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Carrier-free Gene Silencing by Amphiphilic Nucleic Acid Conjugates in Differentiated Intestinal Cells

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Nucleic acid therapy can be beneficial for the local treatment of gastrointestinal diseases that currently lack appropriate treatments. Indeed, several oligonucleotides (ONs) are currently progressing through clinical trials as potential treatments for inflammatory bowel diseases. However, due to low uptake of carrier-free ONs by mucosal cells, strategies aimed at increasing the potency of orally administered ONs would be highly desirable. In this work, we explored the silencing properties of chemically modified and highly resistant ONs derivatized with hydrophobic alkyl chain on intestinal epithelial cells. We screened a set of lipid-ON conjugates for the silencing of model Bcl-2 mRNA and selected 2′-deoxy-2′-fluoro-arabinonucleic acid modified ON bearing docosanoyl moiety (L-FANA) as the most potent candidate with lowest toxicity. The efficacy of L-FANA conjugate was preserved in simulated intestinal fluids and in the inverted transfection setup. Importantly, L-FANA conjugate was able to downregulate target gene expression at both mRNA and protein levels in a difficult-to-transfect polarized epithelial cell monolayer in the absence of delivery devices and membrane disturbing agents. These findings indicate that lipid-ON conjugates could be promising therapeutics for the treatment of intestinal diseases as well as a valuable tool for the discovery of new therapeutic targets.

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Introduction

Nucleic acids are highly attractive class of therapeutics due to their potential to regulate any selected gene of interest. Given their capacity to modulate conventionally undruggable targets, oligonucleotides (ONs) have been extensively investigated as potential therapeutics to treat cancer, viral infections, genetic diseases, and immunological disorders.[1,2] Despite the clear therapeutic potential of ONs, their poor permeability across cellular membranes (due to their intrinsic polyanionic nature and high molecular weight) and susceptibility to degradation by ubiquitous nucleases hamper their clinical translation.[1] Recent advances in the development of various delivery vehicles (*e.g.*, polymer-, lipid-, peptide-, nano/microparticles-, or viral-based) have helped overcoming some of the ON delivery problems; however, issues such as systemic toxicity, low concentration at target sites and pharmaceutical complexity of the delivery systems still represent obstacles to the clinical translation of ON therapeutics.[3,4] The conception of stable ONs with enhanced affinity via various chemical modifications is one of the most remarkable achievements in this field. For example, the combination of phosphorothioate (PS) backbone modification with 2′-*O*-methyl (OMe) and 2′-*O*-(2-methoxyethyl) (MOE) moieties in the sugar units or bicyclic ribonucleosides are the most widely used chemical strategies under clinical investigation.[5–7] Another approach is modification with 2′-deoxy-2′-fluoro-arabinonucleic acid (FANA), which upon binding to the target mRNA induces its RNase H-mediated degradation.[8,9] Several chemically stabilized ONs are already marketed (*e.g.*, mipomersen for homozygous familial hypercholesterolemia[7]) or in late-phase clinical trials.[5]

Nucleic acid therapy could be especially beneficial for several disorders of the gastrointestinal (GI) system that currently lack appropriate treatments such as inflammatory bowel diseases, colon cancer, and familial adenomatous polyposis.[10] The delivery of nucleic acids directly to the GI mucosa is ideal to achieve high local concentrations while minimizing systemic exposure[11] and subsequent side-effects.[12] Indeed, a group of carrier-free ONs targeting GI mucosa for inflammatory bowel disease therapy is progressing through clinical trials, although high doses of ONs are required to obtain the positive therapeutic effects.[10,11,13] Therefore, a safe and efficient delivery approach to the GI mucosa would be desirable to facilitate the cellular uptake of ON and to decrease dosing. Two major strategies are currently investigated to improve the ON delivery efficacy to the intestinal tissue: particle-based systems (*e.g.*, polyplex- or lipoplex-based[2,14]) and single-molecule-based conjugates (*e.g.*, free ON or cell-penetrating peptide-ON conjugates[13,15]). ON incorporated in particles can be taken up primarily by M cells in the Peyer's patches of the intestine and eventually by mucosal macrophages via phagocytosis.[16] Therefore, particle-based systems would be suitable for the targeting of disease-related

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genes expressed mainly in the immune cells, such as TNF-α. In contrast, single-molecule-based system can be taken up not only by lamina propria immune cells but also by epithelial cells.[13] Considering that the dysregulated expression of genes causing intestinal disease is often localized on the gut epithelium,[17–19] single-molecule-based systems could open promising therapeutic avenues for previously inaccessible epithelium-specific targets. In addition, these systems possess the advantage of reduced carrier toxicity and immunogenicity,[20–22] as well as simpler characterization processes, compared with particle-based systems.[21,22]

Recently, we have shown that the conjugation of a long-chain lipid moiety such as docosanoyl (DSA) group to chemically modified ONs enabled significant target mRNA knockdown in a prostate cancer cell line in the absence of transfection reagents.[23] Their binding to serum proteins, however, suggested that amphiphilic ON conjugates might constitute an effective delivery approach primarily for topical applications or liver targeting.[23] However, their applicability to the intestinal mucosa remains questionable since the harsh GI environment containing digestive enzymes coupled with the known difficulties transfecting differentiated epithelium *in vitro* suggests that ON delivery will be limited.[24–32] In this work, DSA was conjugated to various chemically modified ONs targeting a model Bcl-2 mRNA, and the transfecting properties of the resulting conjugates were evaluated *in vitro*. The integrity and transfection efficiency of the most potent conjugate with lowest toxicity were assessed under various conditions mimicking the intestinal environment (inclusion of food-derived fats and digestive enzymes). This conjugate was then further investigated for transfection of differentiated intestinal epithelium.

