eTOC synopsis – 50 words or less

Subcutaneous administration of mRNA formulated within LNPs results in measurable plasma exposure of a secreted protein albeit with dose-limiting local inflammatory responses. Inclusion of a steroid prodrug in mRNA LNPs resulted in increased level and duration of protein expression as well as improved tolerability of the LNP following subcutaneous administration.

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

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Short title: Subcutaneous administration of mRNA LNPs

Abstract

Lipid nanoparticles (LNPs) are the most clinically advanced delivery system for RNA-based drugs but have predominantly been investigated for intravenous and intramuscular administration. Subcutaneous administration opens the possibility of patient self-administration and hence long-term chronic treatment that could enable mRNA to be used as a novel modality for protein replacement or regenerative therapies. Here, we show that subcutaneous administration of mRNA formulated within LNPs can result in measurable plasma exposure of a secreted protein. However, subcutaneous administration of mRNA formulated within LNPs was observed to be associated with dose-limiting inflammatory responses. To overcome this limitation, we investigated the concept of incorporating aliphatic ester prodrugs of anti-inflammatory steroids within LNPs i.e. functionalized LNPs to suppress the inflammatory response. We show that the effectiveness of this approach depends on the alkyl chain length of the ester prodrug which determines its retention at the site of administration. An unexpected additional benefit to this approach is the prolongation observed in duration of protein expression. Our results demonstrate that subcutaneous administration of mRNA formulated in functionalized LNPs is a viable approach to achieving systemic levels of therapeutic proteins, which has the added benefits of being amenable to self-administration when chronic treatment is required.

INTRODUCTION

Chemically modified messenger RNA (modified mRNA) is an emerging class of nucleic acid-based therapeutics, that is able to encode for both wild type and engineered intracellular, transmembrane and secreted proteins [1]. Modified mRNA is chemically engineered to structurally resemble natural, mature and processed mRNA in the cytoplasm, while not eliciting an immunological response when administered. The endogenous machinery of the transfected cell is utilized for *in vivo* translation of the message to the corresponding protein that undergoes post-transcriptional modifications and folding prior to secretion [1, 2].

Substantial investment has been made in the last decades to modify the structural elements of the mRNA (including modifications of the nucleotides and cap structure) to enable increased protein expression and reduced immunogenicity. However, there are still some fundamental challenges with the use of mRNA as therapeutics, including stability, duration of action, *in vivo* pharmacokinetic/pharmacodynamic (PK/PD) and effective delivery to the target cell type or tissue. In general, the technology has progressed significantly since the first non-clinical studies in the 1990s and has to date been explored for vaccines, protein-replacement therapies and in regenerative medicine applications [1-10].

One of the major obstacles facing the successful development of mRNA-based therapies is the identification of a safe and effective delivery system that can offer protection of the mRNA from endo- and exo-nucleases and effectively deliver the mRNA into the cells in a manner that is acceptable to patients. Broadly speaking, RNA delivery can be mediated by viral and non-viral vectors [2, 11, 12]. Lipid nanoparticles (LNPs), initially developed for *in vivo* delivery of siRNA, have also been investigated for delivery of mRNA and have shown promise as a non-viral delivery system [13-16].

LNPs are multi-component systems which typically consist of an ionizable amino lipid, a phospholipid, cholesterol, and a PEG-lipid, with all of the components contributing to efficient

delivery of the nucleic acid drug cargo and stability of the particle [17]. The cationic lipid electrostatically condenses the negatively charged RNA into nanoparticles and the use of ionizable lipids that are positively charged at acidic pH is thought to enhance endosomal escape. The most explored formulations for delivery of siRNA both clinically and non-clinically are predominantly based on cationic lipids such as Dlin-MC3-DMA (MC3) [11, 18]. Recently, the therapeutic siRNA ONPATTRO™ (Patisiran) in MC3 LNPs was awarded break through approval by the Food and Drug Administration for the treatment of the polyneuropathy of hereditary transthyretin-mediated (hATTR) amyloidosis in adults [19].

Building on the experience of LNP delivery of siRNA, several groups have demonstrated effective and tolerable delivery of mRNA using LNPs for transient expression of vaccine antigens as well as of secreted proteins following intravenous (i.v.) or intramuscular (i.m.) administration [4-7, 20-22]. Consequently, a number of mRNA constructs have recently progressed into clinical trials [23]. Whilst great progress has been made in achieving efficient and tolerable LNPs for delivery of mRNA for i.v. or i.m. administration, the challenge remains with regards to subcutaneous (s.c.) self-administration of therapeutic mRNA required for chronic/sub chronic treatment of diseases. Following s.c. administration, LNPs and their mRNA cargo are expected to be largely retained at the site of injection resulting in high local concentrations. Since LNPs are known to be pro-inflammatory, largely attributed to the ionizable lipid present in the LNPs [14], then it would not be unexpected that s.c. administration of mRNA formulated in LNPs would be associated with dose-limiting inflammatory responses. Previous work has shown that co-administration of dexamethasone with LNP reduces immune-inflammatory response following i.v. administration and recently Chen et al reported on reduced immune stimulation following systemic administration by incorporating lipophilic dexamethasone prodrugs within LNP containing nucleic acids [24, 25]. Here, we report the concept of incorporating hydrophobic prodrugs of anti-inflammatory compounds into mRNA-

loaded LNPs i.e. functionalized LNPs to minimize the inflammatory response and maintain protein expression [26]. We report, for the first time, the use of functionalized LNPs *in vivo* that enable tolerable subcutaneous administration of mRNA encoding for a model secreted protein (human Fibroblast Growth Factor 21 (hFGF21)) to demonstrate the utility of this approach for systemic protein-replacement therapies.

RESULTS

Systemic protein exposure and tolerability following i.v. and s.c. administration of mRNA formulated within LNPs

A chemically modified mRNA encoding for the secreted human Fibroblast Growth Factor 21 protein (hFGF21 mRNA) was synthesized and formulated within LNPs prepared using DLin-MC3-DMA as the amino, ionizable lipid. The MC3 LNP formulated hFGF21 mRNA was administered to CD1 mice either i.v. or s.c. at a dose of 0.3 mg/kg of mRNA.

Plasma exposure profiles of the secreted hFGF21 protein are reported in **Figure 1a**. Following s.c. administration, plasma exposure was approximately 20-fold lower compared to i.v. administration exhibiting a delayed C_{max} observed at approximately 8 hours as compared to a much earlier C_{max} for i.v. administration (**Table S1; Figure 1b).**

To assess tolerability, plasma levels of the acute phase protein, haptoglobin and selected inflammatory cytokines/chemokines (interleukin-6 (IL-6), murine interleukin-8 homologue (KC), interferon gamma induced protein 10 (IP-10) and Monocyte chemoattractant protein 1 (MCP-1)) were measured at termination of the study (24 hours post-dosing) and are reported in **Table S1** and **Figure 1c-i.** Haptoglobin, IL-6, IP-10 and MCP-1 levels were elevated following i.v. administration when compared with PBS control. However, after s.c. administration, haptoglobin levels were greatly increased and at the time point measured were 13-fold higher

than after the equivalent i.v. dose. IL-6, IP-10 and MCP-1 were also higher compared with i.v. administration. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured as biomarkers of liver toxicity, however they were not elevated above PBS control levels when hFGF21 mRNA LNPs were administered by either i.v. or s.c. administration (**Table S1**).

The results described using MC3 LNPs for the s.c. delivery of mRNA demonstrate the potential of s.c. route of administration for achieving systemic exposures of protein. However, the observations also clearly highlight the need to improve the tolerability of the mRNA LNP formulation if this is to be a viable path forward for mRNA-based therapeutics which can be self-administered via s.c. injection.

