OMTN, Volume *24*

Supplemental information

Functionalized lipid nanoparticles for subcutaneous administration of mRNA to achieve systemic exposures of a therapeutic protein

Nigel Davies, Daniel Hovdal, Nicholas Edmunds, Peter Nordberg, Anders Dahlén, Aleksandra Dabkowska, Marianna Yanez Arteta, Aurel Radulescu, Tomas Kjellman, Andreas Höijer, Frank Seeliger, Elin Holmedal, Elisabeth Andihn, Nils Bergenhem, Ann-Sofie Sandinge, Camilla Johansson, Leif Hultin, Marie Johansson, Johnny Lindqvist, Liselotte Björsson, Yujia Jing, Stefano Bartesaghi, Lennart Lindfors, and Shalini Andersson

SUPPLEMENTAL METHODS

In vitro studies to investigate kinetics of prodrug cleavage using human adipocytes

Human adipose stem cells (hASCs) were collected from patients undergoing elective surgical fat-removal at Sahlgrenska University Hospital in Gothenburg, Sweden and cryo-preserved. All study subjects received written and oral information before giving written informed consent for the use of their tissue. The studies were approved by The Regional Ethical Review Board in Gothenburg, Sweden. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

An optimized protocol was used to differentiate hASCs to mature "white-like" adipocytes. Briefly, cryo-preserved hASCs were resuspended in EGM-2 medium (endothelial cell growth medium-2) and centrifuged at 200xg for 5min. EGM-2 medium was prepared according to the manufacturer's protocol using EGM^{TM}-2 MV BulletKit^{TM} (Lonza, Basel, Switzerland). Cells were counted and seeded at 11,000 cells per well in 100 µl EGM-2 medium containing 50 U/ml penicillin and 50µg/ml streptomycin (P/S; Gibco, Waltham, MA, USA, cat.no. 15140-122) into 96 well plates (Greiner Bio-One, Kremsmünster, Austria, cat.no. 781092). The cells were incubated at 37°C and 5% CO_2 for 3 to 4 days. For adipocyte differentiation, 90% confluent cells were incubated for 1 week with Basal Medium (BM-1, Zenbio, Research Triangle Park, NC, USA) supplemented with 3% FBS Superior (Sigma Aldrich, St Louis, MO, USA), 1µM dexamethasone (Sigma Aldrich, St Louis, MO, USA), 500µM 3-isobutyl-1-methyxanthine (Sigma Aldrich, St Louis, MO, USA), 1µM pioglitazone (provided by AstraZeneca), P/S and 100 nM insulin (Actrapid Novonordisk, Bagsværd, Denmark). Medium was replaced with BM-1 medium supplemented with 3% FBS Superior, 1µM dexamethasone, P/S and 100nM insulin and cells were incubated for another 5 days. hASCs were tested to ensure no contamination with mycoplasma.

For *in vitro* studies to investigate kinetics of prodrug cleavage, hFGF21 mRNA LNPs containing steroid prodrugs at a final mRNA concentration of 1.25ng/ml were incubated together with the mature human "white-like" adipocytes at 37°C and 5% CO_2. Cells were transfected in the presence of fresh BM-1 medium supplemented with 1% human serum (Sigma Aldrich, St Louis, MO, USA, cat.no. H4522) to mimic the subcutaneous environment.

Culture supernatant was collected at various time points up to 24 hours and analysed for parent steroid, hFGF21 and various inflammatory markers using the same bioanalytical methods as used for plasma samples collected in in *vivo* studies. First-order rate constants for formation of the parent steroid from the different prodrug were estimated using Phoenix 6.4 (Certara, St. Louis, MO, USA).

Table S1. Plasma hFGF21 protein exposure in mice after s.c. and i.v. administration of hFGF21 mRNA in MC3 LNPs and plasma chemistry measured at termination, 24h after dosing (mean ± SEM).

Parameter[a]	PBS control i.v. (n=5)	0.3 mg/kg s.c. (n=5)	0.3 mg/kg i.v. (n=5)
$AUC_{2\text{-}24h}$ (nmol·h/L)	n/a	0.55±0.14	10.3±0.9
Haptoglobin (ug/ml)	31±11	1730±484	129±30
IL-6 (pg/ml)	n.c.[b]	432±111	36±6
KC (pg/ml)	627±587	654±69	207±30
IP-10 (pg/ml)	115±8	922±96	760±237
MCP-1 (pg/ml)	33±5	866±105	280±106
ALT (U/L)	60±19	57±10	79±8
AST (U/L)	219±83	258±48	212±47

[a] $AUC_{2\text{-}24h}$: Area under the plasma drug concentration-time curve over the time interval 2 to 24 hours after dosing, IL-6: interleukin-6, KC: murine interleukin-8 homologue, IP-10: interferon gamma induced protein 10, MCP-1: Monocyte chemoattractant protein 1, ALT: alanine transaminase; AST aspartate transaminase.

[b] n.c.: not calculated, 3 out of 5 values below limit of detection

Table S2. First-order rate constants (h^{-1}) for the formation of parent steroid following incubation of mRNA L608 LNPs containing different steroid prodrugs with primary human adipocytes.

Prodrug	Rofleponide	Budesonide
C5	0.480	
C8		0.170
C14	0.010	0.015
C16	0.003	
C18	0.005	0.009

Table S3. LNP size (intensity average (Z-avg)), polydispersity index (PDI) and mRNA encapsulation efficiency (EE) of LNPs used in studies reported in the manuscript.

