS; the nuclei of the cells were stained with DAPI (10 nM) for 20 min. Subsequently, cells were washed twice with PBS, mounted using MobiGLOW Mounting Medium and placed on microscope slides. Observation by fluorescence microscopy was performed as described above, and confocal microscopy was done using a Zeiss LSM 510 Meta microscope (Carl Zeiss MicroImaging GmbH, Germany). Photographs were taken using a 100x/1.4 NA objective and analysed with LSM Image Browser Rel. 4.2.

Gene Expression Analysis by Real-Time PCR

Kelly cells were seeded in a 24-well plate 24 h prior to the experiment. Anti-GD2-DDAB-liposomes containing VEGF-A siRNA or scrambled siRNA and their uncoupled controls were incubated with the cells for 72 h at a concentration of 1,500 pmol of siRNA per 1 ml. To stop the incubation, the cells were washed with PBS, and total RNA was isolated using the RNeasy® Mini Plus Kit (Qiagen) according to the protocol of the manufacturer. The concentration of RNA was measured using a NanoDrop ND-100 spectrophotometer, and first-strand cDNA synthesis was done with SuperScript™ III RNase H-Reverse Transcriptase (Invitrogen) in a volume of 20 μl, containing 40 U RNaseOut inhibitor (Invitrogen) and 250 ng random hexamers (Promega). The real-time PCR reaction mixture was carried out in a total volume of 25 μl and contained 20 ng of cDNA, primers for VEGF-A (Hs00173626_m1) or GAPDH (Hs99999905_m1) purchased as an Assay-on-Demand from Applied Biosystems and Taq Man Universal PCR MasterMix (Applied Biosystems). The amplification reaction was performed in a Mastercycler® ep realplex (Eppendorf) using the following program: 10 min 95°C, followed by 40 cycles of 15 s 95°C and 1 min 60°C. For each sample the real-time PCR reaction was performed in duplicate, and the average of threshold cycle values (C_t) was used for the calculations. Gene expression levels were normalized by using GAPDH as a reference gene and then subtracted from non-treated Kelly cells.

RESULTS

Characterisation of Liposomes

In order to obtain liposomes for the delivery of siRNA, we tested two lipid compositions, HSPC/Chol/DSPE-PEG (HSPC-liposomes), which represent rather rigid particles, and POPC/DDAB/DSPE-PEG (DDAB-liposomes), producing vesicles with relatively fluid membranes. Particles containing siRNA were prepared using a novel DAC method which was reported as being highly efficient in the encapsulation of siRNA (8). For specific neuroblastoma cell uptake, anti-GD2 antibody was coupled to the surface of liposomes using the SPIT method recently optimised in our laboratory (12). The liposomes thus obtained were then characterised with regard to several parameters. Cryo-TEM analysis of freshly prepared liposomes revealed that HSPC-liposomes were primarily multilamellar particles (Figs. 1 and 2).

In contrast, mostly unilamellar vesicles were observed in the case of DDAB-liposomes. Both types of liposomes appeared as rather heterogeneous populations of particles. The encapsulation of siRNA into liposomes as well as the coupling of antibodies did not influence the morphology of these particles (Figs. 1 and 2).

Size measurements using dynamic light scattering showed that HSPC-liposomes were slightly bigger than DDAB-liposomes (Table I), but both types of liposomes were stable with regard to size during storage at 4°C up to 4 weeks as demonstrated by weekly size determination (Fig. 3). Again, neither the presence of siRNA nor the attachment of antibody to liposomes influenced the size of either the HSPC- or the DDAB-liposomes (Table I). An almost two-fold higher encapsulation efficiency of siRNA was measured in DDAB-liposomes compared to HSPC-liposomes. In addition, the determination of ζ-potential for both types of liposomes showed that DDAB-liposomes were characterised by a positive charge and HSPC-liposomes by a negative. The results of anti-GD2 antibody coupling are presented in Table II. Although similar concentrations of antibodies present in the liposomal preparation (μg protein per μmol lipids) were measured in HSPC- and DDAB-liposomes, the calculated number of antibody molecules coupled to single particle liposomes was higher for HSPC-liposomes then for DDAB-liposomes.