Biodistribution of transfection following i.v. and s.c. administration of mRNA formulated within MC3 LNPs

To better understand protein expression following s.c compared to i.v. administration of mRNA formulated in LNPs, an in vivo imaging study (IVIS) using Luciferase (Luc) mRNA was conducted to identify where protein was being expressed. The study was conducted using the same dose of mRNA as used for the FGF21 protein systemic exposure study as detailed above (0.3 mg/kg).

Luc protein expression following s.c. administration was predominantly confined to the site of administration with some expression being observed in the local axial and brachial draining lymph nodes (**Figure 2**). Protein expression from the liver contributed to less than 1% of total luminescence following s.c. administration of the Luc mRNA formulated in MC3 LNPs (**Table 1**). This distribution of protein expression did not change with time up to 48 hours post administration and hence is assumed to represent where protein expressions occurs following

s.c. administration of mRNA formulated in LNPs. In contrast, and as expected for MC3 LNPs having this composition [27], the majority of protein expression (>95%) following i.v. administration was observed to occur in the liver (**Figure 2b and d, Table 1**).

To further explore s.c. administration of mRNA formulated in LNPs, histological evaluation of the injection site and surrounding tissues was undertaken to identify the cellular distribution of transfection. Luc protein expression (semi-quantitative scoring) was predominantly expressed by adipocytes with limited expression in fibroblasts and macrophages (**Figure 2e-g**). This cellular distribution of protein expression again did not change with time (up to 48 hours, data not shown) and hence is assumed to represent the cellular distribution of where protein expressions occurs following s.c. administration of mRNA formulated in LNPs.

Incorporation of an anti-inflammatory steroid within LNPs.

Considering that tolerability of the mRNA LNP formulation could be the limiting determinant to the success of s.c. self-administration for systemic protein-replacement therapies based on mRNA, it was decided to investigate the concept of incorporating an anti-inflammatory steroid within the LNP. Steroids are potent anti-inflammatory compounds (AICs) which interact with numerous pathways involved with inflammatory responses, and hence have widespread anti-inflammatory effects [28]. Two steroids, namely rofleponide and budesonide were hence investigated for incorporation within LNPs as an approach to improve the tolerability of the mRNA LNP formulation following s.c. administration.

To enable efficient entrapment of the steroid within the LNP, aliphatic ester prodrugs with varying alkyl chain lengths (C5 (rofleponide only), C8 (budesonide only), C14, C16 and C18) were synthesized to increase the lipophilicity/decrease aqueous solubility of the steroids and

thereby promote their incorporation within LNPs. For all steroid prodrugs investigated, entrapment within LNPs was greater than 75%.

The ester prodrugs of rofleponide and budesonide require enzymatic cleavage to release the active parent steroid to have an anti-inflammatory effect [29]. For this, and because of their low aqueous solubility, it was considered that the ester prodrug would likely need to be located on the surface of the LNP to be accessible for such enzymatic cleavage.

The location of the rofleponide-C14 prodrug within the MC3 LNP was therefore investigated by small angle neutron scattering (SANS) with isotropic contrast variation as previously described [30]. In brief, the distribution of the rofleponide-C14 prodrug was elucidated by varying the content of deuterated water (D_2O) to match the scattering length densities of different regions of the particle **(Figure 3a)**. Simultaneous fitting of the SANS data (**Figure 3b)** proposes that a core-shell particle model, where the deuterium-labelled rofleponide-C14 predominantly distributes into the outer shell of the particle, agrees best with the experimental results. In comparison, models that assume more AIC is introduced into the core ("AIC throughout" or "AIC in core") show progressively poorer fit (**Figure 3b**). This strongly supports that the rofleponide-C14 prodrug is not homogeneously distributed within the MC3 LNP, but rather is preferentially located in the outer shell region. Simultaneous fitting of five isotropic contrast data sets estimates that approximately 85 ± 5% of the total mole fraction of the rofleponide-C14 prodrug in the LNP is located at the surface (shell) of the LNP.

Effect of incorporating steroid esters with varying alkyl chain lengths within hFGF21 mRNA LNPs on protein expression and tolerability following s.c. administration

The impact of incorporating steroid prodrugs within LNP formulations of hFGF21 mRNA on systemic protein exposure and tolerability following s.c. administration was investigated in

CD1 mice. Further, steroid prodrugs having fatty acid esters of various chain lengths were investigated to evaluate whether the activity of the steroid was influenced by the carbon chain length of the ester prodrugs. To investigate the broader applicability of this concept to different LNPs, two steroids, namely rofleponide and budesonide, were respectively incorporated within LNPs formulated using two different amino lipids being either DLin-MC3-DMA or L608.

Incorporation of rofleponide ester prodrugs within MC3 LNPs

Fatty acid ester prodrugs of rofleponide having alkyl chain lengths of C5 (valerate), C14 (myristate), C16 (palmitate) and C18 (stearate) were synthesized and incorporated within hFGF21 MC3 LNPs and compared to LNPs not containing steroid.

Consistent with the previous results, s.c. administration of hFGF21 mRNA formulated in MC3 LNPs without steroid resulted in measurable systemic protein exposures but was again associated with systemic inflammatory responses (**Figure 4a-j and Table 2)**. Further, edema and focal neutrophilic inflammation at the site of s.c. injection was observed in all mice (**Figure 4c and l**).

Inclusion of rofleponide prodrugs within hFGF21 mRNA MC3 LNPs reduced both local (edema) as well as systemic inflammatory responses (**Figure 4 and Table 2**). Interestingly, the reduction in inflammatory response seemed to be more pronounced for the longer carbon chain ester prodrugs (C14, C16 and C18) as compared to the C5 ester prodrug, where fewer or no mice were observed to have edema at the site of administration. Plasma haptoglobin and cytokines were lower when rofleponide prodrugs were incorporated within MC3 LNPs, with levels approaching those observed following administration of a phosphate buffered saline, particularly for the longer chain-length prodrugs. Histological evaluation of the injection site also showed significantly reduced neutrophilic inflammation when the longer chain length rofleponide prodrugs were incorporated into the hFGF21 mRNA MC3 LNPs (**Figure 4m)**.

An unexpected, additional benefit of incorporating rofleponide prodrugs within hFGF21 mRNA MC3 LNPs was prolonged protein expression and elevated plasma protein exposures. For example, systemic exposure of hFGF21 ($AUC_{2\text{-}24hours}$) was increased 3.8- and 2.3-fold respectively when rofleponide-C14 or budesonide-C16 was incorporated into the MC3 LNP (**Figure 4a, b; Table 2**).

Incorporation of budesonide ester prodrugs within L608 LNPs

To investigate whether the effects observed when incorporating steroid prodrugs within LNPs were generally applicable, ester prodrugs of a second steroid, namely budesonide, were incorporated within an alternative LNP prepared using L608 as the ionizable amino lipid. For this, fatty acid ester prodrugs of budesonide having alkyl chain of C8 (caprylate), C14 (myristate), C16 (palmitate) and C18:1 (oleate) were synthesized, incorporated within hFGF21 mRNA L608 LNPs and compared to L608 LNPs not containing steroid.

Similar to MC3 LNPs, hFGF21 mRNA formulated in L608 LNPs resulted in measurable plasma exposures of hFGF21 protein following s.c. administration (although plasma levels in 3 out of 5 mice were below the limit of quantification of the assay at the 24-hour termination time point). Further, s.c. administration of the L608 LNPs not containing steroid was again associated with both local and systemic inflammatory responses (**Figure 5**).

