Glycosylated Nucleoside Lipid promotes the liposome internalization in stem cells

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[b] *INSERM U869.*
[b] *INSERM U1026.*

Experimental

(4S)-2,2-Dimethyl-4-[(prop2-yn-1-yloxy)methyl]-1,3-dioxolane 5

To a solution of 2,3-isopropylidene-*sn*-glycerol (4.8 g, 36.5 mmol) in toluene (75 mL) at 0°C, was added 60% sodium hydride (1.6 g, 40.0 mmol) over a period of 30 min, followed by propargyl bromide (5.9 g, 40.0 mmol). The orange suspension was stirred overnight at room temperature under argon. The reaction mixture was poured into water (50 mL) and the organic phase separated. The aqueous phase was extracted with dichloromethane (3x50 mL), the organic extracts combined, dried ($MgSO_4$) and the solvent removed *in vacuo*. The product was purified by distillation (Kugelhröhr, 50-60°C/3 mm Hg) and obtained as a colorless liquid (4.8 g, 78%). R_f 0.8 (pentane-AcOEt, 1 :1) ; ^{1}H NMR (300 MHz, $CDCl_3$) : 1.35 (s, 3H), 1.42 (s, 3H), 2.45 (t, *J*= 2.4 Hz, 1H), 3.58 (dd, *J*= 5.7, 1.2 Hz, 2H), 3.71-3.76 (m, 1H), 4.04-4.09 (m, 1H), 4.20 (t, *J*= 2.4 Hz, 2H), 4.29 (tt, *J*= 5.7 Hz, 1H) , ^{13}C NMR (75 MHz, $CDCl_3$) 25.34, 26.75, 58.65, 66.64, 70.70, 74.47, 74.86, 79.32, 109.54.

[(2S)-2-Octadecanoyloxy-3-prop-2-ynoxypropyl]octadecanoate 6

A solution of compound **5** (1.44 g, 8.4 mmol) in methanol (50 mL) was treated with Dowex 50X2 (4.5 g) at room temperature until completion of the reaction as evidenced by TLC (pentane/AcOEt 1:1). After removal of the solvent under reduced pressure, the crude deprotected acetylenic glycerol was dissolved in dichloromethane (130 mL). To this solution was added stearic acid (6 g, 21.0 mmol), DCC (4.3 g, 21.0 mmol) and DMAP (1.6 g, 21.0 mmol), and the mixture was stirred for 72 h at room temperature. The reaction mixture was filtered and the filtrate concentrated under reduced pressure. The crude yellowish solid was purified by column chromatography on silica gel eluting with dichloromethane-ethyl acetate (94:6) and then dichloromethane-ethyl acetate (90:10), to give the title compound which was obtained as a white solid (4.5 g, 81%), R_f 0.8 (CH_2Cl_2/AcOEt 8:2); ^{1}H NMR (300 MHz, $CDCl_3$) 0.89 (t, *J*= 6.6 Hz, 6H), 1.27 (s, 46H), 1.58-1.65 (m, 4H), 2.27-2.37 (m, 4H), 2.45 (t, *J*= 2.4 Hz, 1H), 3.69 (d, *J*= 6.0 Hz, 2H), 4.18-4.22 (m, 3H), 4.32-4.38 (m, 1H), 5.21-5.28 (m, 1H), ^{13}C NMR (75 MHz, $CDCl_3$) 14.11, 22.70, 24.90, 24.98, 29.08, 29.14, 29.30, 29.38, 29.50, 29.71, 31.94, 34.29, 34.37, 58.50, 60.10, 67.95, 69.77, 74.96, 79.01, 173.04, 173.34.