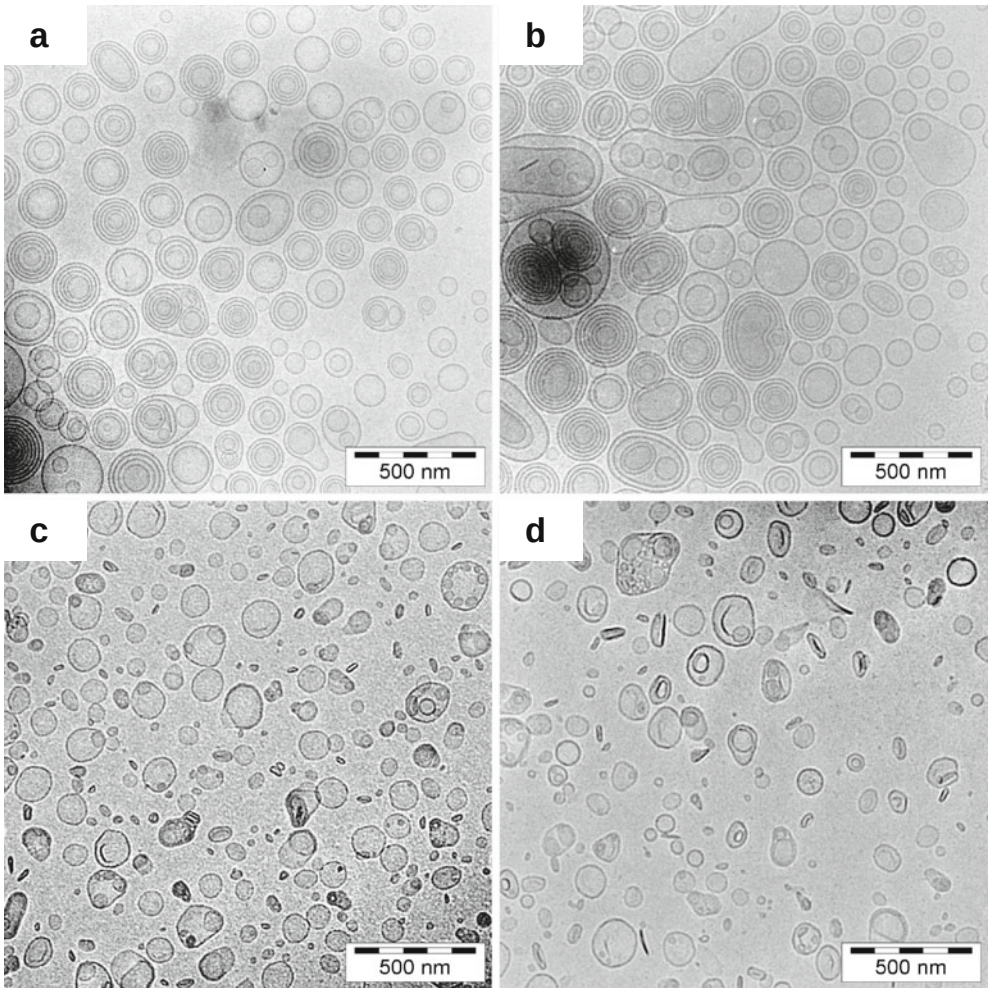

Fig. 1 Morphology of HSCP- and DDAB-liposomes. HSPC- and DDAB-liposomes were made using the DAC method and coupled with anti-GD2 antibody by SPIT. Particles were analysed by means of cryo-TEM, as described in "Materials and Methods." (**a**) HSPC-liposomes, (**b**) anti-GD2-HSCP-liposomes, (**c**) DDAB-liposomes, (**d**) anti-GD2-DDAB-liposomes.