Similar to the incorporation of rofleponide prodrugs within MC3 LNPs, inclusion of budesonide prodrugs within L608 LNPs were able to maintain plasma protein levels of hFGF21 over the 24-hour duration of the study (**Figure 5a**). Incorporation of the shorter chain length prodrug budesonide-C8 within L608 LNPs failed to protect from local inflammatory responses with edema being observed in 4 of the 5 mice treated (similar to the incorporation of the shorter chain length prodrug rofleponide-C5 within MC3 LNPs). Better local tolerability was observed upon incorporating the longer chain length esters of budesonide-C16 and budesonide-C18:1 where edema was only observed in 1 or 2 mice (**Figure 5c**). Interestingly, and in contrast to the

observation for MC3 LNPs with rofleponide esters, incorporation of the C14 prodrug of budesonide did not improve local tolerability with all mice showing edema at the site of injection. The improved local tolerability upon incorporation of budesonide prodrugs having longer alkyl chain lengths into L608 LNPs was confirmed by histological evaluation of the injection site, where less cell infiltrates, fibrin build-up and cell necrosis were observed (exemplified by histological section of hFGF21 mRNA L608 LNPs ± B-C16, **Figure 5d, e**).

In terms of systemic inflammatory responses, there was a trend of decreasing plasma haptoglobin with increasing the ester prodrug chain length of budesonide, with levels for the C16 and C18:1 being significantly lower compared the L608 LNP not containing steroid (**Figure 5b**). Incorporation of budesonide prodrugs however had little effect on cytokine levels which were overall close to baseline and not particularly elevated for hFGF21 mRNA L608 LNPs (data not shown).

Effect of steroid ester prodrug on duration of protein expression and comparison to parent steroid

Investigations to identify the most suitable prodrug ester of the two steroids, indicated that incorporation of the steroid prodrugs within MC3 or L608 mRNA LNPs resulted in prolonged protein expression, at least over the 24-hour period investigated in these studies (**Figure 4a and Figure 5a**). To further investigate this phenomenon, follow-up studies were conducted to extend the duration of pharmacokinetic sampling to 72 hours.

Due to the limited amount of blood that could be sampled, and the plasma volumes required for various analyses, 3 parallel groups of mice were used in these studies, wherein one group was sampled over the period 0-24 hours, a second group over the period 24-48 hours and a third group over the period 48-72 hours. Separate studies were carried out comparing either the

steroid ester prodrug (rofleponide-C16 or budesonide-C16) or parent steroid, all formulated in MC3 LNPs to MC3 LNP's not containing steroid.

Incorporation of both rofleponide-C16 and budesonide-C16 within the mRNA MC3 LNP formulation at an equivalent parent steroid:mRNA weight ratio of 2:3 resulted in prolonged protein expression and plasma exposures compared to an MC3 LNP formulation containing no steroid ($AUC_{2\text{-}58h}$ being 2.6-fold and 3-fold greater for rofleponide-C16 and budesonide-C16 respectively; **Figure 6a**). Incorporation of the parent steroid within the mRNA MC3 LNP formulation at the same parent steroid:mRNA weight ratio also yielded some benefits in terms of plasma exposures compared to an MC3 LNP formulation containing no steroid with $AUC_{2\text{-}58h}$ being 1.2-fold and 1.9-fold greater for rofleponide and budesonide respectively (**Figure 6d**).

Compared to inclusion of parent steroid within the mRNA MC3 LNP formulation, the steroid ester prodrugs also offered benefits in terms of tolerability. Plasma haptoglobin levels (**Figure 6b, e)** were markedly lower upon incorporation of the C16 prodrugs compared to the parent steroid, as were levels of inflammatory cytokines levels, as exemplified by IL-6 (**Figure 6c, f**).

Effect of steroid prodrug:mRNA weight ratio on protein expression and inflammation

hFGF21 mRNA MC3 LNPs were formulated to incorporate rofleponide-C16 at steroid prodrug:mRNA ratios of 1:1, 1:10 or 1:30 w:w respectively, approximating 8, 0.8 and 0.3 mol% of the lipidic LNP components. As most evident in the edema scores (**Figure 7c**), there is an inverse correlation between the dose of prodrug and the degree of local inflammation. Decreasing the amount of rofleponide-C16 in the LNPs however had little effects on prolongation of protein expression (at least when the ratio was 10:1 or less) or systemic haptoglobin levels (**Figure 7a, b**).

Steroid pharmacokinetics

To better understand why incorporation of the steroid prodrugs into LNPs both improves the tolerability and prolongs protein expression compared to inclusion of the parent steroid within mRNA LNP formulations, the systemic pharmacokinetics of the parent steroid and steroid prodrugs following s.c. administration of the respective hFGF21 mRNA MC3 LNPs were evaluated and compared with the pharmacokinetic properties of the respective parent steroid following i.v. administration.

The plasma concentrations-time profiles of rofleponide and budesonide following i.v. administration, as well as following s.c administration of rofleponide and budesonide or their respective prodrugs formulated in hFGF21 mRNA MC3 LNPs are shown in **Figure 8**. The corresponding calculated pharmacokinetic parameters are reported in **Table 3**.

As can be seen, both rofleponide and budesonide were rapidly eliminated from plasma following i.v. administration, exhibiting a half-life of about 20 and 40 minutes respectively. Following s.c. administration of the parent rofleponide and budesonide included in the mRNA MC3 LNP formulation, systemic absorption of the steroid was rapid, with peak plasma concentrations being observed at 30 and 10 minutes for rofleponide and budesonide respectively. Elimination half-lives for both steroids were similar to that observed following i.v. administration resulting in similar plasma profiles for s.c. and i.v. administration of the parent steroid.

In contrast, appearance of the parent steroid in plasma is delayed and more sustained when administered as the ester prodrug incorporated within the hFGF21 mRNA MC3 LNPs (**Figure 8**). As shown for rofleponide (**Figure 8a)**, a clear relationship is observed between the pharmacokinetic properties and the length of the alkyl chain of the prodrug. Increasing alkyl chain length results in a delayed t_{max}, reduced C_{max} and a prolonged half-life (**Table 3**). Pharmacokinetic parameters were also observed to be similar between the C16 ester prodrug of

rofleponide and budesonide, exhibiting a similar delayed t_{max} and elimination half-lives. It would thus seem that the rate of hydrolysis of the steroid prodrugs is inversely proportional to the alkyl chain length of the steroid prodrug, which in turn determines its retention at the site of administration.

This was further confirmed by incubating the steroid prodrugs *in vitro* with human adipocytes. Conversion of the steroid prodrugs *in vitro* was observed to be in the same rank order as observed for the appearance of parent steroid in plasma, with the longer chain-length produgs having a slower rate of conversion (**Figure S1, Table S3**).

DISCUSSION

In the current study, we have developed a viable and tolerable formulation for effective murine delivery of mRNA encoding hFGF21 via the s.c. route of administration that results in sustained increases in plasma hFGF21 levels. S.c. administered hFGF21 mRNA formulated in MC3 LNP alone resulted in measurable systemic plasma concentrations of the secreted hFGF21 protein over a duration of 24 hours, although the observed plasma exposures were much lower compared to i.v. administration of the same dose of mRNA formulated in LNPs (**Figure 1a; Table S1**). Further, s.c. administration of hFGF21 mRNA formulated in MC3 LNP was associated with dose limiting inflammatory responses, that appeared to be exaggerated when compared with i.v. administration of the same dose (**Figure 1c-i; Table S1**). The dose limiting inflammatory responses observed following s.c. administration is likely a result of the high local concentration of the mRNA LNPs at the site of administration, which are known to be pro-inflammatory and attributed to the ionizable amino lipid present in the LNPs [14]. The inflammatory response following s.c. administration of such LNPs manifests itself in the form of localized inflammation and edema at the injection site, as well as systemic responses

345 including elevated levels of haptoglobin (an acute phase marker of an inflammatory response
346 [31]) as well as various cyto/chemokines (**Figure 4; Table 2**).