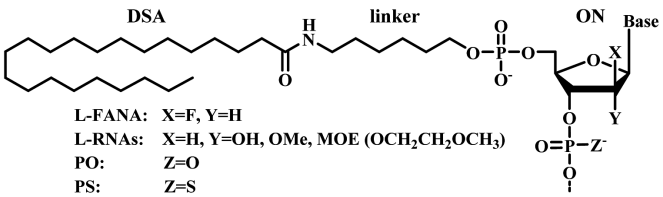
Results

Screening of a set of L-ON conjugates for Bcl-2 mRNA knockdown efficacy

In a previously published study, we reported that lipophilic docosanoic acid (L; DSA) conjugated to ONs modified with 2′-F-arabinonucleosides were more potent than nucleic acids derivatized with cholesterol or docosahexaenoic acid to transfect prostate cancer cells.[23] In a search for novel and safe amphiphilic ONs capable of modulating gene expression in intestinal cells, we synthesized and characterized a set of conjugates by linking DSA to different single-stranded antisense ONs and double-stranded small interfering RNAs (siRNAs) targeting the mRNA of the oncoprotein Bcl-2 (**Table 1**) as previously described.[23] The antisense ONs were based on a single parent DNA sequence (oblimersen[23,33]) and were modified by incorporating OMe, MOE-ribonucleosides, or 2′-F-modified arabinonucleosides connected via PS linkages (L-DNA, L-OMe, L-MOE, and L-FANA, respectively). These chemical modifications of nucleotides are commonly employed to enhance the binding affinity of ONs for target mRNAs as well as to increase their stability against nuclease digestion.[34,35] Typically, antisense ONs' mode of action involves recruitment of RNase H, which recognizes the thus formed DNA:RNA heteroduplex leading to a processive cleavage of the mRNA strand in a catalytic fashion.[33] Since oligoribonucleotides and their 2′-*O*-modified analogues generally do not support RNase H binding, a "gapmer" design, in which a central DNA stretch is flanked by modified ribonucleotides (OMe and MOE) at both termini, is preferred for preserving an RNase H binding site, while providing the ON with higher nuclease resistance.[36,37] In contrast to 2′-*O*-modified ribonucleotides, the FANA modification can be incorporated throughout an ON sequence, since upon binding to complementary mRNA, the FANA nucleotide adopts a conformation similar to that of DNA and therefore supports RNase H recognition.[8,38] Since an "altimer" design showed higher potency compared with "gapmer" design in our previous work,[23] it was chosen for the FANA-modified ON in this study. In order to test whether DSA is capable of facilitating the intracellular delivery of double-stranded siRNAs, it was conjugated to the unmodified phosphodiester siRNA (L-siRNA) and its fully phosphorothioated counterpart (L-PS siRNA).[39,40]

Table 1 The sequences and modification strategies of oligonucleotides

Designation	Sequence[b]	Backbone[c]	Reference
DNA	*5′-TCTCCCAGCGTGCGCCAT-3′	PS	33
OMe	*5′-***TCTC**CCAGCGTGCG***CCAT**-3′	PS	6
MOE	*5′-***TC^{m}TC^{m}**CCAGCGTGCG***C^{m}C^{m}AT*-3′	PS	7
FANA	*5′-TCT**ccc**AGC**gtg**CGC**cat**-3′	PS	9
FANAnc[a]	*5′-CGC**aga**TTA**gaa**ACC**ttt**-3′	PS	38
siRNA	**5′-GCAUGCGGCCUCUGUUUGAUU-3′;* *3′-UUCGUACGCCGGAGACAAACU-5′*	PO	23
PS siRNA	**5′-GCAUGCGGCCUCUGUUUGAUU-3′;* *3′-UUCGUACGCCGGAGACAAACU-5′*	PS	39
siRNAnc	**5′-GUACGACAACCGGGAGAUAUU-3′;* *3′-UUCAUGCUGUUGGCCCUCUAU-5′*	PO	40



[a]nc – negative control. [b]ATGC are DNA, *AUGC* are RNA, ***ATC*** are 2′-*O*-methyl- or 2′-*O*-(2-methoxyethyl)-RNA, **atgc** are 2′-F-ANA. [c]PS – all phosphorothioate linkages and PO – all phosphodiester linkages. C^{m} – 5-methylcytosine, upper sequence of siRNA – sense strand, lower sequence – antisense strand. * – site of DSA conjugation, DSA, docosanoyl; FANA, 2′-deoxy-2′-fluoro-arabinonucleic acid; siRNA, small interfering RNAs; ON, oligonucleotide; OMe, 2′-*O*-methyl; MOE, 2′-*O*-(2-methoxyethyl).

As shown in **Figure 1a**,**b**, both single- and double-stranded L-ONs decreased target mRNA levels in colon carcinoma cells in the absence of transfecting reagent. L-ON conjugates showed the following order of efficacy (based on statistically significant differences at various concentrations): L-FANA > L-OMe > L-siRNA >> L-DNA, where L-DNA did not exhibit a statistically significant reduction. There was no significant difference between the efficacies of L-FANA and L-MOE or L-PS siRNA and L-OMe at any of the concentrations tested. L-MOE, L-PS siRNA, and L-OMe caused a small but significant decrease in cell viability 24 hours after transfection (**Figure 1a**, dot plot). However, the difference in cell viability upon treatment with the different L-ON conjugates was negligible for longer incubation times (2.5 days post-transfection) (see **Supplementary Figure S1**). L-ON conjugates downregulated their target mRNA expression in a dose-dependent

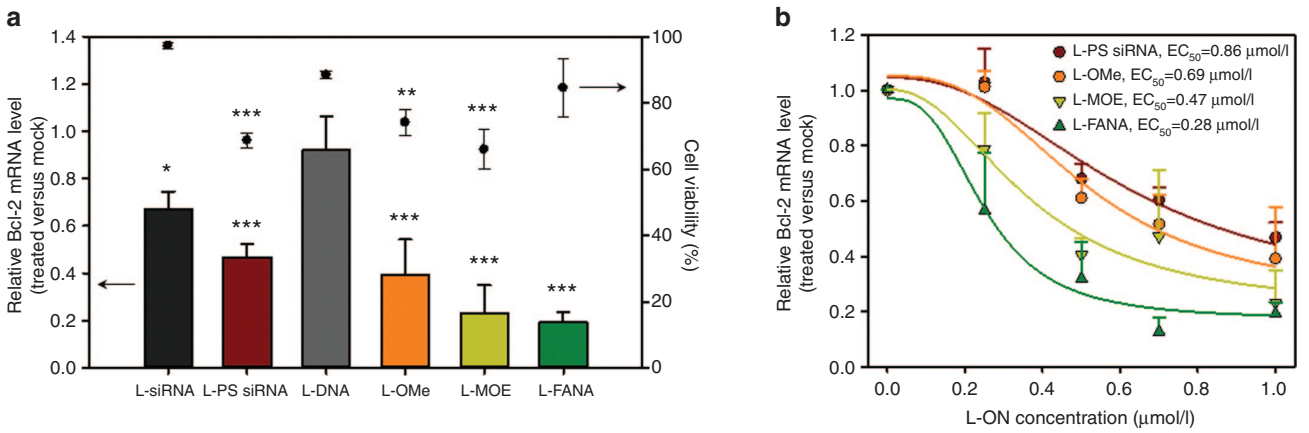


Figure 1 The silencing efficacy and cytotoxicity of L-ON conjugates. (**a**) Bcl-2 mRNA silencing (bar, left y-axis) and viability (closed dot, right y-axis) of HCT-116 cells after transfection with various L-ON conjugates at a concentration of 1 µmol/l. Results are expressed as mean ± SD ($n = 3 – 5$). ***$P < 0.001$, **$P < 0.01$, or *$P < 0.05$ versus medium treated cells. (**b**) Dose-dependent silencing of Bcl-2 mRNA in HCT-116 cells by transfection with L-PS siRNA, L-OMe, L-MOE, and L-FANA conjugates. Results are expressed as mean + SD ($n = 3 – 4$). PS, phosphorothioate; ON, oligonucleotide; OMe, 2′-*O*-methyl; MOE, 2′-*O*-(2-methoxyethyl); FANA, 2′-deoxy-2′-fluoro-arabinonucleic acid; siRNA, small interfering RNAs; SD, standard deviation.

manner, and the EC_{50} values of L-PS siRNA, L-OMe, L-MOE, and L-FANA were 0.86, 0.69, 0.47, and 0.28 µmol/l, respectively (**Figure 1b**). These results indicate that the silencing potency and effect on cell viability of the L-ON conjugate depends on the type of ON used and on its chemical modification. DSA coupled to the unmodified siRNA was less efficient than L-PS siRNA in the absence of transfection reagent. Since L-PS siRNA possessed slightly lower silencing potency compared with L-siRNA upon the LF-mediated transfection (see **Supplementary Figure S2**), the superior silencing properties of carrier-free L-PS siRNA could have been due to its higher resistance to extra- and intracellular nucleases[39] and/or higher uptake of PS-modified oligonucleotides.[41] We selected the L-FANA conjugate as a promising candidate for further investigation based on a combination of lowest EC_{50} with a favorable cytotoxicity profile.