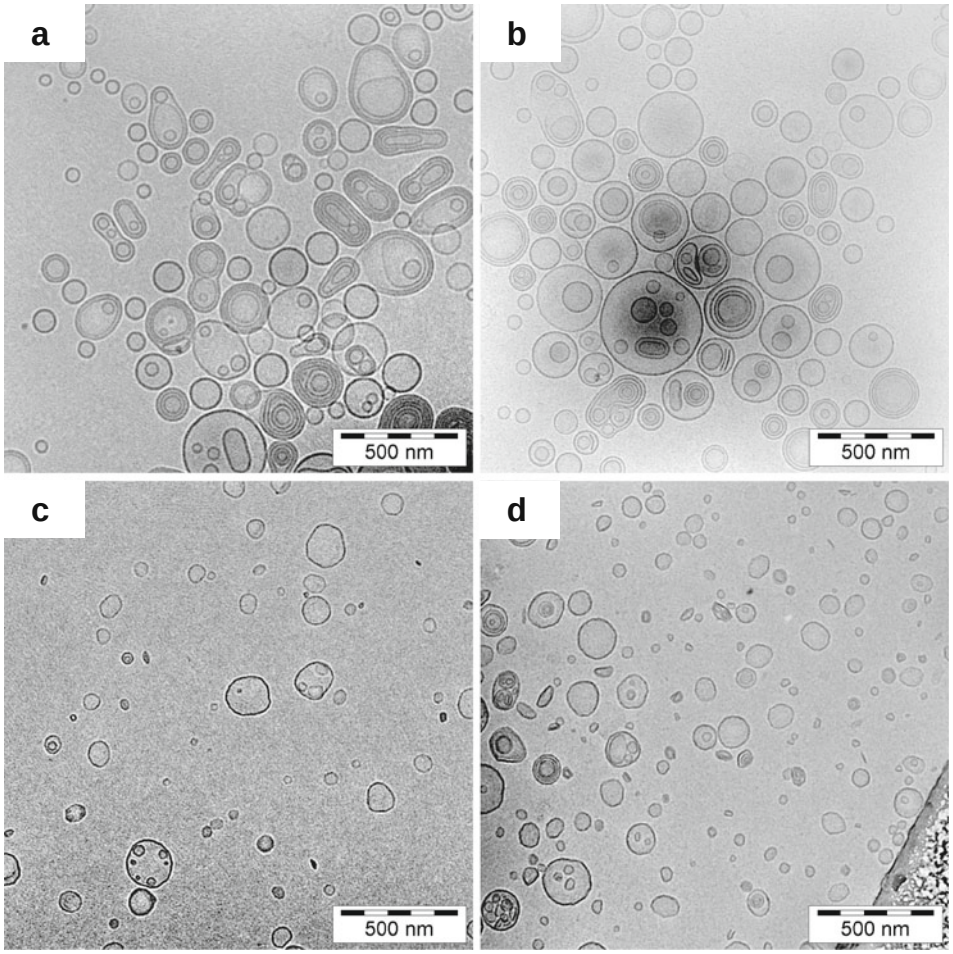

Fig. 2 Morphology of HSPC- and DDAB-liposomes containing siRNA. siRNA was encapsulated into HSPC- and DDAB-liposomes using the DAC method and coupled with anti-GD2 antibody by SPIT. Particles were analysed by means of cryo-TEM as described in "Materials and Methods." (**a**) HSPC-liposomes/siRNA, (**b**) anti-GD2-HSCP-liposomes/siRNA, (**c**) DDAB-liposomes/siRNA, (**d**) anti-GD2-DDAB-liposomes/siRNA.

Uptake of Anti-GD2 Liposomes by Neuroblastoma Cells

Specific overexpression of GD2 on the cell membrane of human neuroblastoma cells has been previously reported (13,14). We confirmed the expression of GD2 molecules on the membrane of Kelly, SK-N-AS and IMR-32 human neuroblastoma cells lines by flow cytometry and immunostaining (Fig. 4A). Each of the cell lines tested expressed GD2, but the expression level differed between cell lines, and showed the highest number of positive cells for Kelly and IMR-32 cells.

To determine the uptake of anti-GD2-HSPC- and anti-GD2-DDAB-liposomes by Kelly, SK-N-AS and IMR-32 cells, these liposomes were labelled on their membrane with rhodamine-PE and analysed by FACS. Coupling of anti-GD2 antibody to the surface of HSPC- and DDAB-liposomes resulted in a several-fold increase in the association of these particles with each of the tested neuroblastoma cell lines (Fig. 4C and D). Uptake of control liposomes which did not

Table I Characterisation of HSCP- and DDAB-liposomes prepared by DAC

	-siRNA	+siRNA	-siRNA	+siRNA
	HSPC-L		DDAB-L	
size [nm]	241 ± 11	236 ± 25	192 ± 27	185 ± 21
ζ-potential [mV]	-16.4 ± 1.9	-15.2 ± 1.2	17.9 ± 6.4	11.7 ± 0.8
siRNA EE [%]	–	21 ± 5	–	47 ± 10
	anti-GD2-HSPC-L		anti-GD2-DDAB-L	
size [nm]	241 ± 8	234 ± 26	192 ± 22	191 ± 20
ζ -potential [mV]	-15.8 ± 0.8	-14.7 ± 0.2	9.4 ± 3.9	6.6 ± 0.3
siRNA EE [%]	–	26 ± 10	–	43 ± 12

Size, ζ-potential and siRNA encapsulation efficiency (EE) were measured in HSCP- and DDAB-liposomes as described in "Material and Methods." Data are presented as ±SD of 5 to 13 preparations. *L* liposomes.