347 Investigation of the biodistribution of mRNA LNPs following s.c. and i.v. administration using
348 Luc mRNA (expressing the non-secreted Luc protein), showed that following s.c.
349 administration, protein expression was predominantly confined to the site of administration
350 (**Figure 2; Table 1**). In contrast, following i.v. administration of mRNA in MC3 LNPs, the
351 majority of protein expression (>95%) was observed to occur in the liver, consistent with
352 previous observations for these types of LNPs [32]. This likely explains the almost 20-fold
353 lower hFGF21 protein systemic exposure observed following s.c. administration compared to
354 i.v. Following s.c. administration, mRNA LNPs are largely confined to the injection site, with
355 minimal drainage occurring to the systemic circulation as indicated by the very low liver
356 expression of Luc protein. As such, the number of cells transfected in the vicinity of the
357 injection site is likely to be much lower following s.c. compared to i.v. administration, where
358 the entire cell population of the liver are able to take up mRNA LNPs and express protein.
359 Immunohistochemical evaluation of the s.c. injection site showed that the predominant cell type
360 transfected was adipocytes. This may not be surprising, as the type of LNP used in the current
361 investigations, incorporating an ionizable lipid such as MC3, are known to be internalized by
362 cells (such as hepatocytes) via the low density lipoprotein (LDL) receptor following adsorption
363 of Apolipoprotein E onto the surface of LNPs [33]. Adipocytes are known to also express LDL
364 receptors [34], which may explain why adipocytes are efficient translators of mRNA following
365 s.c. administration when using ionizable LNPs as a delivery vector [35].

366 Prodrugs of rofleponide and budesonide with various alkyl chain lengths were synthesized to
367 enable incorporation into the LNP in an attempt to control the inflammatory responses and
368 enable delivery of mRNA via s.c. administration. The resulting LNP formulations were well
369 tolerated at the doses investigated, particularly when incorporating prodrugs having longer

alkyl chain lengths (**Figure 4 & 5; Table 2**). Interestingly, incorporation of steroid within the LNPs altered the dynamics of protein production when compared with LNP formulations that did not include steroid. Systemic protein exposures were increased up to 3-fold when the steroid prodrugs were incorporated into the LNPs and this was accompanied by a prolongation in hFGF21 protein exposure of up to 72 hours (**Figure 6**). Importantly these finding were not limited to MC3 LNPs and were repeated with L608 LNPs (**Figure 5)**, demonstrating potential for broad application of this strategy across LNP delivery systems. These results demonstrate that s.c. administration of mRNA can be a viable route of administration for clinical application of mRNA-protein replacement and regenerative therapies, enabling self-administration and thus expanding potential impact of these exciting emerging therapeutics.

LNP formulations have been successfully applied to support clinical development of siRNA therapeutics due to their ability to both encapsulate RNA molecules and protect them from degradation by systemic RNase [36] These same considerations make them attractive formulations to be explored for mRNA delivery [13]. Indeed, Pardi et al [37] demonstrated that mRNA, encoding the intracellular protein luciferase encapsulated within LNPs, could be successfully translated to protein after administration using various routes of administration, including i.v. and s.c.. Doses administered in this study were relatively low, up to 5 μg/injection (<150 μg/kg), and tolerability was not discussed.

LNPs however are recognized to have an immunostimulatory profile that is distinct from their cargo [14, 38], and dose-limiting systemic inflammatory responses have been described for LNP formulations administered i.v. [24]. Indeed, recently de Groot et al [39] described a potential interaction of cationic lipids with Toll-like receptor 4 as a potential initiating factor in the immunogenic response to these types of nanoparticulate formulation. While the s.c. route of therapeutic administration has the benefits of convenience and allowing for self-administration when compared with i.v., this administration route has the potential to display

exaggerated inflammation due to the presence of multiple active defense mechanisms utilized by the skin to protect against microbial pathogens. These include components of both the innate and adaptive immune systems across a variety of cells capable of mounting a formidable inflammatory response [40]. The response is likely amplified by the high local concentrations of mRNA LNPs at the s.c. injection site which appear to largely remain at the site of administration and not drained into the systemic circulation (**Figure 2**). Therefore, it is perhaps not unexpected that in the current study, we observed a dose-limiting and pronounced local and systemic inflammation upon s.c. injection of mRNA LNP formulations, an effect that appeared exaggerated when compared with i.v. administration. Interestingly, the profile of cytokines measured systemically following s.c. administration was similar to that observed by others after i.v. administration of siRNA LNP formulations; elevated levels primarily of IL-6, IP-10, KC and MCP-1 suggesting similar underlying mechanisms [24, 32, 41].

Pre-treatment with anti-inflammatory agents, both dexamethasone and Janus kinase inhibitor, have previously been shown to control adverse effects of siRNA LNPs after i.v. administration [24, 42]. Moreover, this strategy has been applied clinically to enable siRNA LNP delivery to patients where clinical trials for Patisiran (ONPATTRO™) included pretreatment with dexamethasone, paracetamol, histamine H1 and H2 antagonists [43]. However, this strategy is problematic, particularly when sub-chronic/chronic therapy is required. Steroids have been reported to induce hyperglycemia in non-diabetic patients potentially leading to steroid induced diabetes mellitus [44-46]. Furthermore, steroids are also known to have unwanted side effects such as hypertension, cataracts and increase risk of fractures [47]. We therefore hypothesized that formulation of a steroidal anti-inflammatory prodrug within an LNP would lead to precision delivery of the steroid compound to the precise time and location at which its action is required, i.e. the cells that are exposed to the mRNA LNP formulation.

We explored the relationship between inflammation, protein synthesis and systemic steroid exposure when either rofleponide or budesonide were incorporated into the LNP formulation as either parent drug or ester prodrugs having different alkyl chain lengths to determine the value of the ester modification. We have shown that when the parent steroid is incorporated into the LNP formulation without any modifications, it is rapidly absorbed into the systemic circulation from the site of administration and then rapidly eliminated (**Figure 8**). As therefore might be expected, incorporation of the parent steroid within the mRNA LNP formulation gave limited protection, with an inflammatory response to this LNP formulation still being apparent (**Figure 6**). When the ester prodrugs of rofleponide and budesonide are incorporated within the LNP formulation, appearance of the parent steroid in the plasma is delayed and is associated with a lower C_{max} and is sustained for a longer duration (**Figure 8**). Interestingly, there also appeared to be a relationship between exposure profile of the parent steroid in plasma and length of the alkyl chain of the prodrug, where a longer alkyl chain appeared to both delay and reduce C_{max}, and extend the half-life ($t_{1/2}$). It would thus seem that the rate of hydrolysis of the steroid prodrugs is inversely proportional to the alkyl chain length of the steroid prodrug, which in turn determines its retention at the site of administration. This was confirmed by measuring conversion rates of the steroid prodrug to the active parent *in vitro* using human adipocyte cultures (**Figure S1; Table S2**) and is consistent with what is known regarding hydrolysis rates of glycerides by lipases where cleavage of the ester and release of the fatty acid is reduced with increasing alkyl chain length of the acid [48].