Target Bcl-2 mRNA silencing by L-FANA conjugate

We further investigated the target mRNA silencing effect of L-FANA by transfecting two human colorectal carcinoma cell lines, HCT-116 and Caco-2, with L-FANA and its control groups. At a concentration of 1 µmol/l, L-FANA downregulated Bcl-2 mRNA in HCT-116 and Caco-2 cells by 81 and 82%, respectively (**Figure 2**). Monitoring the Bcl-2 mRNA silencing over time showed that the knockdown efficacy of L-FANA was constant from 1 to 3.5 days post-transfection (see **Supplementary Figure S3**). Transfection of free FANA and negative control conjugate with an irrelevant nucleic acid sequence (L-FANAnc) did not change the Bcl-2 mRNA expression levels, indicating that target mRNA inhibition is caused by a sequence-specific antisense mechanism and that lipid conjugation contributes to the improved intracellular delivery of FANA.

In order to assess whether sedimentation influenced the transfection efficiency, experiments with cells in inverted position were performed (**Figure 3**). Recent studies with nanoparticles demonstrated that cellular uptake can be dramatically reduced in the inverted configuration (cells on

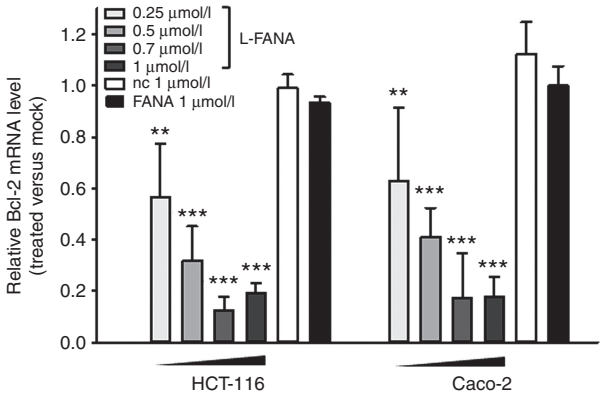


Figure 2 Dose-dependent silencing of Bcl-2 mRNA in HCT-116 and Caco-2 cells by L-FANA conjugate compared with the silencing efficacy of the FANA without lipid and to L-FANAnc with nontargeting sequence (nc). Results are expressed as mean + SD ($n = 3 – 4$). ***$P < 0.001$ or **$P < 0.01$ versus medium treated cells. FANA, 2′-deoxy-2′-fluoro-arabinonucleic acid; SD, standard deviation.

top), as opposed to the conventional *in vitro* setup where cells are on the bottom of the culture plate and sedimentation can artificially promote internalization.[15,40,42] Indeed, we observed that transfection efficiency of siRNA/LF lipoplex was significantly reduced using the inverted setup, whereas the silencing efficiency of L-FANA conjugate was the same in both cell setups. These results indicated that L-FANA was predominantly taken up by cells via sedimentation-independent uptake routes, which was in line with the outcome of our previous work on peptide-nucleic acid conjugates.[15] In the case of the siRNA/LF lipoplex, it is likely that the rapid onset of silencing was a consequence of the fast uptake of sedimented particles (see **Supplementary Figure S4**).[43] This was in contrast to the behavior of L-FANA, where increasing the exposure time to the oligonucleotide conjugate from 5 to 15 hours resulted in significantly higher knockdown efficacy

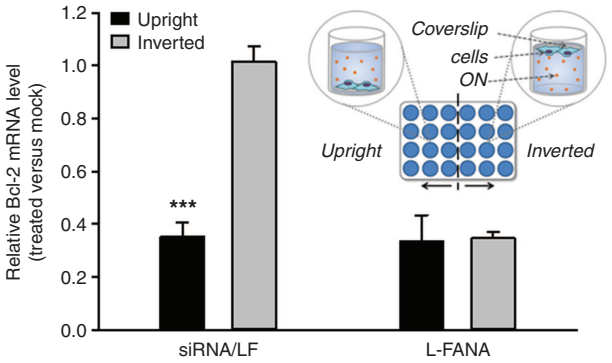


Figure 3 Comparison of transfection efficiency of siRNA/LF lipoplex and L-FANA conjugate at concentrations of 0.05 µmol/l and 1 µmol/l, respectively, in HCT-116 cells with upright and inverted setup. Results are expressed as mean + SD ($n = 3 – 4$). ***$P < 0.001$ versus siRNA/LF in inverted setup. FANA, 2′-deoxy-2′-fluoro-arabinonucleic acid; SD, standard deviation; siRNA, small interfering RNA.

(38 versus 65%, respectively, at 0.5 µmol/l; see **Supplementary Figure S4**), further supporting a presumed sedimentation-independent uptake.

In vitro evaluation of L-FANA silencing properties for intestinal nucleic acid delivery

An efficient gene silencing in intestinal epithelium first requires that the nucleic acid drugs be stable against the attack of intestinal enzymes. To this end, we tested whether the preincubation of L-FANA conjugate in simulated intestinal fluid (SIF) containing pancreatic digestive enzymes would impair its transfection efficacy (**Figure 4**). Importantly, L-FANA conjugate was stable even after 15 hours of incubation with SIF, as assessed by polyacrylamide gel electrophoresis analysis (**Figure 4a**), and the lipid moiety was not cleaved off upon incubation with pancreatic enzymes containing lipases (**Figure 4b**). For the silencing experiments, L-FANA and its negative control (L-FANAnc) conjugates were incubated with pancreatic enzymes at pH 6.8 for 2 hours at 37°C, and after the heat-inactivation of the digestive enzymes and dilution with cell culture medium, the mixture was directly added to the HCT-116 cells. The difference between relative expression levels of target Bcl-2 mRNA after the treatment with L-FANA with or without pancreatin preincubation was not statistically significant (31 and 19%, respectively; **Figure 4c**). This result shows that the preincubation with pancreatin did not strongly affect the silencing efficacy of the L-FANA conjugate due to the enzymatic degradation or unspecific protein binding. Treatments with pancreatin alone or pancreatin with L-FANAnc did not change the target mRNA expression, indicating that the silencing effect was caused by L-FANA in a sequence-specific manner.

It was previously demonstrated that amphiphilic ON conjugates can bind to lipid-containing particles (*e.g.*, low-density lipoprotein or chylomicrons[44,45]), implying the possibility of interaction of L-ON conjugates with food-derived fats in the intestine. Using a gel-based binding assay, we observed that L-FANA indeed bound to soybean oil in a concentration-dependent manner, while the migration of unconjugated FANA was not retarded in the presence of oil (**Figure 4a** and **Supplementary Figure S5**). The decrease of silencing efficacy observed for L-FANA in the presence of oil emulsion could be attributed to the interaction between the oil and the lipid moiety of the conjugate interfering with the cellular uptake (**Figure 4c**). However, the interaction of L-FANA with the oil emulsion could be disrupted after the digestion of the oil by the SIF (**Figure 4a**). Importantly, the released L-FANA from SIF-digested oil emulsion fully preserved its silencing capacity (**Figure 4c**). This finding suggests that the interaction of amphiphilic ONs with food-derived fat would not hamper their cellular uptake and subsequent silencing activity.