5'-[4-((1,2-Distearoyl-*sn*-glycer-1-ol)methyl)-1H-1,2,3-triazol-1-yl]thymidine 7

A stirred suspension of compound **6** (995 mg, 1.5 mmol) and 5'-deoxy-5'-azidothymidine (401 mg, 1.5 mmol) in water (25 mL) and THF (25 mL) was treated with copper sulfate (24.0 mg, 0.15 mmol) and sodium ascorbate (59.4 mg, 0.3 mmol) at 60°C for 7 h. After cooling, the reaction mixture was filtered, the remaining solid washed with water then ethanol before drying for 1 h at 50°C under vacuo. The crude product was purified by column chromatography on silica gel eluting with dichloromethane-methanol (9:1) to give the title compound as a white solid (0.9 g, 64%), R_f 0.63 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (300 MHz, DMSO-d_6, 343K) 0.87 (t, *J*= 6.7 Hz, 6H), 1.25 (s, 46H), 1.47-1.56 (m, 4H), 1.80 (d, *J*= 1.2 Hz, 3H), 2.12-2.17 (m, 2H), 2.12-2.28 (m, 4H), 3.61 (d, *J*= 5.4 Hz, 2H), 4.05-4.12 (m, 2H), 4.23-4.32 (m, 1H), 4.55-4.74 (2m, 5H), 5.08-5.15 (1H), 6.15 (t, *J*= 6.9 Hz, 1H), 7.27 (d, *J*= 1.2 Hz, 1H), 8.00 (s, 1H).

5'-[4-((1,2-Distearoyl-*sn*-glycer-1-yl)methyl)-1H-1,2,3-triazol-1-yl]-N-3-propargylthymidine 8

A solution of compound **7** (310 mg, 0.33 mmol) and propargyl bromide (79 mg, 0.66 mmol) in anhydrous DMF (25 mL) was stirred overnight at room temperature with potassium carbonate (100 mg, 0.66 mmol) under argon. The solvent was removed in vacuo, the resulting solid was dissolved in dichloromethane (80 mL) and the solution washed with water (2x50 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure and the product was purified by column chromatography on silica gel eluting with dichloromethane-methanol (99:1) to afford the title compound as a white solid (260 mg, 71%), R_f 0.65 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) 0.89 (t, *J*= 6.7 Hz, 6H), 1.26 (s, 46H), 1.61 (m, 4H), 1.95 (s, 3H), 2.22 (t, *J*= 2.4 Hz, 1H), 2.28-2.35 (m, 4H), 2.36-2.41 (m, 2H), 3.66 (dd, *J*= 1.5, 5.2 Hz, 2H), 4.12 (m, 1H), 4.24 (dd, *J*=5.1, 9.9 Hz, 1H), 4.30 (m, 1H), 4.55 (m, 1H), 4.66 (d, *J*= 5.1, 2H), 4.71 (s, 2H), 4.72 (d, *J*= 2.4 Hz, 2H), 5.15-5.22 (m, 1H), 6,21 (dd, *J*= 6.6 Hz, 1H), 6.81 (d, *J*= 1.2 Hz, 1H), 7.66 (s, 1H),), ^{13}C NMR (75 MHz, $CDCl_3$) 13.16, 14.16, 22.72, 24.90, 24.95, 29.12, 29.16, 29.33, 29.40, 29.54, 29.69, 30.46, 31.95, 34.14, 34.31, 38.68, 51.14, 62.63, 64.60, 68.77, 69.86, 71.63, 78.11, 83.79, 87.24, 124.57, 134.77.

5'-[4-((1,2-Distearoyl-*sn*-glycer-1-yl)methyl)-1H-1,2,3-triazol-1-yl]-N-3-(1-(β-D-glucopyranoside)-1H-1,2,3-triazol-4-yl)methyl]thymidine 9