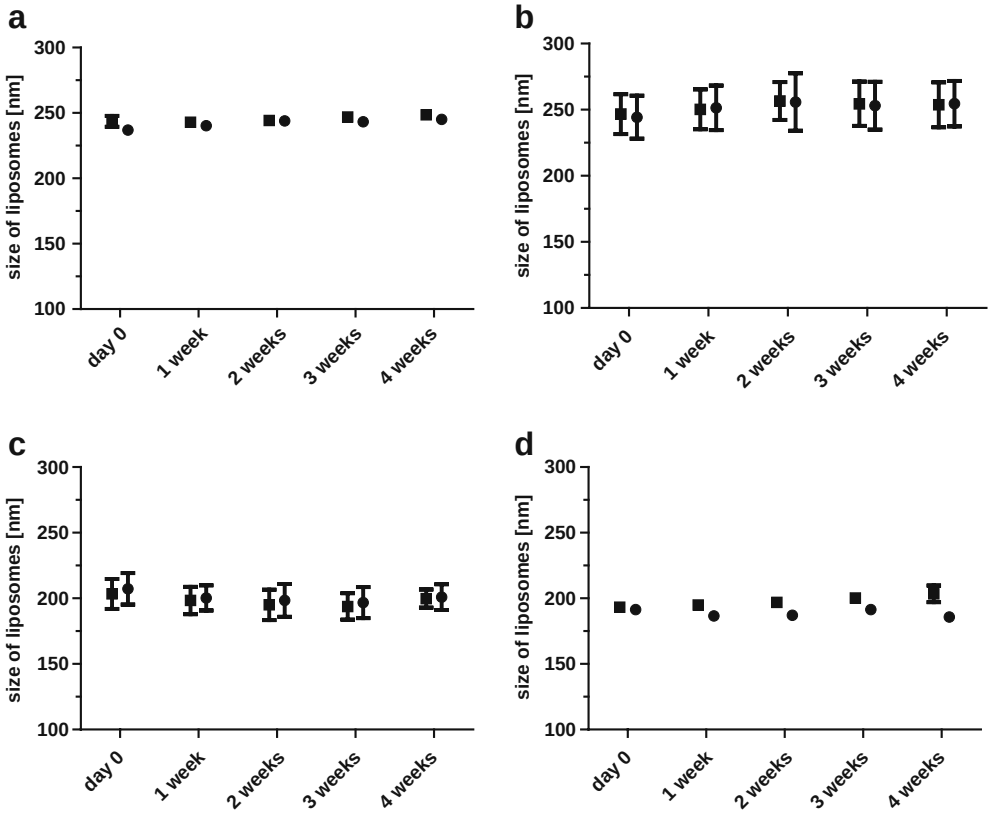

Fig. 3 Size stability of HSPC- and DDAB-liposomes, with and without encapsulated siRNA. The size of HSPC- (**a**, **b**) and DDAB-liposomes (**c**, **d**) coupled with anti-GD2 antibody (■) and uncoupled (●) particles, either containing siRNA (**b**, **d**) or empty (**a**, **c**), was measured weekly for a period of 1 month, as described in "Materials and Methods." Data are presented as ±SEM of 3 to 4 preparations.

contain anti-GD2 antibody was very limited. Interestingly, we observed that the association of anti-GD2-DDAB-liposomes with these three cell lines was also significantly higher than with anti-GD2-HSPC-liposomes, reaching values of more than 80% positive cells in the case of Kelly and IMR-32 cells. In addition, the uptake of anti-GD2-HSPC and anti-GD2-DDAB-liposomes correlated with the presence of GD2 molecules on the surface of neuroblastoma cell lines. The lowest association of both types of liposomes was measured for SK-N-AS cells and the highest for Kelly and IMR-32 cells. Based on the high association of anti-GD2-liposomes with Kelly cells and the fact that these cells are able to grow tumours in mouse models, Kelly cells were chosen for further *in vitro* experiments.

Table II Efficiency of Anti-GD2-Antibody coupling to HSCP- and DDAB-Liposomes

	anti-GD2-HSPC-L		anti-GD2-DDAB-L	
	-siRNA	+siRNA	-siRNA	+siRNA
μg Ab/μmol TL	51 ± 12	54 ± 12	67 ± 15	58 ± 13
Ab/L	84 ± 17	95 ± 29	66 ± 31	63 ± 21

Anti-GD2-antibody was coupled to the surface of HSPC- and DDAB-liposomes applying sterol-based post-insertion method. The amount of attached antibody to liposomes was determined using protein assay according to the protocol described in "Material and Methods." Data are presented as ±SD of 4 to 6 preparations. *Ab* antibody, *TL* total lipid, *L* liposome.

Delivery of siRNA to Neuroblastoma Cells by means of Anti-GD2-Liposomes

In order to measure the uptake of siRNA delivered to neuroblastoma by anti-GD2-liposomes, fluorescently labelled siRNA with Alexa488 (green signal) was encapsulated into anti-GD2-HSPC- and anti-GD2-DDAB-liposomes. In parallel to the uptake of these particles containing siRNA, the uptake of empty anti-GD2-liposomes labelled with rhodamine-PE (red signal) in Kelly cells was studied. The results of the flow cytometry experiments showed that the association of DDAB-liposomes was higher than HSPC-liposomes, but in principle the uptake patterns of these two types of liposomes was the same for particles labelled with rhodamine-PE as for those containing Alexa488 tagged siRNA (Figs. 5A and B). The delivery of siRNA by both types of liposomes coupled with anti-GD2 antibody was significantly increased compared to uncoupled liposomes. The association of siRNA delivered by anti-GD2-DDAB-liposomes was twice as high as that of anti-GD2-HSPC-liposomes. To check whether the delivery of siRNA by anti-GD2-liposomes occurs via the GD2 pathway, Kelly cells were incubated with a mixture containing liposomes and free anti-GD2 antibody. The association of anti-GD2-HSPC- and anti-GD2-DDAB-liposomes, as well as the