Intestinal epithelial cells which are differentiated have reduced proliferative and endocytic capacity which makes their *in vivo* transfection rather challenging.[46–50] To compare the delivery of modified ON into proliferating cells and difficult-to-transfect epithelial cell monolayers, the L-FANA conjugate was tested on differentiated Caco-2 cells. To illustrate the resistance to transfection of fully differentiated Caco-2 cell monolayers, experiments were performed with Bcl-2 targeting siRNA complexed with LF (**Figure 5a**,**b**). Transfection of siRNA/LF at a concentration of 0.2 µmol/l elicited a small decrease of Bcl-2 mRNA expression (21%, **Figure 5a**), which was not accompanied by its downregulation at the protein level (**Figure 5b**). We could not achieve dose-dependent Bcl-2 mRNA knockdown even with siRNA concentrations as high as 0.4 µmol/l, at which the control siRNAnc possessing a target-unrelated sequence caused appreciable suppression of Bcl-2 mRNA, probably due to the toxicity caused by high concentrations of LF.[28] In contrast, Bcl-2 silencing was successfully achieved in proliferating Caco-2 cells at a dose of 0.05 µmol/l of siRNA (see **Supplementary Figure S6**). Strikingly, the single-molecule-based L-FANA conjugate was able to knockdown Bcl-2 mRNA in differentiated Caco-2 cell monolayers in a sequence-specific and dose-dependent manner (**Figure 5a**). At L-FANA concentrations of 1.25, 2.5, and 5 µmol/l the target Bcl-2 mRNA expression was reduced to 56, 32, and 26%, respectively. At 5 µmol/l the negative control L-FANAnc was inactive. Conjugation of DSA was found to be essential for successful delivery, as unconjugated FANA did not have any effect on Bcl-2 mRNA expression even at 5 µmol/l. Consistent with mRNA silencing, western blot analysis demonstrated that L-FANA reduced efficiently the target Bcl-2 protein level (by 53% at 2.5 µmol/l; **Figure 5b**). Importantly, the treatment with relatively high dose of L-FANA neither caused cytotoxicity (**Figure 5c**) nor decreased the transepithelial electrical resistance (TEER) of Caco-2 monolayers (**Figure 5d**), which is a measurable indicator of the monolayer integrity.[51] In comparison, the treatment with sodium decanoate, a widely used absorbefacient disrupting the tight junctions,[10] caused a pronounced drop in TEER. Based on these results, it is likely that L-FANA conjugates will be taken up primarily by epithelial cells *in vivo* with minimal systemic translocation via the paracellular route.

Discussion

Intestinal delivery of nucleic acid drugs is a promising therapeutic strategy for treatment of several diseases but faces numerous challenges associated with the complexity of the

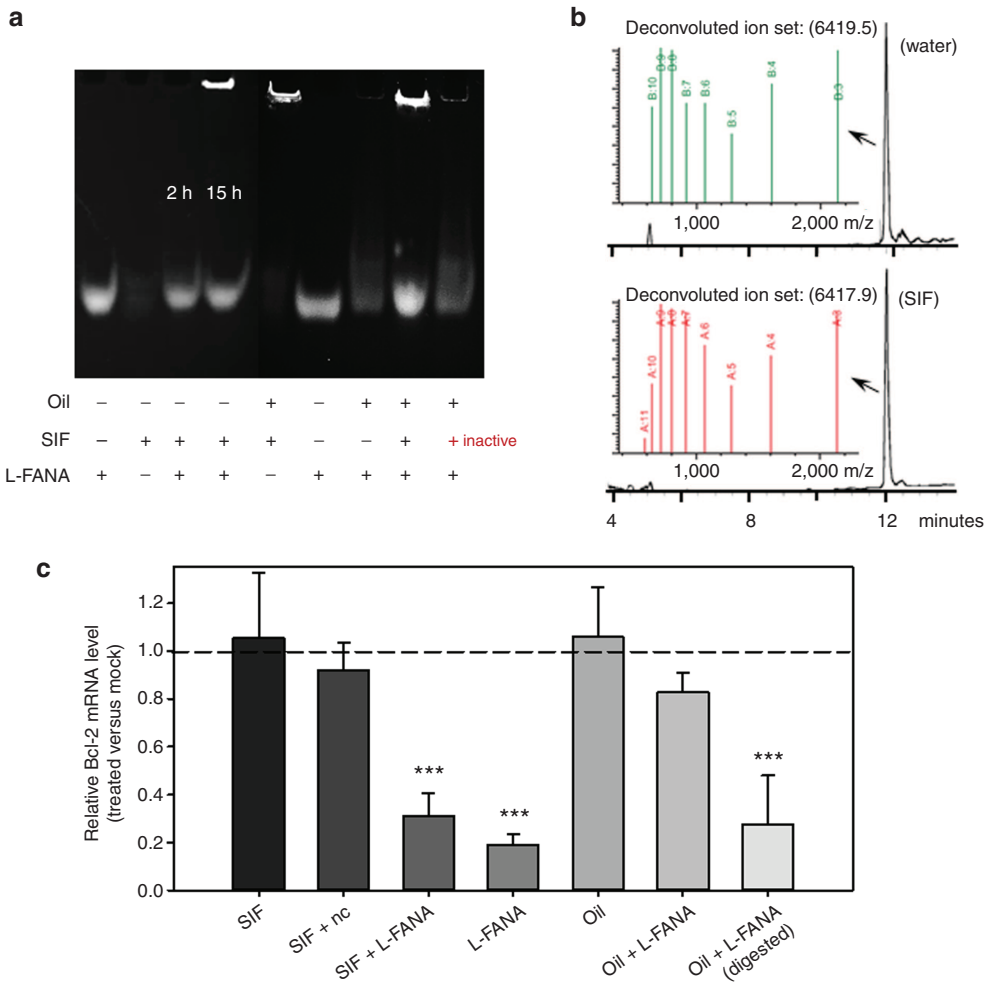


Figure 4 The stability and silencing properties of L-FANA in SIF (pancreatin 1X USP) and 0.5% soybean oil emulsion (Oil). (**a**) Polyacrylamide gel electrophoresis images of L-FANA conjugate after incubation with SIF for 2 hours and 15 hours, or with a mixture of soybean oil and SIF at 37°C for 2 hours. $+_{inactive}$ – SIF inactivated at 95°C for 15 minutes before incubation with L-FANA and oil emulsion. Bands at the top of the gels could correspond to the unspecific staining of insoluble material from pancreatin. (**b**) Results of LC-MS analysis of L-FANAnc conjugate after 2 hours incubation in water or with SIF. L-FANAnc conjugate: retention time 12 minutes; mass 6,417.9 and 6,419.5 Da in water and SIF, respectively (theoretical 6,419.3 Da). (**c**) Target Bcl-2 mRNA knockdown efficiency after the transfection of HCT-116 cells with L-FANA preincubated with SIF for 2 hours or with soybean oil in Opti-MEM for 30 minutes. Oil + L-FANA (digested) – released L-FANA after preincubation with oil emulsion and SIF-mediated digestion; nc – L-FANAnc conjugate with nonspecific sequence. Results are expressed as mean + SD ($n = 3 – 4$). ***$P < 0.001$ versus Opti-MEM medium treated cells. FANA, 2′-deoxy-2′-fluoro-arabinonucleic acid; SIF, simulated intestinal fluid; SD, standard deviation; LC-MS, liquid chromatography-mass spectroscopy.