Another observation was that addition of rofleponide or budesonide prodrugs to the mRNA LNP formulations resulted in a sustained boost in the protein production levels and higher overall systemic protein exposures (**Figure 6**). It is recognized that transfected mRNA is able to activate cellular innate immune responses through pattern recognition receptors that detect nucleic acids as part of the cellular viral defense response [49]. It is also known that LNPs can

activate the innate immune system via interaction with the TLR4 receptor, thereby potentially initiating a cellular immune response to these nanoparticles [39]. Coupled with observations that cellular innate immune activation is associated with down regulation of cellular translation [50, 51], this could perhaps explain the unexpected increased and sustained protein levels resulting from inclusion of anti-inflammatory steroidal agents within LNPs that are able to suppress local inflammatory responses. Similar improvements in protein production with exogenous mRNA have recently been reported when dexamethasone was incorporated into LNPs and administered intravenously [52].

To summarize, incorporation of either rofleponide or budesonide ester-prodrugs into two different LNP formulations was able to significantly improve the tolerability of mRNA LNPs, oftentimes entirely preventing the local edema-response and the increase in systemic cytokine concentrations seen following s.c administration. Due to the robust retention of steroid prodrug in the LNP, we believe that the protection afforded by the steroid is likely due to a highly localized anti-inflammatory mechanism of action, probably at the cellular site of LNP uptake. Moreover, we demonstrate that inclusion of a steroid prodrug in a mRNA LNP formulation was able to dramatically increase the level and duration of protein production following s.c. administration. Together these findings demonstrate that successful systemic protein exposure can be achieved through mRNA administered via the s.c. route, an observation that if repeated in humans could increase therapeutic application opportunities for this important emerging platform.

MATERIALS AND METHODS

mRNA synthesis

Modified mRNA encoding human Fibroblast Growth Factor 21 (hFGF21) or Luciferase (Luc) were synthesized as previously described [4]. Briefly, the mRNA was codon optimized and synthesized *in vitro* by T7 RNA polymerase-mediated transcription. The uridine-5' triphosphate (UTP) was substituted with 1-methylpseudo-UTP, using a linearized DNA template, which also incorporates 5' and 3' UTRs, including a poly A tail. A donor methyl group from S-adenosylmethionine was added to methylated capped RNA (cap-0), resulting in a cap-1 modification to increase mRNA translation efficiency. These chemical modifications to the mRNA are designed to both improve protein translation and reduce immunogenicity and are the same as used by Carlsson et al [9] and An et al [4].

Synthesis of rofleponide and budesonide prodrugs

Rofleponide and budesonide were obtained from AstraZeneca. Rofleponide is a pure enantiomer, while budesonide is a diastereomeric mixture of approximately 55:45 at the acetal carbon. Rofleponide and budesonide prodrugs (in addition to the deuterated rofleponide-C14 prodrug used for SANS) were synthesized using a general procedure as exemplified in the following for the synthesis of partially deuterated rofleponide-C14 (d27). The chemical structures of rofleponide and budesonide and their respective prodrugs are illustrated in **Figure 9**.

d27-Myristoic acid (75 mg, 0.29 mmol) was dissolved in dichloromethane (1.5 mL) and one drop of dimethylformamide. Oxalyl chloride (0.031 mL, 0.35 mmol) was added dropwise at room temperature and the mixture was left stirring overnight. Dichloromethane and excess oxalyl chloride were removed by evaporation to obtain the light-yellow, oily d27-myristoyl chloride.

Triethylamine (0.045 mL, 0.32 mmol) was added to a solution of rofleponide (60 mg, 0.13 mmol) in dichloromethane (0.5 mL). d27-Myristoyl chloride (70.2 mg, 0.26 mmol) was dissolved in dichloromethane (0.5 mL) and then added to the rofleponide solution. The reaction mixture was allowed to stir at room temperature for 3 h. Liquid chromatography/mass spectrometry indicated >99% conversion to the desired product. All chemicals used for the manufacture and purification of the steroid prodrugs were purchased from Sigma Aldrich, St Louis, MO, USA.

The product was purified by automated silica flash column chromatography (10g SNAP column, Biotage, Uppsala, Sweden) using an ethyl acetate:dichloromethane gradient (0% to 10% respectively over 8 x column volume) and a collection wavelength of 228 nm. The fractions containing product were pooled and evaporated, followed by co-evaporation with dichloromethane to remove any residual ethyl acetate. The identity of the deuterated (d27) rofleponide-C14 product (85 mg, 94 %) was confirmed by ^{1}H NMR (in deuterated chloroform, Bruker 500 MHz, Billerica, MA, USA) and electrospray ionization mass spectrometery (positive ion mode, Waters Acquity UPC2, Milford, MA, USA): δ 0.85 - 0.98 (m, 6H), 1.44 (h, 2H), 1.55 - 1.86 (m, 7H), 1.98 (dd, 1H), 2.14 - 2.24 (m, 1H), 2.28 (m, 1H), 2.45 (m, 6H), 4.39 (d, 1H), 4.62 - 4.75 (m, 2H), 4.86 (d, 1H), 4.95 (d, 1H), 5.26 (m, 1H), 6.14 (s, 1H); m/z of 707.

LNP formulation

LNPs were prepared by microfluidic mixing as described previously [30]. Briefly, an ethanolic solution of the lipid components and a solution of the mRNA in RNAse free citrate buffer pH 3 (50 mM) were mixed at a ratio of 1:3 respectively at a total flow rate of 12 ml/min using a NanoAssemblr™ (Precision Nanosystems, Vancouver, Canada). Following microfluidic mixing, the LNPs were dialyzed overnight against 500 x sample volume of phosphate-buffered

saline (pH 7.4) using Slide-A-Lyzer™ G2 dialysis cassettes with a molecular weight cut-off of 10k (Thermo Fisher Scientific, Waltham, MA, USA).

MC3 LNPs encapsulating hFGF21 or Luc mRNA were formulated using the amino lipid dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA, synthesized as previously described [53]), cholesterol (Chol), distearylphosphatidylcholine (DSPC) and dimyristoylphosphatidylethanolamine polyethyleneglycol 2000 (DMPE-PEG2000) at a % molar composition of 50:38.5:10:1.5 respectively and at a total lipid:mRNA weight ratio of 10:1 (nitrogen:phosphate (N:P) ratio of 3). L608 LNPs were formulated using the amino lipid (12Z, 15Z)-N,N-dimethyl—2-nonylhenicosa-12-15-dien-l-amine (L608, synthesized as described for compound 32 in [54]), Chol, DSPC and DMPE-PEG2000 at the same % molar composition but at a total lipid:mRNA ratio of 17:1 (N:P ratio of 6). For incorporation of steroid prodrugs within the LNPs, the compounds were dissolved in the ethanolic solution of lipids prior to microfluidic mixing. To avoid losses during formulation, parent steroid was added to the formulation post LNP manufacture.

The size and polydispersity of LNPs was determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) and the encapsulation and concentration of mRNA in the LNP formulations were determined using the RiboGreen assay [55]. Typically, LNPs had a particle size (Z-average) of 70-100 nm with a polydispersity index of < 0.2 (which was not affected by the type of ionizable cationic lipid used or incorporation of anti-inflammatory compound) and encapsulation of mRNA was >90% (see **Table S3**). Endotoxin levels within the mRNA LNP formulations were typically less than 1 EU/ml (Endosafe-PTS™, Charles River Laboratories, Wilmington, MA, USA) but were not routinely measured since values were consistently low. All formulations were prepared within one week of testing to ensure the chemical stability of the components.