A stirred suspension of compound **8** (230 mg, 0.23 mmol) and 1-azido-1-deoxy-β-D-glucopyranoside (47 mg, 0.23 mmol) in water (12 mL) and THF (12 mL) was treated with copper sulfate (3.6 mg, 0.023 mmol) and sodium ascorbate (9 mg, 0.046 mmol) at 65°C for 24 h. After cooling, the reaction mixture was filtered, the remaining solid washed with water then ethanol before drying for 1 h at 50°C under vacuo. The crude product was purified by column chromatography on silica gel eluting with dichloromethane-methanol (9:1) to give the title compound as a white solid (134 mg, 49%), R_f 0.35 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (300 MHz, DMSO-d_6) 0.85 (t, *J*= 6.7 Hz, 6H), 1.23 (s, 46H), 1.41-1.54 (m, 4H), 1.86 (s, 3H), 2.14-2.20 (m, 2H), 2.22-2.28 (m, 4H), 3.19 (dd, *J*= 9.0 Hz, 1H), 3.34 (dd, *J*= 9.0 Hz, 1H), 3.43 (dd, *J*= 9.0 Hz, 1H), 3.58 (d, *J*= 5.4 Hz, 2H), 3.66 (d, *J*= 9.0 Hz, 2H), 3.74 (dd, *J*= 9.0 Hz, 1H), 4.03-4.12 (m, 1H), 4.22-4,30 (m, 2H), 4.53 (s, 2H), 4.59-4.71 (m, 2H), 5.04 (s, 2H), 5.08-5.15 (m, 1H), 5.47 (d, *J*= 9.7, 1H), 6,22 (dd, *J*= 6.9 Hz, 1H), 7.45 (s, 1H), 8.09 (s, 1H), 8.11 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) 13.17, 14.43, 22.59, 24.89, 24.97, 28.85, 28.92, 29.21, 29.54, 31.78, 33.87, 34.04, 36.59, 38.55, 51.56, 61.13, 62.78, 64.16, 68.44, 69.98, 70.21, 71.16, 72.33, 77.45, 80.44, 84.61, 85.65, 87.87, 122.94,125.23, 135.54. HRMS (ESI, positive) calcd for $C_{61}H_{104}N_8O_{14}Na$ 1195.7564, found 1195.7525.

1-[(1,2-Distearoyl-*sn*-glycer-1-ol)methyl-1H-1,2,3-triazol-1-yl]-β-D-glucopyranoside 10

A stirred suspension of compound **6** (500 mg, 0.756 mmol) and 1-azido-1-deoxy-β-D-glucopyranoside (156 mg, 0.756 mmol) in water (30 mL) and THF (30 mL) was treated with copper sulfate (13 mg, 0.0756 mmol) and sodium ascorbate (30 mg, 0.151 mmol) at 65 °C for 24 h. The mixture was cooled at room temperature, the THF was evaporated under reduced pressure and remaining reaction mixture was filtered. The solid was washed with water, dichloromethane and air dried, then washed again with methanol and air dried. This solid material was dissolved in dichloromethane:methanol (9:1) mixture and filtered. The filtrate was concentrated under reduced pressure and dried under vacuum to give the title compound as a white solid (439 mg, 66 %), R_f 0.4 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD 9:1) 0.82 (t, *J*=6.7 Hz, 6H), 1.2 (s, 56H), 1.46-1.63 (m, 4H), 2.21-2.28 (q, *J*=7.2 Hz, 4H), 3.48-3.54 (m, 1H), 3.57-3.63 (m, 4H), 3.71-3.76 (dd, *J*=3.8 Hz, 1H), 3.80-3.89 (m, 2H), 4.03-4.11 (m, 1H), 4.23-4.28 (dd, *J*=3.2 Hz, 1H), 4.60 (s, 2H), 5.14 (bs, 1H), 5.47 (d, *J*=8.9 Hz, 1H), 7.87 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$:CD_3OD 9:1) 13.9, 22.5, 24.8, 29.0, 29.2, 29.4, 29.5, 29.6, 31.8, 34.2, 61.2, 62.6, 64.5, 68.7, 69.2, 70.0, 72.6, 76.8, 77.3, 79.1, 88.1, 173.4, 173.8. HRMS (ESI, positive) calcd for $C_{48}H_{89}N_3O_{10}Na$ 890.6440, found 890.6423.

Liposomes preparation

Liposomes **L1**, **L2**, **L3** and **L4** were prepared from a dichloromethane solution (0.5 mL) of soya lecithin and GNL **9** in the following proportions: 5.0 mg/0 mg, 4.5 mg/0.5 mg, 4.0 mg/1.0 mg, and 0 mg/5.0 mg respectively. For each formulation, a 100 mL aliquot was concentrated with stirring under a flow of nitrogen until a dry film was obtained. The lipidic film was

then suspended in 1 mL aqueous NaCl 9‰ solution (vortex for 1 min), then sonicated in a bath sonicator at r.t. for 1 min. The resulting dispersion was extruded through polycarbonate filters (Whatman nucleopore Track-Etch membrane, pore size 200 nm) mounted in an extruder from Avanti Polar Lipids Inc., Alabaster, AL, USA. For the biological assays, 1 mL of a 0.1 mM dichloromethane solution of DIL (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) was added to each formulation.