GI environment. To identify a potent delivery platform, a set of L-ON conjugates was prepared using chemically modified antisense ONs and siRNAs. Although all the L-ON conjugates carried the same DSA lipid moiety, they induced different degrees of cytotoxicity. This could be attributed to the modification-dependent protein binding of ONs[52,53] or Bcl-2-level-dependent cell death.[33] Superior silencing efficacy of L-FANA and L-MOE conjugates compared to those of L-DNA and L-OMe could result from a combination of factors including higher nuclease resistance and higher affinity for the complementary mRNA.[35,54,55]

A fully phosphorothioated L-PS siRNA conjugate showed significant Bcl-2 silencing upon carrier-free transfection, while PS siRNA without a DSA group did not (see **Supplementary Figure S2**), suggesting that the uptake of L-PS siRNA was mediated by the lipid moiety. A phenomenon called gymnosis was recently reported in which target genes are sequence-specifically suppressed in cells by chemically modified ONs, such as locked nucleic acids and FANA, in the absence of transfection reagents or delivery moiety conjugation. For an efficient and nontoxic gymnotic transfection, prolonged exposure of proliferating cells (6 – 10 days) to high (μmol/l) concentrations of ONs is required.[38,56] Considering that the average transit times in the small and large intestines of healthy humans are 3 hours and 27 hours, respectively (as reviewed in refs. 57,58), gymnotic ON delivery is likely to be too slow for intestinal tissues, at least in the absence of frequent dosing. Moreover, the renewal cycle of intestinal epithelial cells lasts barely 3 – 5 days,[49] and therefore an intestinal ON therapy needs an efficient delivery method to facilitate the target mRNA and protein knockdown. In this study, DSA conjugation to ONs promoted the delivery of

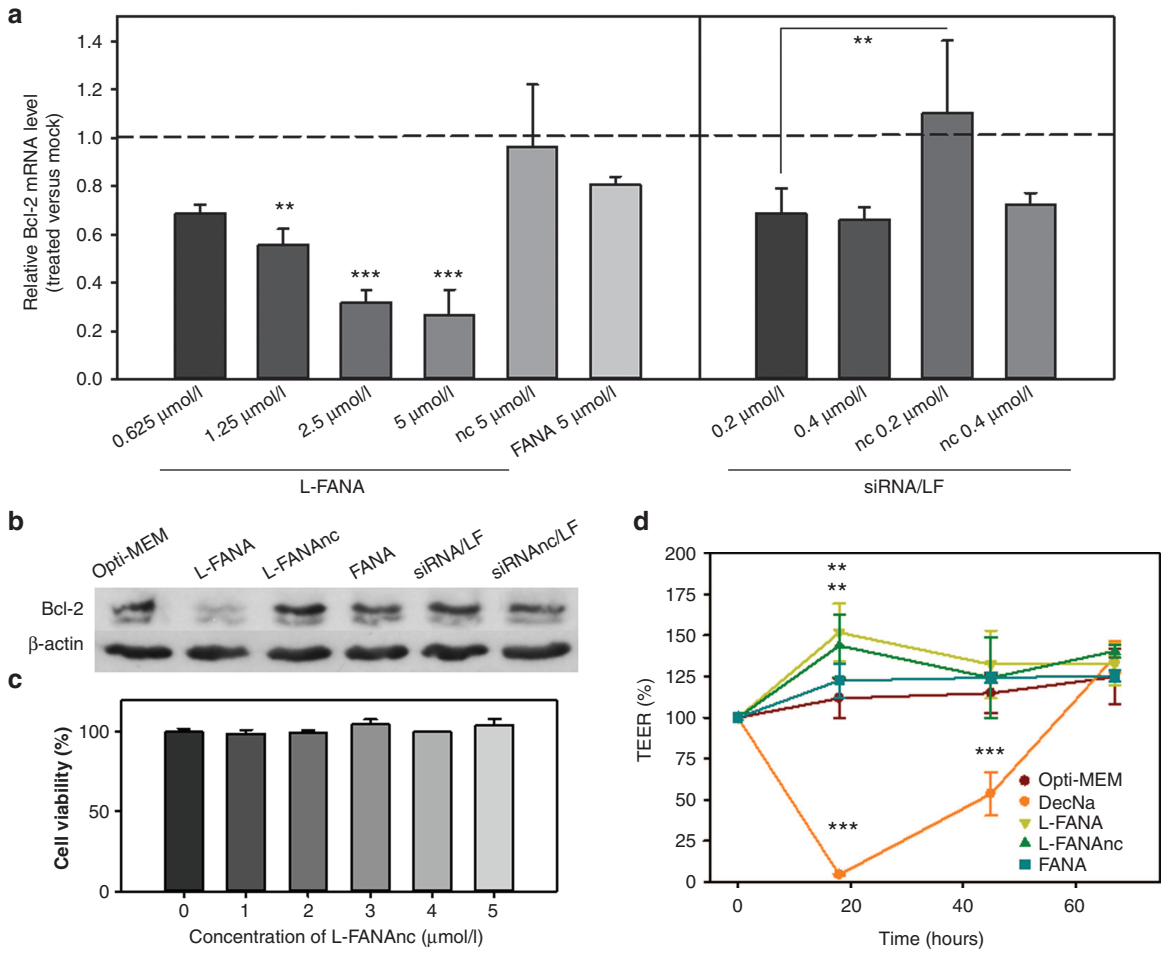


Figure 5 ***In vitro*** **evaluation of L-FANA silencing properties for differentiated intestinal monolayer delivery.** (**a**) Target Bcl-2 mRNA knockdown efficiency by transfection of differentiated Caco-2 cell monolayers with L-FANA and siRNA/LF lipoplex in Opti-MEM medium, nc –corresponding negative controls L-FANAnc and siRNAnc with nonspecific sequences. Results are expressed as mean + SD ($n = 3 – 5$). (**b**) Western blot assay of Bcl-2 and β-actin protein expression after transfection of differentiated Caco-2 cell monolayers with L-FANA (2.5 µmol/l), siRNA/LF (0.2 µmol/l), and their control groups in Opti-MEM medium. (**c**) Viability of differentiated Caco-2 monolayer cells after treatment with 1 – 5 µmol/l of L-FANAnc in Opti-MEM medium. Results are expressed as mean + SD ($n = 3$). (**d**) TEER change upon overnight treatment (15 hours) with FANA derivatives (5 µmol/l) or permeation enhancer sodium decanoate (DecNa; 4 mmol/l) in Opti-MEM medium. Results are expressed as mean ± SD ($n = 3 – 4$). ***$P < 0.001$, ** $P \leq 0.01$ versus Opti-MEM medium treated cells, ** $P < 0.01$ between two treatment groups. FANA, 2′-deoxy-2′-fluoro-arabinonucleic acid; SD, standard deviation; siRNA, small interfering RNAs.

single-stranded antisense ONs and even double-stranded siRNAs during limited exposure times. Several groups have reported that the conjugation of neutral lipids, such as cholesterol and aliphatic fatty acids, to ONs improves their uptake and silencing efficacy, both *in vitro* and *in vivo*.[23,44,59] However, the mechanism of cellular uptake of amphiphilic ONs and their trafficking remain poorly understood and appear to be system-dependent.[60–62]

For a successful *in vivo* delivery of ON to intestinal epithelium, L-ON conjugates must remain functional in the intestinal environment, and they should be able to silence target genes in difficult-to-transfect epithelial cell monolayers. One concern regarding their biological activity in the intestine was that amphiphilic ON conjugates might bind to intestinal lipophilic content, potentially leading to reduced activity. Here, in a model system, we demonstrated that free L-FANA is released from oil phase after digestion by pancreatic enzymes and retains its silencing ability. The combination of the extreme stability of FANA-modified ONs toward nucleases[35] and hydrolysis at low pH[63], together with the high stability of DSA under similar conditions found in the GI tract[64] makes DSA-FANA conjugates particularly suitable for GI applications.