Figure/Study description	LNP (nm) [PDI]	EE (%)
Figure 1		
MC3 LNPs with hFGF21 mRNA administered IV and SC @ 0.3 mg/kg	98 [0.1]	93
Figure 2		
MC3 LNPs with luciferase mRNA administered IV and SC @ 0.3 mg/kg	76 [0.1]	97
Figure 3		
MC3 LNPs with hFGF21 mRNA containing partially deuterated rofleponide-C14 prodrug at a rofleponide:mRNA weight ratio of 1:1	86 [0.1]	96
Figure 4		
MC3 LNPs with hFGF21 mRNA; no steroid	96 [0.1]	96
MC3 LNPs with hFGF21 mRNA; rofleponide-C5	101 [0.1]	95
MC3 LNPs with hFGF21 mRNA; rofleponide-C14	93 [0.1]	96
MC3 LNPs with hFGF21 mRNA; rofleponide-C16	93 [0.1]	95
MC3 LNPs with hFGF21 mRNA; rofleponide-C18	86 [0.1]	96
Figure 5		
L608 LNPs with hFGF21 mRNA; no steroid	74 [0.1]	97
L608 LNPs with hFGF21 mRNA; budesonide-C8	78 [0.1]	97
L608 LNPs with hFGF21 mRNA; budesonide-C14	79 [0.1]	93
L608 LNPs with hFGF21 mRNA; budesonide-C16	78 [0.1]	97
L608 LNPs with hFGF21 mRNA; budesonide-C18:1	78 [0.1]	97
Figure 6 a-c		
MC3 LNPs with hFGF21 mRNA; no steroid	81 [0.04]	99
MC3 LNPs with hFGF21 mRNA; rofleponide-C16	70 [0.04]	99
MC3 LNPs with hFGF21 mRNA; budesonide-C16	73 [0.05]	99
Figure 6 d-f		
MC3 LNPs with hFGF21 mRNA; no steroid	79 [0.02]	96
MC3 LNPs with hFGF21 mRNA; rofleponide	80 [0.03]	95
MC3 LNPs with hFGF21 mRNA; budesonide	80 [0.01]	95
Figure 7		
MC3 LNPs with hFGF21 mRNA; no steroid	91 [0.1]	92
MC3 LNPs with hFGF21 mRNA; rofleponide-C16 (1:1)	84 [0.07]	97
MC3 LNPs with hFGF21 mRNA; rofleponide-C16 (1:10)	85 [0.1]	98
MC3 LNPs with hFGF21 mRNA; rofleponide-C16 (1:30)	89 [0.2]	98
Figure 8a		
MC3 LNPs with FGF21 mRNA; rofleponide	N. R.[#]	N. R.[#]
MC3 LNPs with FGF21 mRNA; rofleponide-C5	92 [0.1]	94
MC3 LNPs with FGF21 mRNA; rofleponide-C14	81 [0.01]	97
MC3 LNPs with FGF21 mRNA; rofleponide-C16	81 [0.06]	97
Figure 8b		
MC3 LNPs with FGF21 mRNA; budesonide	113 [0.03]	66
MC3 LNPs with FGF21 mRNA; budesonide-C16	82 [0.02]	97

[#] Not recorded

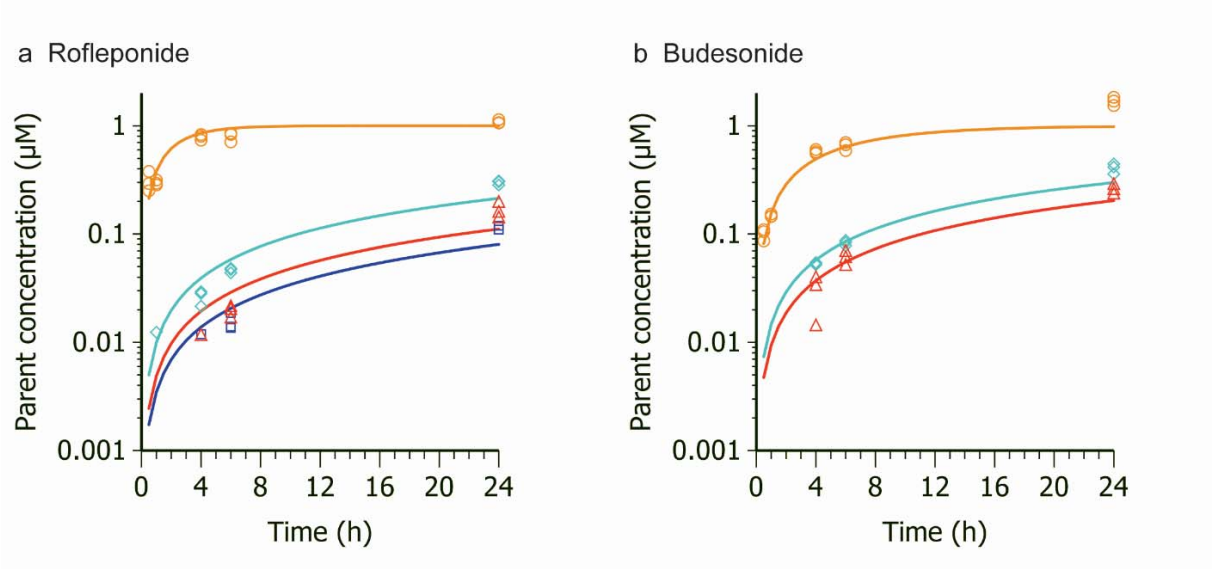

Figure S1. Formation of parent steroid (rofleponide and budesonide) following incubation of mRNA L608 LNPs containing different anti-inflammatory prodrugs with human primary adipocytes. Rofleponide-C5 or budesonide-C8 (○), rofleponide-C14 or budesonide-C14 (◇), rofleponide-C16 (▫) and rofleponide-C18 or budesonide-C18 (△).