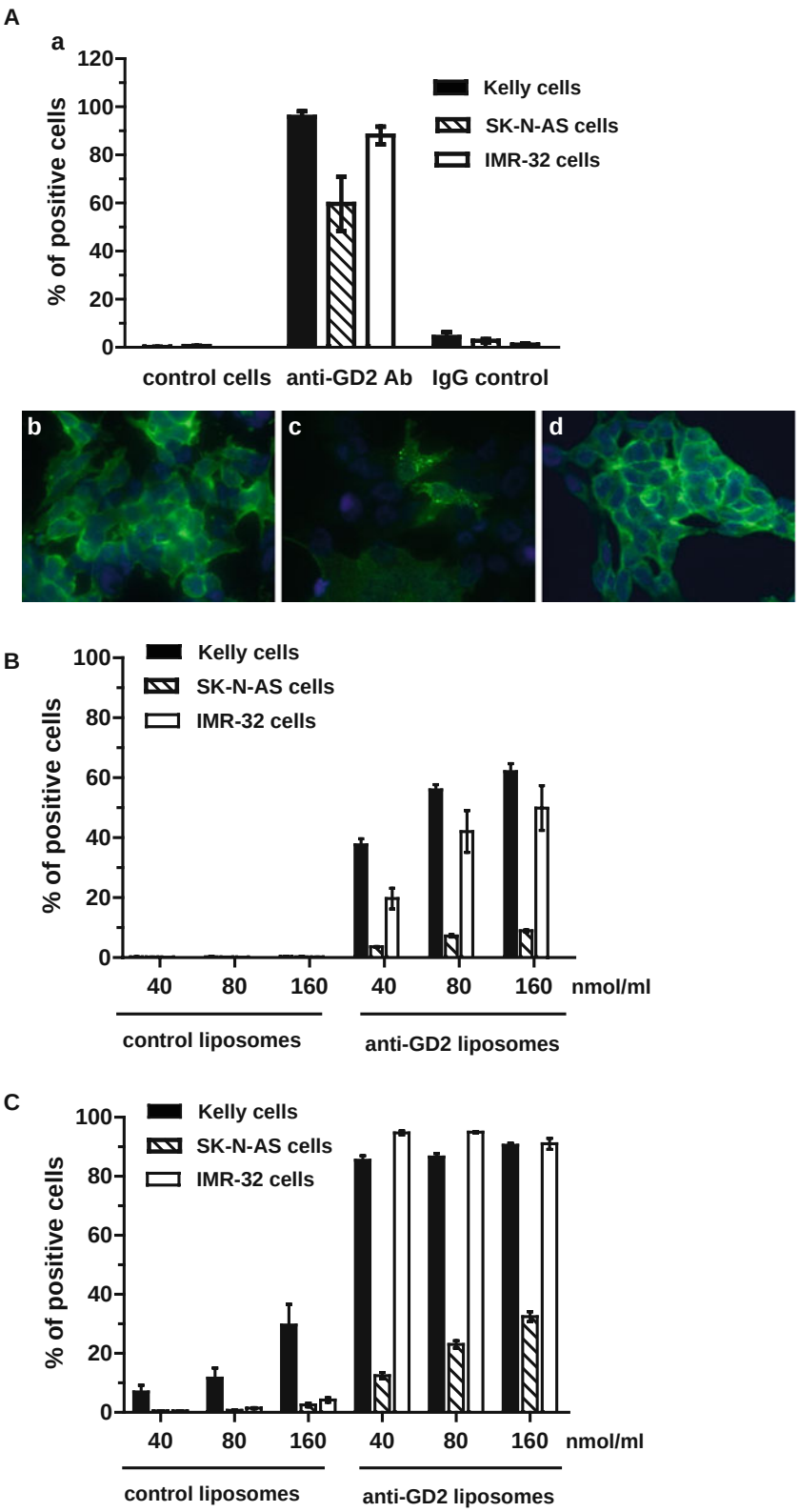

Fig. 4 Association of anti-GD2-HSPC- and anti-GD2-DDAB-liposomes with neuroblastoma cells lines. (**A**) The expression of GD2 by Kelly cells (**b**), SK-N-AS (**c**) and IMR-32 (**d**) cells was detected using a monoclonal antibody, measured by FACS (**a**) and visualized by fluorescence microscopy (green; **b**, **c**, **d**, blue; nuclei of the cells stained with DAPI) as described in "Material and Methods." Association of anti-GD2-HSPC-, HSPC-liposomes (**B**) and anti-GD2-DDAB-, DDAB-liposomes (**C**) with neuroblastoma cells. Kelly, SK-N-AS and IMR-32 cells were incubated with rhodamine-PE-labelled liposomes for 1 h, at concentrations of 40, 80 and 160 nmol/ml, washed and analysed with FACS as described in "Materials and Methods." Data are presented as ±SEM of 4 to 5 measurements.

uptake of siRNA mediated by these liposomes, were inhibited by free anti-GD2 antibody (Fig. 5A and B). However, in the case of anti-GD2-HSPC-liposomes, we observed 84% and 75% inhibition of the uptake of rhodamine-PE-labelled liposomes and siRNA Alexa488-containing particles, respectively. In contrast, anti-GD2 antibody blocked the association of anti-GD2-DDAB-liposomes labelled with rhodamine-PE by 30% and the delivery of siRNA by 40%.

The delivery of siRNA to neuroblastoma cells by anti-GD2-liposomes was visualised by fluorescence microscopy (Fig. 5C). As in the FACS experiments, the uptake of anti-GD2-HSPC- and anti-GD2-DDAB-liposomes containing Alexa488-labelled siRNA was compared to particles labelled with rhodamine-PE. A higher association of anti-GD2-HSPC- and anti-GD2-DDAB-liposomes with Kelly cells was observed for both rhodamine-PE and Alexa488 siRNA-containing particles compared to their uncoupled controls. In addition, stronger fluorescent signals were measured with the Kelly cells incubated with anti-GD2-DDAB-liposomes than with anti-GD2-HSPC-liposomes. Intracellular delivery of siRNA by anti-GD2-liposomes of both lipid formulations into Kelly cells was confirmed by confocal microscopy. In addition, stronger rhodamine-PE and Alexa488 siRNA signals were detected in the cells which were incubated with anti-GD2-DDAB-liposomes.