Differentiated intestinal epithelial cells featuring microvilli and expressing tight junctions represent a difficult target for nucleic acid delivery.[24–31] Their reduced proliferation and endocytosis rate in comparison to undifferentiated cells are largely responsible for poor transfection efficacy.[48,50] Regarding particle-based delivery systems, the resistance to transfection observed with differentiated cells may be partially attributed to the structure of their apical membrane featuring microvilli, which limits access to the absorptive membrane.[31,32] Several studies have described a variety of attempts to overcome this barrier by, for example, pretreatment with membrane-disturbing agents,[31] transfection of proliferating Caco-2 in the suspension state followed by accelerated differentiation,[25]

formulation with small lipid nanoparticles,[32] use of β_1-integrin-mediated endocytosis[65] or electroporation.[24] As previously reported for siRNA/LF lipoplexes, we were unable to efficiently downregulate target Bcl-2 gene in polarized Caco-2 cell monolayers. In contrast, the single-molecule-based L-FANA conjugate was able to effectively silence the Bcl-2 gene expression at both mRNA and protein levels in differentiated monolayers. Importantly, despite lower potency of carrier-free amphiphilic conjugates in comparison to siRNA/LF lipoplexes in classical *in vitro* assays based on rapidly proliferating cancer cells, they by far outperform particulate delivery vehicles under more stringent conditions, such as the inverted setup or using differentiated cells. The involvement of cell surface receptors, such as fatty acid receptors, better access of L-FANA to the adsorptive cell membrane due to the smaller size, or other uptake routes different from LF-mediated endocytosis may explain the efficient target silencing by L-FANA conjugate in a nondividing polarized epithelium. Although several studies have extrapolated the possible internalization routes of amphiphilic ON conjugates in proliferating cells,[60,61] it is possible that the uptake mechanism and intracellular trafficking can vary among different cell types and states of cell differentiation.[29,60,66]

The present report demonstrates that amphiphilic ON conjugates can be efficiently delivered to colon carcinoma cells in the absence of delivery agents, causing target gene silencing. Conjugation of DSA and chemical modification of the ON backbone were essential for efficient delivery and enzymatic resistance, respectively. Target mRNA knockdown was not significantly altered in simulated intestinal environment containing pancreatic enzymes. The most encouraging finding was that an L-ON conjugate was able to silence Bcl-2 mRNA and protein in fully differentiated Caco-2 cell monolayers. This well-defined single-molecule-based approach proved to be superior to conventionally employed lipoplexes, which were ineffective for transfecting the epithelium. To the best of our knowledge, this is the first study showing the efficient gene silencing in differentiated epithelial cells by an oligonucleotide conjugate in the absence of complexation, delivery device, or membrane-disturbing agents. We believe that L-FANA represents a suitable approach to further improve the efficacy of ON enteral treatments currently under clinical investigation. In addition, L-FANA would also provide a carrier-free tool for molecular biologists studying gene functions in differentiated intestinal epithelium. Future work will focus on evaluation of the proposed delivery platform in intestinal disease relevant targets *in vivo*.

Materials and methods

Materials. HCT-116 cell line was kindly provided by Prof. Azzalin's group at ETH Zurich and Caco-2 cells were purchased from ATCC (Manassas, VA). Dulbecco's modified essential medium (DMEM) with GlutaMAX, Opti-MEM medium, fetal bovine serum (FBS), nonessential amino acids, penicillin-streptomycin solution, trypsin, Lipofectamine 2000 (LF), phosphate-buffered saline (PBS; 1 mmol/l KH_2PO_4, 3 mmol/l Na_2HPO_4, 155 mmol/l NaCl, pH 7.4), and RNase-free distilled water were obtained from Invitrogen (Carlsbad, CA). Porcine pancreatin (4×USP), ammonium persulfate, triethylammonium acetate (TEAA) buffer 1 mol/l, Intralipid (20% w/v of soybean oil, 1.2% of egg yolk phospholipids, 2.25% of glycerol, pH 6–8.9; mimic of high-fat meal), chloroform ($CHCl_3$), Triton X-100, NaF, Na_3VO_4, Tris, and skim milk powder were obtained from Sigma-Aldrich (Buchs, Switzerland). Sodium decanoate was purchased from TCI (Tokyo, Japan). Duplex annealing buffer (100 mmol/l potassium acetate, 30 mmol/l HEPES, pH 7.5) was purchased from Integrated DNA Technologies (IDT, Coralville, IA). Potassium dihydrogen phosphate was obtained from Merck (Kenilworth, NJ). Methanol (MeOH), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Acros Organics (Geel, Belgium). Hexafluoroisopropanol (HFIP) was obtained from Fluorochem (Hadfield, UK). Ethylenediaminetetraacetic acid (EDTA) and polysorbate 20 were purchased from AppliChem (Darmstadt, Germany). Gel Red nucleic acid gel stain was obtained from Biotium (Hayward, CA). MycoAlert PLUS Mycoplasma Detection Kit was purchased from Lonza (Basel, Switzerland). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Rockville, MD). Thermanox coverslips, DNA loading dye, NaCl, and micro BCA protein assay kit were purchased from Thermo Fisher Scientific (Waltham, MA). Transwell inserts were obtained from Corning (Corning, NY). Low-binding microcentrifugation tubes (DNA Lobind) were purchased from Eppendorf-Vaudaux (Schönenbuch, Switzerland). RNeasy Mini kit and specific primers for α-splicing variant of human Bcl-2 mRNA (Hs_BCL2_1_SG; QT00025011) and human β-actin (Hs_ACTB_2_SG; QT01680476) were obtained from Qiagen (Valencia, CA). High-capacity cDNA reverse transcription kit and Power SYBR Green polymerase chain reaction (PCR) Master Mix were purchased from Applied Biosystems (Foster City, CA). Complete EDTA-free protease inhibitors' cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Poly(vinylidene difluoride) membranes (PVDF) were obtained from Bio-Rad Laboratories (Hercules, CA). Mouse antihuman Bcl-2 monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody were purchased from Dako (Glostrup, Denmark). Rabbit anti-β-actin polyclonal antibody and HRP-conjugated goat antirabbit IgG polyclonal antibody were obtained from Abcam (Cambridge, UK). ImmunoCruz Western blotting luminol reagent was purchased from Santa Cruz Biotechnology (Dallas, TX). Super RX X-Ray films were obtained from Fujifilm (Tokyo, Japan).