Biological assays

ADSCs culture

Adipose tissue derived stem cells (ADSCs) were isolated from human adipose tissue. Human subcutaneous fat was obtained from healthy patients aged 20 to 80 years old who underwent hip surgery in Bordeaux Pellegrin CHU (Bordeaux, France). Fat mass was separated from other tissue, washed with sterilized PBS, finely cut and incubated with 0.1% collagenase (type I, Sigma, St Louis, MO) at 37°C with gentle agitation for 1 hour. Collagense activity was stopped with an equal volume of Dulbecco's Modified Eagle's Medium/F12 (DMEM-F12, SigmaAldrich, France) supplemented with 10% fetal bovin serum (FBS, Sigma, France), 100µg/ml penicillin and 100µg/ml streptomycin (PS). After a centrifugation at 1000 rpm for 10 min a cellular pellet was obtained, resuspended in DMEM-F12/10% FBS/PS and filtered through a 100 µm mesh filter in order to remove debris. The filtrate was centrifuged and cell suspension seeded onto conventional culture flask in controlled atmosphere (100% humidity, 37°C, 5% CO2). Culture medium was refreshed every three days and cells were passed when confluence reached 80%.

Liposome assays

ADSCs were prepared and seeded at a density of 10000 cells/cm² in 4-well plates (Nunc, Danemark) and cultured in DMEM-F12/10%FBS in controlled atmosphere. When cells reached confluency, 10µl of DIL-labelled liposome suspension (**L1**, **L2**, **L3** and **L4**) were added in the culture medium. After 48 hours medium was removed, cells were washed and observed under fluorescence microscope (Zeiss Axiovert 25 CFL microscope, excitation and emission wavelengths 550 nm and 581 nm, respectively). To improve intracellular localization of liposomes, Hoechst 33342 (Thermo Scientific, France) solution was added at 1µg/mL for 10 minutes to stain nuclei. Double-labelled cells were observed by confocal microscopy (Leica DMI6000 TCS SP5 AOBS).

DLS data for compound 9

Zeta Potential Report

v2.2

Malvern

Sample Details

Sample Name: ll10018 zeta cc 2
SOP Name: zetaliposomeLL.sop
General Notes:

File Name:	LL-liposomes-Zeta.dts	**Dispersant Name:**	Water
Record Number:	14	**Dispersant RI:**	1.330
Date and Time:	mardi 8 mars 2011 13:49:22	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

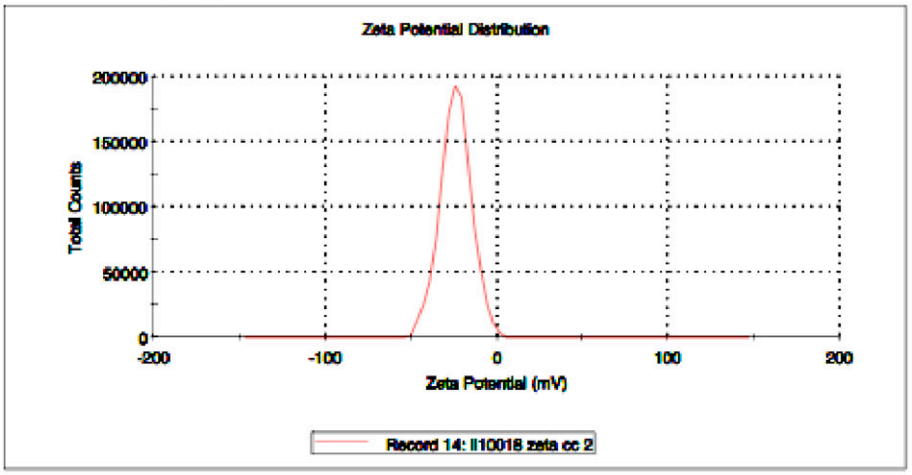
System

Temperature (°C):	25.0	**Zeta Runs:**	12
Count Rate (kcps):	31.8	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	11