Effects of Inhibitors of Endocytosis on the Delivery of siRNA to Neuroblastoma Using Anti-GD2-Liposomes

GD2 is a ganglioside that builds up the cell membrane of neuronal cells. The removal of GD2 from the membrane and trafficking into the cell occurs via endocytosis (21). Anti-GD2-liposomes containing siRNA bound to the GD2 molecule on the surface of the cell would also follow the GD2 pathway and enter the cell using the same endocytotic pathway. In order to verify this hypothesis, we used the following chemical inhibitors of endocytosis: chlorpromazine (28 μM), genistein (200 μM) and methyl-β-cyclodextrin (5 mM) to block the delivery of siRNA into the Kelly cells by means of anti-GD2-liposomes. Neither anti-GD2-HSPC-liposomes nor anti-GD2-DDAB-liposomes showed inhibition with regard to uptake of siRNA in the cells treated with chlorpromazine (Fig. 6). Genistein decreased the delivery of siRNA for both types of liposomes by about 20%. The strongest levels of inhibitory effects on the uptake of siRNA delivered by anti-GD2-HSPC- and anti-GD2-DDAB-liposomes, 84±6% and 68±16%, respectively, were observed in cells treated with methyl-β-cyclodextrin.

Effects of VEGF-A siRNA Delivered by Anti-GD2-Liposomes in Kelly Cells

The ability of anti-GD2-liposomes to deliver functional siRNA into neuroblastoma cells was tested using siRNA specific for VEGF-A. This molecule is well known as a pro-angiogenic factor which stimulates growth of new blood vessels in the tumour and therefore promotes the development of the tumour mass (22). Recent data showed its relevance in neuroblastoma progression (23). Anti-GD2-DDAB-liposomes were chosen as delivery particles for VEGF-A siRNA, because they showed higher siRNA encapsulation efficiencies compared to HSPC-liposomes (Table I). As a control for VEGF-A siRNA, scrambled siRNA (non-specific to any gene) was used. After 72 h of incubation of Kelly cells with liposomes, the expression of VEGF-A at the gene level was checked by real-time PCR. Uncoupled liposomes containing VEGF-A siRNA or scrambled siRNA did not have any effect on the expression of VEGF-A (Fig. 7). Similarly, anti-GD2-liposomes with scrambled siRNA did not inhibit VEGF-A. Kelly cells treated with anti-GD2-liposomes containing VEGF-A siRNA showed 30% down-regulation of VEGF-A expression.

DISCUSSION

siRNA-based therapeutics specifically targeting genes of proteins responsible for tumour progression are seen as promising new options with high levels of efficacy and reduced toxicity compared to conventional strategies in oncology (11). In our study, we have chosen neuroblastoma, the most common solid tumour in childhood which originates from the peripheral nervous system, as a highly aggressive disease in need of new therapy (10). The molecular features of neuroblastoma such as oncogene amplification, ploidy status, gene mutations or growth factor overexpression have been shown to determine the heterogeneity of the tumour, its behaviour and clinical outcome. Based on these, it is postulated that novel approaches targeting neuroblastoma gene expression could help to ameliorate therapeutic outcome.

We prepared anti-GD2 antibody targeted liposomes for specific delivery of siRNA to neuroblastoma cells using two recently reported methods, DAC (8), which provides high siRNA encapsulation efficiency, and SPIT (12), by which antibody attached to a cholesterol anchor can be inserted into the outer layer of the liposomal membrane after the particles are formed. Our data showed that these liposomes were stable but displayed a rather heterogeneous population of particles. Depending on the lipid composition, DAC produced multilamellar vesicles in the case of "rigid" HSPC-liposomes, or unilamellar as observed in "fluid" DDAB-liposomes. In addition, the average size of the particles seems also to be defined by the lipid composition since DDAB-liposomes were smaller than HSPC-liposomes. A prolonged time of centrifugation during the preparation of the liposomes did not result in smaller particles (data not shown). This finding is also consistent with previously published data (24). As a consequence, there is limited control on the size of the final liposomes produced by DAC. The encapsulation efficiency of siRNA into liposomes was relatively high. However, the presence of positively charged DDAB lipids improved the siRNA loading efficiency almost two-fold in comparison to HSPC-liposomes. This indicates