Entrapment of steroid prodrug within LNPs

To estimate the entrapment of the steroid prodrugs within LNPs, any free prodrug (in solution or crystalline) and LNPs were separated following dialysis using a size exclusion gel column (PD-10; GE Healthcare, Chicago, Il, USA). The excluded volume containing the LNPs was diluted 2-fold with a solution containing 40 mM sodium dodecyl sulphate and 1% triton to solubilize the LNPs. The steroid prodrug and lipid content of the resulting solution was then analyzed using Ultra Performance Liquid Chromatography combined with Charged Aerosol Detection (Corona® CAD, ESA Biosciences Inc, Chelmsford, MA, USA) fitted with a C18 column (Waters Acquity BEH, 1.7 µm, 2.1 x 50 mm; Milford, MA, USA) run at 80°C at a flow rate of 0.5 ml/min. A mobile phase of 100 mM aqueous ammonium acetate (A) and a 50:50 mix of acetonitrile:isopropyl alcohol (B) run at a gradient profile of 90% A for 1 minute, 90% A to 90% B over 4 minutes followed by 5 minutes of 90% B was used for compound elution. The entrapment efficiency of the steroid prodrug was calculated by comparing the ratio of the measured concentration of the steroid prodrug to the measured concentration of the amino lipid (DLin-MC3-DMA or L608) to the theoretical concentration of the two components. In all cases, entrapment of steroid prodrugs within either MC3 or L608 LNPs was greater than 75%.

Distribution of steroid prodrug within LNPs

The location/distribution of one selected AIC, namely rofleponide-C14, within the hFGF21 mRNA MC3 LNP was further evaluated using small angle neutron scattering (SANS) with isotropic contrast variation. The technique of contrast variation SANS is based on the distinct interaction that neutrons have with hydrogen (H) and deuterium (D) atoms, such that by substituting D for H (selective deuteration), it is possible to highlight different regions of the LNP structure [30]. MC3 LNPs incorporating rofleponide-C14 at a prodrug:mRNA ratio of 1:1 w/w were therefore formulated with partially deuterated rofleponide-C14 (d27, synthesized as

described above). SANS measurements were performed using the KWS-2 instrument operated by Jülich Centre for Neutron Science (JCNS) at Forschungs-Neutronenquelle, Heinz Maier-Leibnitz [56]. The measurements were performed at 3 different sample-to-detector distances, 2 m, 8 m and 20 m, and 2 neutron wavelengths, λ = 5 Å and 10 Å, with a wavelength resolution of 10%. These configurations cover the scattering vector (q) range of $0.00133 < q\ (Å^{-1}) < 0.411$. The measurements were done in quartz disc-shaped ('banjo') cuvettes (Hellma, Jena, Germany) of 1 and 2 mm path length, maintained at 25°C. LNPs were diluted in the appropriate solvent ratio of H_2O/D_2O (15-100 %v/v) to a final concentration of 0.3 mg/mL of mRNA (3mg/mL of lipid). The generated data were corrected for detector sensitivity/noise, and solvent/empty cell contribution, taking into account the measured sample transmission.

Scattering profiles obtained in the buffers containing various proportions of D_2O were fitted simultaneously (SasView software, NIST Center for Neutron Research, Gaithersberg, MD, USA) using the "Core-Shell-Sphere" model. The model describes a spherical particle comprised of a hydrated core (composed of DLin-MC3-DMA, Chol, mRNA and 24 vol% water) and a lipid monolayer shell (composed of DSPC, DLin-MC3-DMA, Chol and the DMPE part of DMPE-PEG2000) as previously described for the composition/structure of MC3 LNPs [30]. The parameters fitted were the radius of the core, the thickness of the outer shell and the scattering length densities of the core and the shell. As the presence of deuterated rofleponide-C14 in either the core or in the shell of the LNP alters the scattering length density of that region, it is possible to identify the location and distribution of AIC within the LNP. The experimental SANS profiles were compared to three models generated using different values for the scattering length densities of the core and shell, calculated based on the deuterated rofleponide-C14 being only in the shell ("AIC in the shell"), only in the core ("AIC in the core") or to have no preferential location in the particle ("AIC distributed throughout"). The best fit was obtained for the "AIC in the shell" model, where the core has a radius of approximately 27

589 nm and is surrounded by a shell layer of 2.4 nm which contains the majority of the AIC. No
590 layer representing the diffuse PEG surrounding the particle was included as it did not improve
591 the model. Schultz polydispersity (0.16) was included in the model to describe the size
592 distribution of the LNPs.

593

594 ***In vivo* studies**

595 Twelve-week-old female CD1 mice were obtained from Charles River Laboratories (Sulzfeld,
596 Germany). Mice were kept in communal cages with aspen wood chip bedding in a holding
597 facility with a controlled environment (12 hour light/darkness cycle, room temperature 21-22°C
598 and relative humidity 40-60%, with free access to water and standard rodent chow (R70,
599 Lantmännen)). Mice were acclimatized to these conditions for at least 5 days before use. All in
600 vivo studies were approved by the Local Ethics Review Committee on Animal Experiments
601 (Gothenburg Region).

602 On the day of dosing, mice were lightly anaesthetized with 5% isoflurane and were
603 administered the test treatment either i.v. or s.c. (in the intra-scapular region) at an
604 administration volume of 5 ml/kg. Following dose administration, blood samples were collected
605 from the saphenous vein from alternating legs at various time points up to 24 hours. At
606 termination, mice were anaesthetized using isoflurane, examined for any clinical abnormalities
607 including edema at the injection site and terminal blood samples were collected. Plasma was
608 prepared by centrifugation (3,000 g for 10 minutes at 4°C) and the samples were quantified for
609 hFGF21 protein using a Multiplex®-Luminex® assay (Merck Millipore, Burlington, MA, USA,
610 cat.no. HLPPMAG-57K). The terminal sample was also used for quantification of haptoglobin
611 using the Milliplex map mouse acute phase panel 2 kit (Merck Millipore, Burlington, MA,
612 USA, cat.no. MAP2MAG-76K) and quantification of cytokines/chemokines (IL6, KC, IP10,
613 MCP-1) using a Milliplex®-Luminex® assay (Merck Millipore, Burlington, MA, USA, cat.no.

MCYTOMAG-70K). At termination, the injection site was collected, fixed in formalin and sectioned, stained with haematoxylin and eosin and examined microscopically. Statistical analyses for all the experiments were performed using a one-way ANOVA with unequal variance and adjusted p-values to compare all conditions. The p-values were adjusted to control the false discovery rate using the Benjamini–Hochberg procedure [57].

Biodistribution study.

MC3 LNPs encapsulating Luc mRNA were administered to CD1 mice either i.v. (tail vein) or s.c. (intrascapular) at a dose of 0.3 mg/kg and a dosing volume of 5 ml/kg whilst under light anesthesia (isoflurane). At 8, 12, 24 and 48 hours (8 hours only for i.v. dosing) post-administration, whole body scans of the mice were collected using an IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA, USA). Twenty minutes prior to imaging each mouse received a 150 mg/kg dose of luciferin (Rediject D-Luciferin, PerkinElmer, Waltham, MA, USA) administered s.c. at a dosing volume of 5 ml/kg. At the 8, 24 and 48 hour time points, n=5-8 mice were euthanized, organs extracted (liver, spleen, kidneys, lung, axillary/brachial lymph nodes and tissue surrounding injection site) and imaged ex-vivo using the IVIS Spectrum. After imaging, the organs were separately stored in formalin for immunohistological evaluation to evaluate cellular distribution of transfection.