Results

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-24.1	**Peak 1:**	-24.1	100.0	8.99
Zeta Deviation (mV):	8.99	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0525	**Peak 3:**	0.00	0.0	0.00

Result quality Good



Size Distribution Report by Number

v2.0

Malvern

Sample Details

Sample Name: SizeLiposomesLL 10018 3

SOP Name: SizeLiposomesLL.sop

General Notes:

File Name:	LL-liposomes-Size.dts	**Dispersant Name:**	Water
Record Number:	60	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.01	**Measurement Date and Time:**	mardi 8 mars 2011 13:39:...

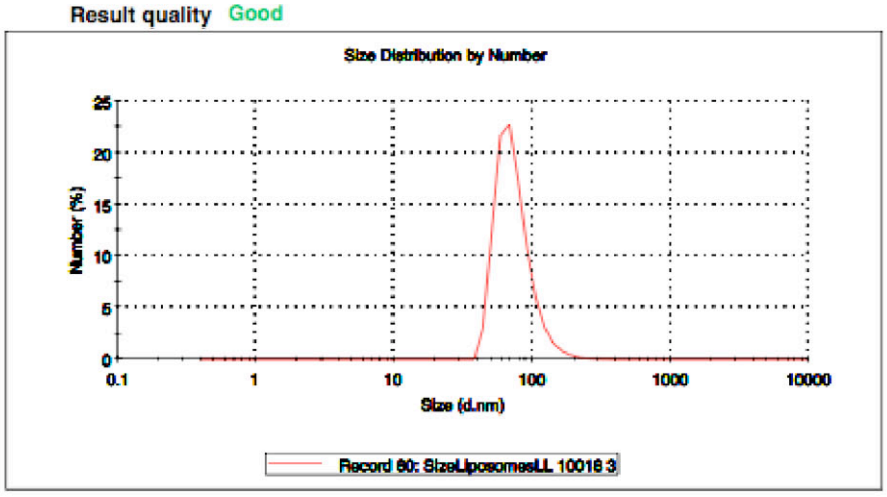
System

Temperature (°C):	25.0	**Duration Used (s):**	70
Count Rate (kcps):	169.9	**Measurement Position (mm):**	4.65
Cell Description:	Low volume disposable ...	**Attenuator:**	8

Results

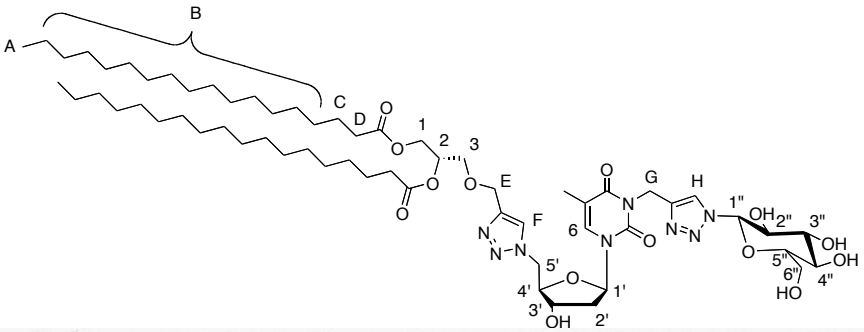
			Diam. (nm)	% Number	Width (nm)
Z-Average (d.nm):	122.8	**Peak 1:**	74.60	100.0	25.03
PdI:	0.158	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.962	**Peak 3:**	0.000	0.0	0.000