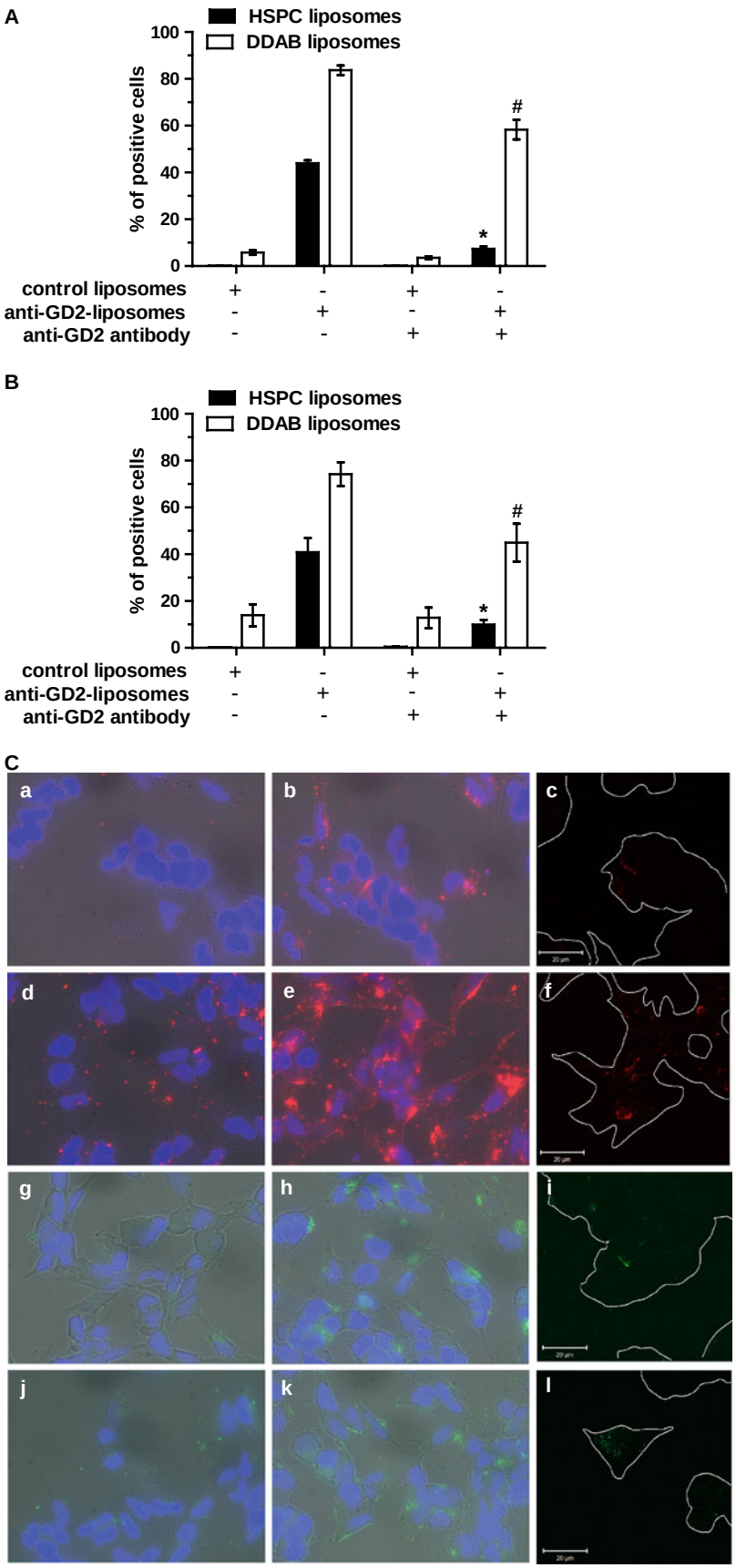

Fig. 5 Delivery of siRNA to Kelly cells using anti-GD2-liposomes. Anti-GD2-HSPC- and anti-GD2-DDAB-liposomes labelled with rhodamine-PE (**A**) and containing Alexa488 siRNA (**B**) were incubated with Kelly cells at a concentration of 80 nmol/ml in the presence and absence of 0.1 mg/ml free anti-GD2 antibody. After 1 h, cells were washed and analysed with FACS, as described in "Material and Methods." Data are presented as ±SEM of 6 to 9 measurements. $^{*}P < 0.05$ *versus* anti-GD2-HSPC-liposomes without free anti-GD2 antibody, $^{\#}P < 0.05$ *versus* anti-GD2-DDAB-liposomes without free anti-GD2 antibody. Visualisation of the uptake of anti-GD2-liposomes containing siRNA using fluorescence (**a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**) and confocal (**c**, **f**, **i**, **l**) microscopy (**C**). Rhodamine-PE (red) labelled HSPC- (**a**), anti-GD2-HSPC- (**b**, **c**), DDAB- (**d**), anti-GD2-DDAB-liposomes (**e**, **f**) and Alexa488 siRNA (green) containing HSPC- (**g**), anti-GD2-HSPC- (**h**, **i**), DDAB- (**j**), anti-GD2-DDAB-liposomes (**k**, **l**) were incubated with Kelly cells for 3 h at a concentration of 80 nmol/ml. Cells were washed and fixed. The nuclei were stained with DAPI (blue); samples were studied using fluorescence or confocal microscopy as described in "Materials and Methods."

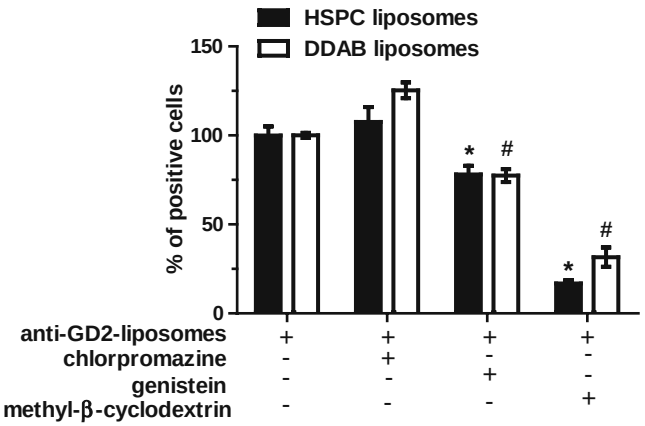

Fig. 6 Effects of inhibitors of endocytosis on the delivery of siRNA to Kelly cells by means of anti-GD2-liposomes. To Kelly cells treated with either chlorpromazine (28 μM) or genistein (200 μM) for 1 h or methyl-β-cyclodextrin (5 mM) for 15 min, anti-GD2-HSPC- and anti-GD2-DDAB-liposomes containing Alexa488 siRNA were added at a concentration of 80 nmol/ml. After 1 h, cells were washed, and the uptake of siRNA was measured by FACS, as described in "Materials and Methods." Data are presented as ±SEM of 3 experiments. $^{*}P < 0.05$ *versus* anti-GD2-HSPC-liposomes without inhibitors, $^{\#}P < 0.05$ *versus* anti-GD2-DDAB-liposomes without inhibitors.

that charged-based interaction of negative siRNA molecules with cationic DDAB lipids take place and increase the number of siRNA molecules entrapped within the liposomes. The efficiency of anti-GD2-antibody coupling was comparable for both lipid compositions. The estimated higher number of antibody molecules attached to a single liposome particle for the HSPC-liposomes is a result of the bigger size of these vesicles compared to the DDAB-liposomes. The presence of siRNA within the liposomes did not interfere with the coupling procedure, and liposomal size did not increase after antibody coupling.