Synthesis of oligonucleotides and their derivatives. Unmodified Bcl-2 targeting siRNA[23] and negative control siRNAnc with nontargeting sequence[40] were synthesized by Bioneer (Daejeon, South Korea). Unmodified antisense strand of siRNA was obtained from Microsynth (Balgach, Switzerland). All ONs were synthesized according to the standard protocol for automated phosphoramidite solid-phase synthesis except for L-OMe conjugate, which was provided by Microsynth. DSA was conjugated via an aminohexanol-linker to the 5′-end of ONs in line with previously described method.[23] All L-ON conjugates were purified by reverse-phase HPLC, analyzed by LC-MS, and quantified via UV spectrophotometry (NanoPhotometer P 330, Implen, Germany). Details of synthetic procedure and analytical data

are presented in the supporting information. The ONs' molar extinction coefficients at 260 nm were calculated using the software of IDT website (OligoAnalyzer tool, www.idtdna.com/calc/analyzer). FANA and MOE extinction coefficients were calculated using DNA and OMe values, respectively. These modifications were assumed to have negligible effect on the extinction coefficients, as previously described.[38,67] The complementary single strands of siRNAs were combined in duplex annealing buffer at a concentration of 50 µmol/l each, heated up to 95°C for 1 minute, and cooled slowly to 4°C overnight to ensure proper annealing. Various ONs and L-ON conjugates were dissolved in RNase-free deionized water at a concentration of 100 µmol/l and stored at –20°C.

Cell culture. HCT-116 cells were maintained in DMEM medium supplemented with GlutaMAX containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5%-CO_2 humidified atmosphere. The cells were seeded in a 24-multiwell plate at a density of 4×10^4 cells/well. For the inverted transfection, the cells were seeded on Thermanox coverslips in a 24-multiwell plate at a density of 1×10^5 cells/well. The cells were incubated for 1 day before experiments. Caco-2 cells were maintained in DMEM medium supplemented with GlutaMAX containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1% of nonessential amino acids at 37°C in a 5%-CO_2 humidified atmosphere. Cells with passage number between 58 and 77 were seeded in a 12-multiwell plate at a density of 7×10^4 cells/well. For the differentiated Caco-2 cell monolayer transfection, the cells were seeded in Transwell inserts with polyester membrane with pore size of 0.4 µm in a 12-multiwell plate at a density of 1.12×10^5 cells/well as previously described.[68] The medium was exchanged every other day, and cells between 13 and 17 days of differentiation were used for the experiments. The differentiation of monolayers was monitored by measuring TEER using an EVOM epithelial voltmeter with STX2 electrode (World Precision Instruments, Sarasota, FL).

All the monolayers achieved TEER higher than 1,000 Ωcm^2 after the 2 weeks of culturing, indicating the completion of differentiation process. TEER values of individual wells measured just before the transfection with ONs were set to 100% and their change was monitored for the three following days.

All experiments were performed on mycoplasma-free cell lines (regularly checked by MycoAlert PLUS Mycoplasma Detection Kit), and only cells in the exponential phase of growth were used for seeding.

Cytotoxicity assay. To compare the cytotoxicities of different L-ON conjugates, HCT-116 cells were seeded in a 96-multiwell plate at a density of 7×10^3 cells/well the day before the experiment. Alternatively, Caco-2 cells were seeded in a 96-multiwell plate, and the culture medium was exchanged every other day for 2 weeks to obtain differentiated monolayers. The cells were treated with various concentrations of L-ON conjugates in 50 µl of serum-deficient Opti-MEM medium for 15 hours. The Opti-MEM medium containing no L-ON was used as a control. The medium was exchanged for 100 µl of DMEM supplemented with 10% FBS, and the cells were further incubated for 24 hours, after which the cell viability was assessed using tetrazolium-based CCK-8 reagent following the manufacturer's instructions.

Screening of Bcl-2 mRNA silencing efficiencies of various L-ONs. To assess the silencing efficiency of various L-ON conjugates, the conjugates at various concentrations (0.25 – 1 µmol/l) were incubated with HCT-116 and Caco-2 cells. Given the absence of intact serum in the intestinal environment, all transfection experiments were performed in serum deficient Opti-MEM medium. The Opti-MEM medium containing no ONs was used as a control. Following overnight incubation (15 hours), the transfection medium was exchanged with fresh DMEM supplemented with 10% FBS. After 2.5 days of further incubation cells were washed with PBS, and total RNA was isolated using RNeasy Mini kit (Abs_{260} /Abs_{230} >1.8) according to the previously optimized method.[23] The expression levels of Bcl-2 mRNA relative to the internal control β-actin mRNA were quantified by two-step quantitative real-time PCR.[23] Briefly, cDNA was synthesized from 1.2 µg of total mRNA using high-capacity cDNA reverse transcription kit according to the manufacturer's instructions. Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix and specific primers for human Bcl-2 and β-actin on a 7900HT Fast Real Time PCR instrument (Applied Biosystems) according to the manufacturer's instructions. Briefly, the reaction mixtures were incubated at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of denaturation (95°C for 15 seconds) and extension/detection (60°C for 1 minute). Relative mRNA expression levels were calculated using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method (the fluorescence threshold was set to 0.4). Results are expressed as the Bcl-2 mRNA level change between ON-treated and ON-free medium treated cells. L-ON potency (effective concentration causing 50% of target mRNA silencing, EC_{50}) was calculated using a four-parameter logistic function to fit the dose-response data via SigmaPlot 13.0 software.

Comparison of transfection efficiencies in upright and inverted transfection. The transfection medium consisted of 250 µl of Opti-MEM containing either 1 µmol/l of carrier-free Bcl-2 targeting L-FANA conjugate or 0.05 µmol/l of Bcl-2 specific siRNA complexed with LF according to the manufacturer's instructions as a particle-mediated delivery control. HCT-116 cells grown on the coverslips were washed with Opti-MEM, and the coverslips were transferred using tweezers into a new 24-multiwell plate for the transfection. For the upright transfection, the cells were placed on the bottom of the multiwell plate followed by the addition of the transfection medium. For the inverted transfection, the transfection medium was added to the empty well, and the cells grown on coverslips were carefully deposited upside down onto the surface of the medium. The coverslips floated on the surface of the medium due to the surface tension of the medium. After overnight incubation (15 hours), the cells were transferred to a new plate and further cultured for 2.5 days in 500 µl of DMEM supplemented with 10% FBS. Subsequently, total RNA was isolated and gene expression levels of Bcl-2 mRNA were assessed as described above.

L-FANA conjugates stability in SIF and 0.5% soybean oil emulsion. To investigate the influence of digestive enzymes and food-derived fats present in intestine on the biological function of L-FANA, its stability and efficacy were tested in simulated intestinal environment containing pancreatic enzymes and lipids. For polyacrylamide gel electrophoresis analysis, 1 µl of L-FANA (100 µmol/l) was mixed with 9 µl of USP SIF (2.5 g/l porcine pancreatin (4 × USP), 50 mmol/l KH_2PO_4, pH 6.8) or water and incubated for 2 hours or 15 hours at 37°C. Samples were kept at 95°C for 15 minutes to heat-inactivate the enzymes and mixed with 1 µl of DNA loading dye. Alternatively, 1 µl of L-FANA was premixed with 1 µl of 5% soybean oil emulsion in Opti-MEM (prepared by diluting 20% Intralipid with Opti-MEM) for 30 minutes followed by incubation at 37°C for 2 hours with 8 µl of active/heat-inactivated SIF or water. Samples were then loaded onto 20% (w/v) polyacrylamide gel prepared in a Tris-acetate-EDTA buffer (TAE: 40 mmol/l Tris-acetate, 1 mmol/l EDTA, pH 8.0) by free-radical polymerization with ammonium persulfate/TEMED as an initiator. The gel was then immersed in TAE buffer and electrophoresed at constant voltage of 150 V for 60 min. L-FANA was revealed following manufacture's protocol for Gel Red nucleic acid gel stain, and fluorescence was recorded on a ChemiDoc XRS (Bio-Rad Laboratories).