*Studies to explore systemic exposure and pharmacokinetics of steroids***.**

The pharmacokinetic properties of rofleponide and budesonide after intravenous tail vein (parent steroid only) or subcutaneous intrascapular injection (parent steroid and selected ester prodrugs) were evaluated in CD1 mice. Serial blood samples were collected from the saphenous vein at varying time points post-dose and a terminal sample collected at 24 hours post-dose. Blood samples were quantified for rofleponide and budesonide using an Acquity UPLC® I-class

system and a Xevo^{TM} TQ-XS triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The lower limit of quantification was 0.1 nmol/L for both rofleponide and budesonide. Pharmacokinetic parameters were calculated using non-compartment analysis in Phoenix 6.4 (Certara, St. Louis, MO, USA).

AUTHOR CONTRIBUTIONS

ND, DH, NE, AD, AH, FS, EH, SB, EA, NB and SA were members of the cross-functional team within AstraZeneca that were involved in conceptualizing and designing all studies, in data analysis/interpretation and overall execution of the project.

NE, AH, EH, AD and SA were additionally responsible for resourcing and coordinating the work.

AD and PN synthesized the ionizable lipids and the rofleponide/budesonide prodrugs used in the investigations

TK, YJ, LL, AH, and ND were responsible for co-ordination, manufacture, characterization and supply of LNP formulations to the work described.

AR, MYA, ADab and LL contributed to the design, preparation of samples and analytics as well as the execution and interpretation of the data for the SANS investigations.

SB was responsible for the design and conduct of all in vitro studies used to support the investigations and summarizing/interpreting the results.

DH, NE, CJ, EH, JL, LB, MJ and A-SS coordinated and conducted in vivo studies and were responsible for bioanalysis and result generation.

LH and MJ were responsible for and conducted the biodistribution study.

FS and NE analyzed and summarized the data from in vivo studies with regards to safety, immunology and histology.

ND was largely responsible for authoring the manuscript, with contribution from all co-authors according to area of expertise.

SA was the project leader and coordinated the project together with EA.

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CONFLICTS OF INTEREST

LL and TK are authors on the patent WO2017/194454 (A1) relating to Lipid Nanoparticles comprising lipophilic anti-inflammatory agents and methods of use thereof. All authors were at the time of study conduct employees of AstraZeneca.

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Figure Titles

Figure 1. Systemic hFGF21 protein exposure and tolerability following i.v. and s.c. administration of hFGF21 mRNA formulated in MC3 LNPs. Plasma concentration-time profiles (mean ± SE) and exposure of hFGF21 protein, haptoglobin, transaminases and cytokines in plasma at 24 hours post-administration in CD1 mice (n=5) following iv administration of PBS (group 1, ✱) or s.c. (group 2, ●) or i.v. (group 3, ◆) administration of 0.3 mg/kg hFGF21 *mRNA* in MC3 lipid nanoparticles. Blood samples were collected at 2, 5, 8 and 24 hours after dose administration for plasma hFGF21 protein exposure (**a**). Calculated individual and geometric mean (✖) exposure ($AUC_{2\text{-}24h}$) of FGF21 protein (**b**). Plasma exposure at 24 hours of haptoglobin (**c**), ALT (**d**), AST (**e**) IL-6 (**f**), IP-10 (**g**), KC (**h**) and MCP-1 (**i**). The PBS group for IL-6 was not included in the statistical evaluation (n.c.: not calculated) as 3 out of 5 values were below limit of detection. Statistics for IL-6 and $AUC_{2\text{-}24h}$ were therefore evaluated using a Welch two sample T-test. Remaining parameters were evaluated using a one-way ANOVA with unequal variance and adjusted p-values. One sample of KC in the PBS groups was a clear outlier and excluded from the statistical analysis. Significance defined as: ns (not significant) $p>0.05$, * p 0.05-0.01, ** $p<0.01$-0.001 and *** $p<0.001$.

Figure 2. Biodistribution of luciferase protein expression following i.v. or s.c. administration of Luc mRNA formulated in MC3 LNPs. Representative whole body and excised tissue IVIS images 8 hours following s.c. (**a**, **c**) and i.v (**b**, **d**) administration of 0.3 mg/kg Luc *mRNA* in MC3 lipid nanoparticles and cellular expression as evaluated by immunohistochemistry (brown staining): adipocytes (**e**), fibroblasts (**f**) and macrophages (arrow head) and neutrophils (arrow) (**g**).

Figure 3. Characterization of steroid prodrug distribution within LNPs. Schematic of the isotopic contrast variation employed to highlight the location of AIC in the LNPs with partially deuterated rofleponide-C14 prodrug depicted as red triangles (**a**). SANS data (symbols) and best fit (lines) shown as scattering intensity as a function of the scattering vector (q) for mRNA-containing MC3 LNPs with a rofleponide-C14:mRNA 1:1 weight ratio (**b**). The data shown is for MC3 LNPs in 15 to 100 vol% D_2O buffer (PBS, pH 7.4). The solid lines show the best fit model, where most AIC molecules are located in the shell. The dotted and broken lines show the models that assume the AIC molecules partitioning in the core or distributed homogenously throughout the particle, respectively. The corresponding models are shown above. The intensity for each data set contrast is offset by an order of magnitude for clarity.

Figure 4. Rofleponide prodrugs formulated in MC3 LNPs improve protein expression and reduce local (edema) as well as systemic inflammatory responses following s.c. administration. Plasma concentration-time profiles (mean ± SE) and exposure of hFGF21 protein, haptoglobin, transaminases, cytokines and histopathology of the injection site at 24 hours post-administration following s.c administration in CD1 mice (n=5) of PBS (group 1, ✸) or hFGF21 *mRNA* in MC3 lipid nanoparticles (0.3 mg/kg *mRNA*) containing no steroid (group 2, ●) or with rofleponide-C5 (group 3, ◆), rofleponide-C14 (group 4, ▲), rofleponide-C16 (group 5, ■) or rofleponide-C18 (group 6, ★) incorporated into the LNP at a prodrug steroid:*mRNA* weight ratio of 1:1. Blood samples were collected at 2, 5, 8 and 24 hours after dose administration for plasma hFGF21 protein exposure (**a**). Individual and geometric mean (✖) exposure ($AUC_{2\text{-}24h}$) of FGF21 protein (**b**) and incidence of edema at site of injection at 24 hours after dosing (**c**). Plasma exposure at 24 hours of haptoglobin (**d**), ALT (**e**), AST (**f**) IL-6 (**g**), IP-10 (**h**), KC (**i**) and MCP-1 (**j**). Statistics evaluated using one-way ANOVA with unequal variance and adjusted p-values. Significance defined as: ns (not significant) p>0.05, * p 0.05-

0.01, ** p<0.01-0.001 and *** p<0.001. Hematoxylin and eosin stained histological sections of injection site at 24 hours after dosing for PBS showing no morphological changes (*) (**k**), MC3 LNPs without steroid showing extensive focal acute inflammation (*) (**l**) and MC3 LNPs containing rofleponide-C16 showing significant reduction of inflammation (*) (**m**). x5 (**k, l**) and x10 (**m**) magnification.