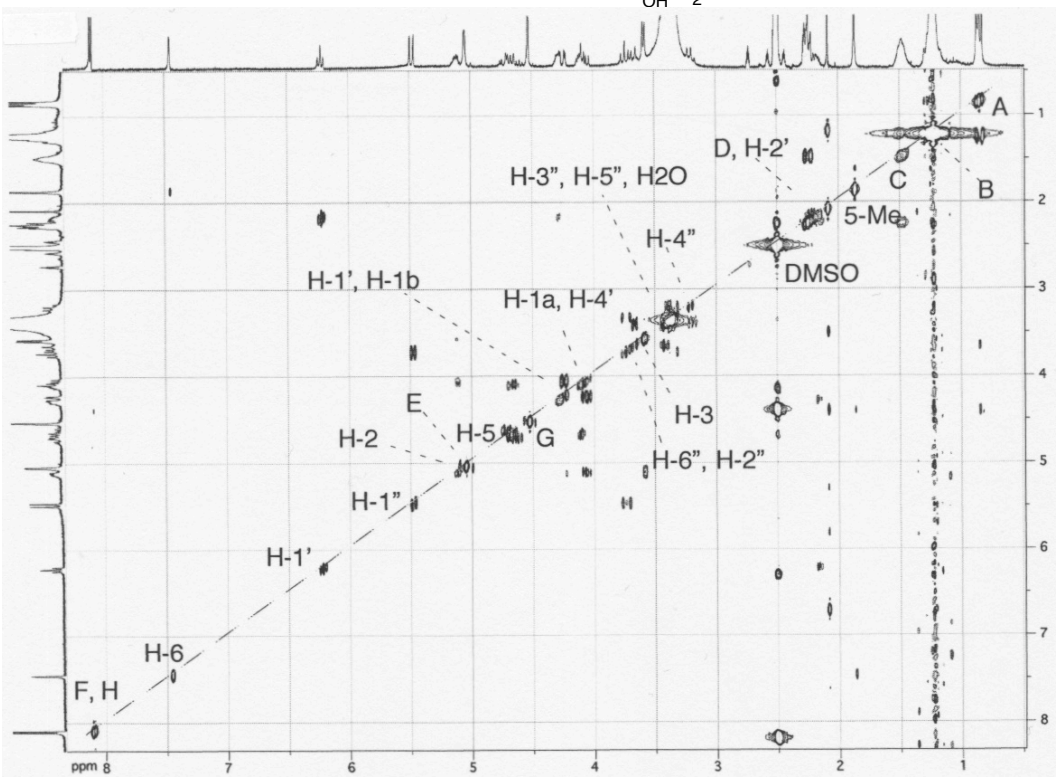
Result quality Good

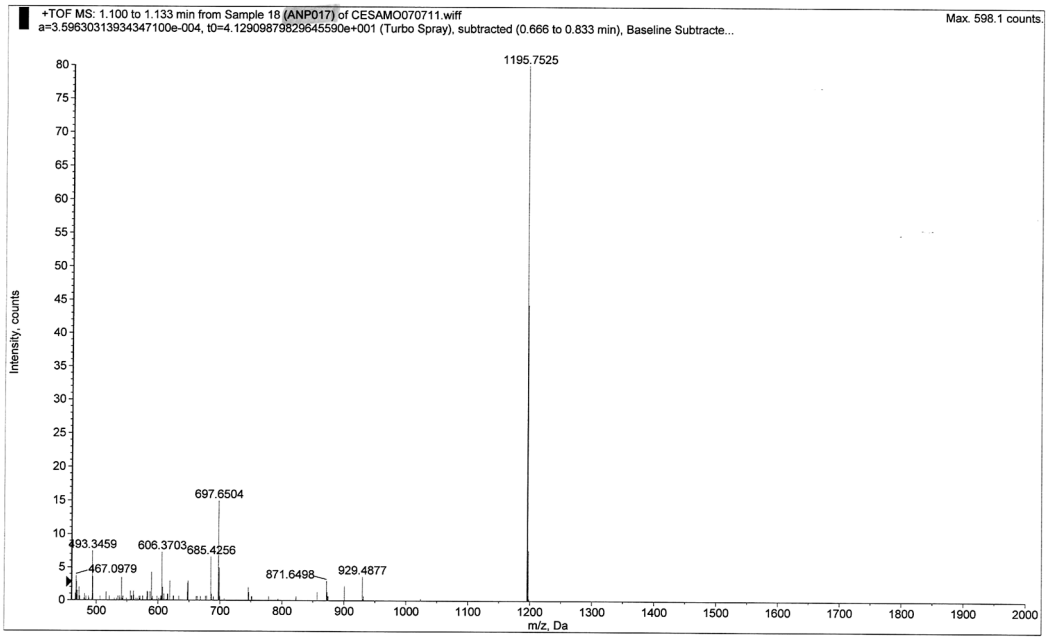


Selected NMR and Mass spectrometry data for compound 9

NMR assignments were made by 1H-1H COSY, DEPT and HSQC data.