For the LC-MS analysis, 2 µl of L-FANA (100 µmol/l) were mixed with 18 µl of SIF or water and incubated for 2 hours at 37°C, then at 95°C for 15 minutes, and centrifuged at 14000 × *g* at room temperature for 10 minutes. The supernatants were mixed with 50 µl of $CHCl_3$, and shaken to extract possible hydrophobic contaminants from SIF. After incubating the mixtures at room temperature for a few minutes, water layers were collected and stored at –20°C until analysis. Analytical liquid chromatography-mass spectrometry (LC-MS) was carried out on Agilent LC-MS using a reverse phase column (Waters Acquity OST C18, 2.1 x 50 mm, 1.7 µm) with solvent A being 400 mmol/l HFIP and 15 mmol/l TEAA in water and solvent B being MeOH. The flow was set at 0.3 ml/min, and a gradient was run at 65°C from 5 to 90% B in 14 minutes.

Transfection of L-FANA preincubated with SIF and 0.5% soybean oil emulsion. The influence of preincubation of L-FANA conjugates with SIF on the transfection efficacy was investigated. HCT-116 cells were seeded one day prior to the experiment in a 24-multiwell plate at a density of 4×10^4 cells/well in DMEM containing 10% FBS. Two and a half microliters of L-FANA were pre-incubated with 22.5 µl of SIF for 2 hours at 37°C followed by heat-inactivation of enzymes for 15 minutes at 95°C. The mixtures were diluted ten times with Opti-MEM to a final L-FANA concentration of 1 µmol/l and incubated with cells overnight (15 hours). ON-free Opti-MEM medium was used as a control. Subsequently, the transfection medium was changed to DMEM supplemented with 10% FBS. After 2.5 days of further incubation, total RNA was isolated, and gene expression levels of Bcl-2 mRNA were assessed as described above. To study the silencing activity of L-FANA released from SIF-digested oil emulsion, 2.5 µl of L-FANA (100 µmol/l) were preincubated with 6.25 µl of 20% Intralipid for 30 minutes at room temperature followed by incubation with 25 µl of SIF for 2 hours at 37°C. The released L-FANA was separated from cytotoxic oil digestion products by preparative polyacrylamide gel electrophoresis before transfection experiments. Gels were prepared and electrophoresed as described above. After the electrophoresis, the L-FANA band was excised, weighed, and immersed in 250 µl of Opti-MEM. For L-FANA extraction, samples containing gel pieces were flash-frozen in liquid nitrogen, heated at 90°C for 15 minutes, and agitated at room temperature for 3 hours. The concentration of extracted L-FANA in the supernatant was 0.6 µmol/l. The supernatants were incubated with cells overnight (15 hours). The expression levels of Bcl-2 mRNA were assessed as described above.

Transfection of differentiated Caco-2 cell monolayers. The differentiated Caco-2 cell monolayers grown in Transwell inserts for 2 weeks were washed with Opti-MEM medium from apical and basal sides. The 500 µl of Opti-MEM medium containing either naked ON derivative (0.625 – 5 µmol/l) or siRNA (0.2 – 0.4 µmol/l) complexed with LF according to the manufacturer's instructions was added to the apical compartment. The basal chamber was filled with 1.5 ml of Opti-MEM medium. The Opti-MEM medium in both compartments was used as a control. Following the overnight incubation (15 hours), the transfection medium was exchanged with fresh DMEM supplemented with 10% FBS in both compartments. For Bcl-2 mRNA expression analysis by qRT-PCR, cells were lysed 2.5 days after transfection as described above. For Bcl-2 protein expression analysis by western blot, cell monolayers were lysed 4 days after transfection in 25 µl of lysis buffer (20 mmol/l Tris–HCl pH 7.7, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% v/v Triton X-100, 25 mmol/l NaF, 1 mmol/l PMSF, 1 mmol/l Na_3VO_4 supplemented with Complete protease inhibitors) in line with previously described method.[40] Briefly, cell lysates were scraped from the Transwells, centrifuged at 10,000 × *g* for 15 minutes at 4°C to remove cell debris, and protein concentration in the supernatants was determined by the micro BCA assay according to the manufacturer's instructions. 50 µg of total protein per sample were resolved on 12% SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was washed once with TBS-T buffer (20 mmol/l Tris–HCl pH 7.7, 150 mmol/l NaCl, 0.1% v/v polysorbate 20) and blocked with TBS-T containing 5% w/v skim milk (blocking buffer) for 1 hour. The membrane was cut in two at 35 kDa; the lower part containing Bcl-2 (26 kDa) was incubated with anti-Bcl-2 antibody diluted to 1:100 in blocking buffer, and the upper part containing β-actin (42 kDa, loading control) was incubated with anti-β-actin antibody diluted to 1:4,000 overnight at 4°C. Membranes were washed 3 times for 5 minutes with PBS-T followed by 1.5-hour-incubation with the corresponding HRP-conjugated secondary antibodies diluted to 1:4,000 in blocking buffer. Membranes were washed three times with TBS-T, and protein bands were detected with ImmunoCruz luminol reagent and revealed on Super RX X-Ray films using an AGFA Curix 60 film processor (AGFA, Mortsel, Belgium). The relative intensities of the bands were analyzed using Image J software (National Institutes of Health, Bethesda, MD).

Statistical analysis. All treatment groups were compared pairwise using the one-way analysis of variance (ANOVA) test combined with Tukey's (Holm-Sidak) post-hoc test

assuming normal data distribution. The statistical analysis was performed using SigmaPlot 13.0 software. The differences between treatment groups were considered statistically significant at *P*-values <0.05.

Supplementary material

Figure S1. Viability of HCT-116 cells after transfection with various ON derivatives at a dose of 1 µmol/l in Opti-MEM medium overnight. Cell viability was assessed 1 or 2.5 days after medium exchange.
Figure S2. Bcl-2 mRNA silencing and viability of HCT-116 cells after transfection with various siRNA derivatives complexed with LF and free PS siRNA.
Figure S3. The Bcl-2 mRNA silencing kinetics in HCT-116 cells transfected with 1 µmol/l of L-FANA or 0.05 µmol/l of siRNA/LF in Opti-MEM medium.
Figure S4. The influence of incubation time on knockdown efficacy in HCT-116 cells transfected with L-FANA or siRNA/LF lipoplex.
Figure S5. Polyacrylamide gel electrophoresis images of L-FANA conjugate and unconjugated FANA incubated with oil emulsion.
Figure S6. Transfection of proliferating Caco-2 cells with siRNA/LF and negative control siRNA (siRNAnc)/LF lipoplexes.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)