Figure 5. Budesonide prodrugs formulated in L608 LNPs improve protein expression and reduce local (edema) as well as systemic inflammatory responses following s.c. administration. Plasma concentration-time profiles of hFGF21 protein (mean ± SE); plasma haptoglobin, edema and histopathology of the injection site at 24 hours post-administration following s.c. administration in CD1 mice (n=5) of hFGF21 *mRNA* formulated in L608 LNPs (0.3 mg/kg *mRNA*) containing no steroid (group 1, ●) or with budesonide-C8 (group 2, ◆), budesonide-C14 (group 3,), budesonide-C16 (group 4, ■) or budesonide-18:1 (group 5, ★) incorporated into the LNP at an equivalent parent steroid: mRNA weight ratio or 2:3. Blood samples were collected at 4, 7 and 24 hours after dose administration for plasma hFGF21 protein exposure (**a**). Individual and geometric mean (✖) haptoglobin concentrations in plasma 24 hours after dosing (**b**). Incidence of edema at site of injection at 24 hours after dosing (**c**). Histological sections of injection site at 24 hours after dosing for L608 LNPs without steroid showing severe acute inflammation (*) (**d**) and L608 LNPs containing budesonide-C16 showing significant reduction of the inflammation (*) (**e**). Magnification x5. Statistics evaluated using one-way ANOVA with unequal variance and adjusted p-values. Significance defined as: ns (not significant) p>0.05, * p 0.05-0.01, ** p<0.01-0.001 and *** p<0.001.

Figure 6. Incorporation of steroid prodrugs (C16) within MC3 LNPs prolongs duration of protein expression and improves tolerability compared to parent steroid following s.c. administration. Plasma concentration-time profiles of hFGF21 protein (mean ± SE, n = 4-12); plasma haptoglobin and IL-6 levels at 24 hours post-administration following s.c. administration in CD1 mice of hFGF21 *mRNA* formulated in MC3 LNPs (0.3 mg/kg *mRNA*) containing no steroid (◆), rofleponide-C16 (●), budesonide-C16 (▲), rofleponide parent co-administered (●) or budesonide parent co-administrated (▲) at an equivalent parent steroid: *mRNA* weight ratio of 2:3. hFGF21 protein concentration in plasma was determined in blood samples collected up to 72 hours after dose administration (**a, d**). Plasma concentration of haptoglobin (**b, e**) and IL-6 (**c, f**) were determined in blood samples collected at termination (24, 48 or 72 hours post dose).

Figure 7. Effect of steroid prodrug (rofleponide-C16):mRNA weight ratio formulated within MC3 LNPs on hFGF21 protein expression and inflammation following s.c. administration. Plasma concentration-time profiles (mean ± SE) of hFGF21 protein; haptoglobin and edema at 24 hours post-administration following s.c. administration in CD1 mice (n =5) of phosphate buffered saline (group 1, ✱) or hFGF21 *mRNA* formulated in MC3 LNPs (0.3 mg/kg *mRNA*) containing no steroid (group 2, ●) or rofleponide-C16 at a prodrug steroid:*mRNA* ratio of 1:1 (group 3, ◆), 1:10 (group 4, ▲) or 1:30 (group 5, ■). hFGF21 protein concentration in plasma was determined in blood samples collected up to 24 hours after dose administration (**a**). Individual and geometric mean (✖) haptoglobin concentrations in plasma 24 hours after dosing (**b**). Incidence of edema at site of injection at 24 hours after dosing (**c**). Statistics evaluated using one-way ANOVA with unequal variance and adjusted p-values. Significance defined as: ns (not significant) $p>0.05$, * p 0.05-0.01, ** $p<0.01$-0.001 and *** $p<0.001$.

Figure 8. Pharmacokinetics of parent steroid in CD1 mice following i.v. (◆) or s.c. (▲) administration of parent drug or C5 prodrug (○) C14 prodrug (✡) or C16 prodrug (▫). Parent steroid was formulated in 0.6% ethanol in phosphate buffered saline solution; steroid prodrugs were formulated in hFGF21 mRNA MC3 LNPs. Blood was collected up to 24 hours after dose administration for quantification of rofleponide (**a**) or budesonide (**b**) in plasma; line represents group mean and markers show individual values (n=4).

Figure 9. Chemical structures of rofleponide, budesonide (and their respective prodrugs), MC3 and L608 amino-lipids.

Tables

Table 1. Organ distribution of luminescence following s.c. and i.v administration of 0.3 mg/kg Luc mRNA in MC3 LNPs (mean ± SEM).

Route	Time	Injection site/skin	Lymph	Liver	Spleen	Kidneys	Heart	Lung
s.c.	8 hours	99.2 ±0.5%	0.71 ±0.52%	0.07 ±0.03%	0.01 ±0.00%	0.00 ±0.00%	0.00 ±0.00%	0.00 ±0.00%
	24 hours	98.2 ±0.8%	1.72 ±0.84%	0.07 ±0.03%	0.02 ±0.01%	0.00 ±0.00%	0.01 ±0.01%	0.00 ±0.00%
	48 hours	98.2 ±0.9%	1.39 ±0.79%	0.23 ±0.06%	0.09 ±0.02%	0.01 ±0.00%	0.04 ±0.01%	0.00 ±0.00%
i.v.	8 hours	1.11 ±0.44%	0.30 ±0.07%	97.6 ±0.5%	0.96 ±0.16%	0.00 ±0.00%	0.01 ±0.00%	0.00 ±0.00%

Table 2. Plasma hFGF21 protein exposure in mice after s.c. administration of 0.3 mg/kg hFGF21 mRNA in MC3 LNPs (MC3) and plasma chemistry measured at termination, 24 hours after dosing (mean ± SEM)

Parameter[a]	PBS control	MC3	MC3 + Rofleponide - C5	MC3 + Rofleponide - C14	MC3 + Rofleponide - C16	MC3 + Rofleponide - C18
$AUC_{2\text{-}24h}$ (nmol·h/L)	n/a	0.29±0.05	0.41±0.08	1.1±0.35	0.65±0.14	0.70±0.08
Haptoglobin (ug/ml)	84±32	1680±110	607±67	286±53	429±68	318±22
IL-6 (pg/ml)	50±24	301±58	316±79	55±12	51±43	142±49
KC (pg/ml)	163±37	395±42	427±71	78±18	157±100	403±150
IP-10 (pg/ml)	102±17	700±62	742±83	260±53	203±85	389±112
MCP-1 (pg/ml)	69±11	488±53	383±44	53±12	98±62	316±170
ALT (U/L)	90±9	104±9	123±22	114±7	120±10	143±30
AST (U/L)	37±2	34±1	35±2	51±4	51±4	66±9

[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.

Table 3. Steroid pharmacokinetic parameters based on composite mean plasma concentration-time profiles

Parameter[a]	Rofleponide parent		Rofleponide -C5	Rofleponide -C14	Rofleponide -C16	Budesonide parent		Budesonide -C16
Route	i.v.	s.c.	s.c.	s.c.	s.c.	i.v.	s.c.	s.c.
Dose (mg/kg)	0.15	0.2	0.47	0.48	0.46	0.2	0.2	0.47
F (%)	-	48	70	89	83	-	59	62
CL (ml/min/kg)	74.5	-	-	-	-	121	-	-
AUC (nmol·h/L)	72	46	134	142	121	65	38	60
C_{max} (nmol/L)	267	74	182	30	13	240	73	7
t_{max} (h)	0.03	0.5	0.5	1.5	2.5	0.03	0.17	1.5
$t_{½}$ (h)	0.33	0.34	1.8	3.3	7.9	0.73	1.0	7.0

[a] Pharmacokinetic parameters calculated using non compartment analysis. F: bioavailability, CL: clearance, AUC: area under the plasma concentration-time curve, C_{max}: maximal observed plasma concentration, t_{max}: time of C_{max}, $t_{½}$: half-life.