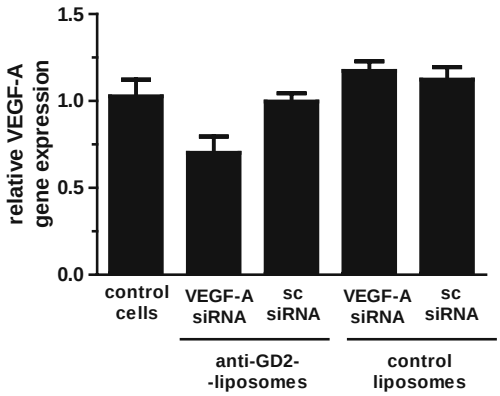

Fig. 7 Effects of VEGF-A siRNA delivered by anti-GD2-liposomes on the expression of VEGF-A in Kelly cells. Anti-GD2-DDAB-liposomes and control uncoupled DDAB-liposomes containing VEGF-A siRNA or scrambled (sc) siRNA were incubated with Kelly cells at a concentration of siRNA of 1,500 pmol/ml for 72 h. Expression of VEGF-A was determined by real-time PCR as described in "Materials and Methods." Data are presented as ±SEM of 4 to 8 measurements.

GD2 is known to be highly expressed on the surface of neuroblastoma tumours (13,14). Our data confirmed the presence of GD2 on the membrane of Kelly, SK-N-AS and IMR-32 neuroblastoma cells tested in the current study. The association of uncoupled liposomes and particles bearing anti-GD2 antibody on their surface with the cells showed that the anti-GD2 antibody is needed for a high uptake of liposomes and siRNA by neuroblastoma cells. The number of cells which internalized anti-GD2 liposomes correlated well with the level of GD2 expression by these cells. Interestingly, the association of anti-GD2-DDAB-liposomes was higher than that achieved with anti-GD2-HSPC-liposomes in all three cell lines. The positive charge of DDAB-liposomes most likely plays a role in this phenomenon by enhancing the GD2-mediated uptake of liposomes. The weaker potential of anti-GD2 antibody to block the association of anti-GD2-DDAB-liposomes and siRNA delivery with neuroblastoma cells compared to anti-GD2-HSPC-liposomes may indicate the presence of mechanisms other than those which are GD2-mediated in the uptake of anti-GD2-DDAB liposomes.

Experiments in which the association of anti-GD2-liposomes labelled with rhodamine-PE was compared with that of anti-GD2-liposomes containing Alexa488 siRNA showed that siRNA delivered by GD2 targeted liposomes follows the same mechanism and pattern of uptake as the carriers. Studies using confocal microscopy proved the intracellular delivery of siRNA into neuroblastoma cells by means of anti-GD2-liposomes. Inhibitors of endocytosis indicate that the uptake of siRNA carried by anti-GD2-liposomes occurred via the endocytotic pathway, but did not define which type of endocytosis was involved. There was no blocking effect of chlorpromazine (clathrin-mediated endocytosis (25)) on the uptake of anti-GD2-liposomes containing siRNA, and genistein (caveolae-mediated pathway (25)) had a mildly inhibiting effect. However, the third compound, methyl-β-cyclodextrin, which inhibits both pathways of endocytosis (clathrin- and caveolae-mediated (25)), effectively decreased the siRNA uptake. Additional experiments would be required to elucidate the exact mechanism and pathway of intracellular routing of siRNA delivered by anti-GD2-liposomes in neuroblastoma cells.

VEGF-A, a growth factor for tumour angiogenesis, plays an important role in highly vascularised neuroblastoma tumours (23) and has been chosen as a target for siRNA-based therapeutics. Anti-GD2-DDAB-liposomes loaded with siRNA specific for VEGF-A successfully reduced expression of this gene in neuroblastoma cell lines by 30%, supporting our strategy of using anti-GD2-liposomes as potential carriers for the specific delivery of siRNA to neuroblastoma cells. In order to increase this down-regulatory effect, experimental conditions or the liposomal preparation could be further optimised.

In the current study, we showed that DAC and SPIT are compatible and effective methods to produce targeted liposomes containing siRNA. The preparation of liposomes for siRNA delivery by these two protocols is fast, straightforward from a technical point of view, easy to perform in sterile and RNase-free conditions, and has the potential for up-scaling. Liposomes coupled with anti-GD2 antibody are readily taken up by neuroblastoma cells and also efficiently deliver biologically active siRNA into these cells. Therefore, anti-GD2-liposomes can serve as carriers of siRNA into neuroblastoma tumours.

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