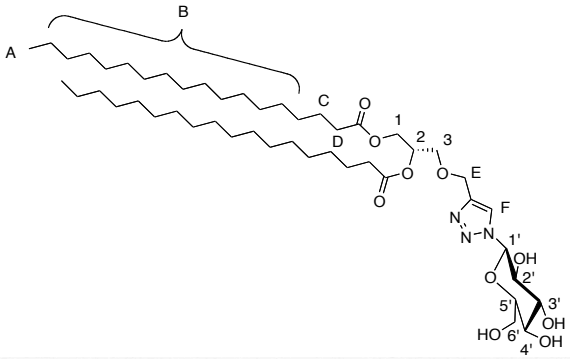


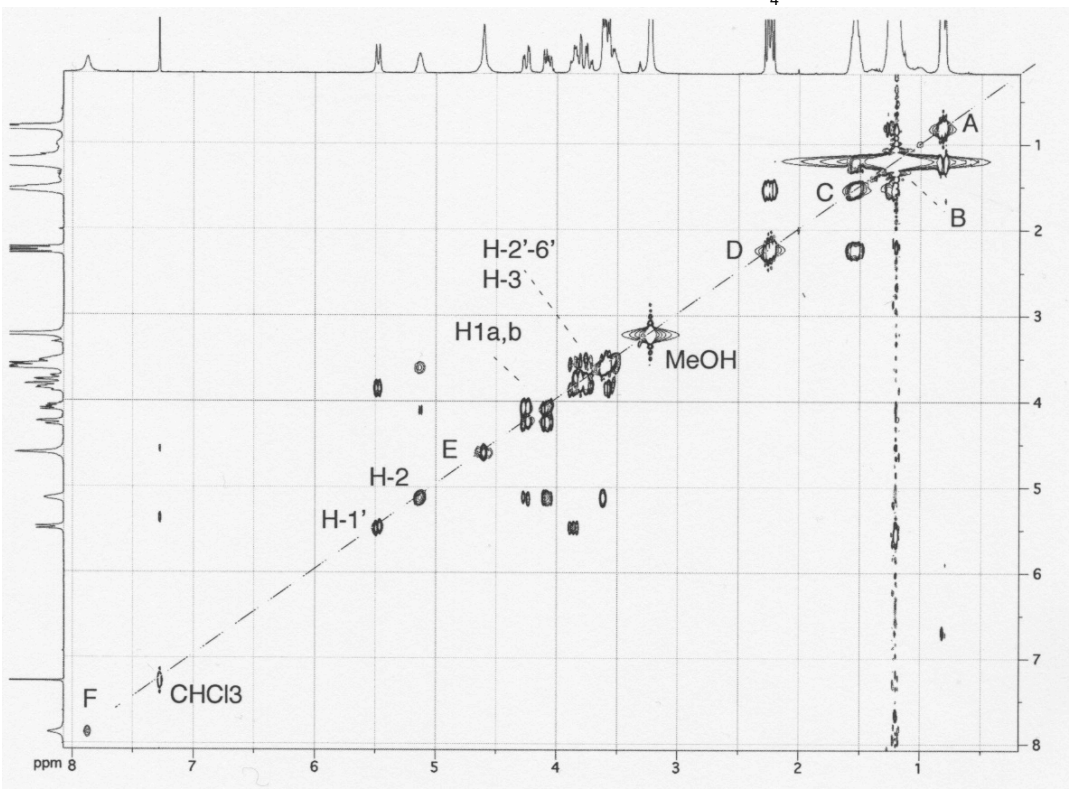


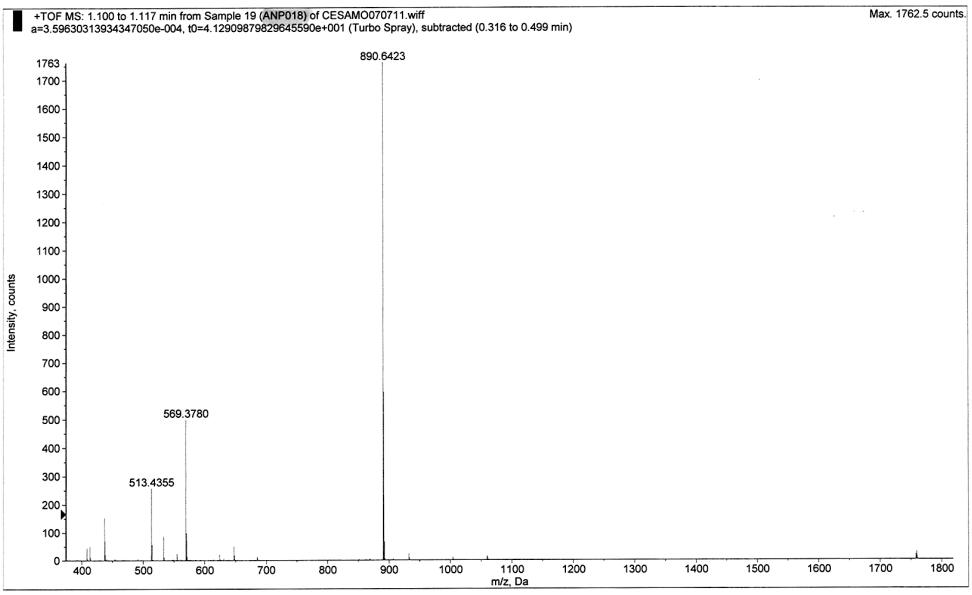


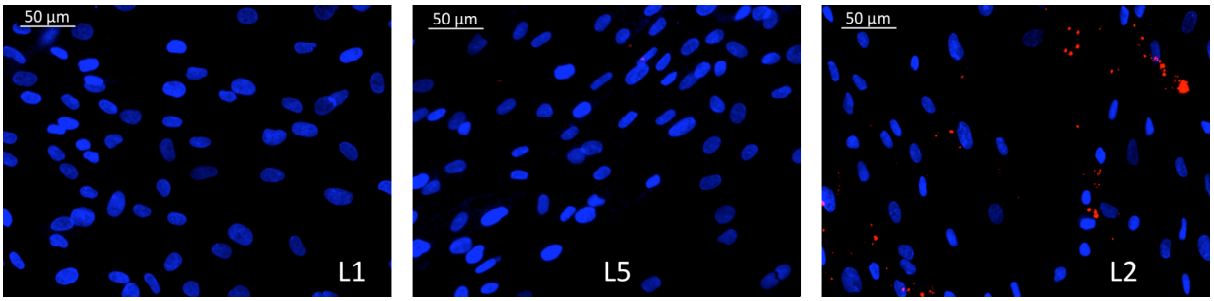
Selected NMR and Mass spectrometry data for compound 10

NMR assignments were made by 1H-1H COSY and DEPT data.

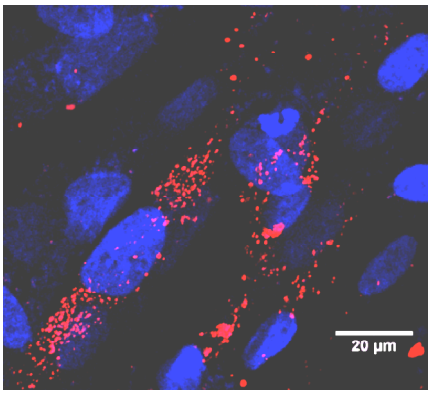








Confocal images of labelled liposome formulations **L1**, **L2** and **L5** incubated in the presence of ADSC stem cells. DIL red staining was observed only with the GNL formulations (**L2)**.



ADSC stem cells incubated in the presence of **L4** formulation (pure GNL).