Lipid-Conjugated Oligonucleotides via "Click Chemistry" Efficiently Inhibit Hepatitis C Virus Translation

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Abstract: Conjugation of a lipid moiety via "click chemistry" potentiates the cellular uptake of oligonucleotides and allows their intracellular delivery. These nontoxic lipid conjugates efficiently inhibit hepatitis C virus internal ribosome entry site (IRES)-mediated translation in human hepatic Huh7 cells. The biological activity of the lipid-conjugated oligonucleotides is not affected by the presence of serum.

There is currently substantial interest in the use of nucleic acids for modifying gene expression for therapeutic purposes.[1] The use of oligonucleotide (ON[a]) analogues as potential therapeutic agents for modulating the expression of specific genes has shown promise for different classes of molecules including aptamers, triplex-forming oligonucleotides, antisense oligonucleotides, small interfering RNAs (siRNAs), and antagomirs.[2] However, one major barrier in the broad utilization of ON analogues as clinical drugs is their reduced ability to transverse cell membranes. Cellular uptake and localization of ONs are crucial problems for their effects on the genetic expressions. Most approaches to their cellular delivery involve cationic lipophilic carriers and/or polymers.[3] While such synthetic carriers can be exceptionally effective for delivering plasmid DNA into the cytoplasm of cells, most are of limited utility because of their toxicity to cells and poor efficiency in the case of ONs.[4] Alternatively, a lipophilic moiety has been covalently tethered to the ON structures with the aim of improving cellular uptake and antisense activity.[5] Despite the different synthetic strategies developed for modification of the oligonucleotides,[6] the synthesis of the lipid−ON conjugates (LON) is anything but trivial and requires extensive expertise in organic chemistry and solid phase synthesis.

A real advance would be to use a bio-orthogonal conjugation reaction that can be utilized for a large variety of lipid and ON sequences. As a modular approach to reliable chemical transformations, the Huisgen 1,3-dipolar cycloaddition of alkynes and azides,[7] a typical example of a "click" reaction,[8] is especially attractive for irreversibly coupling biomolecules with ONs under mild conditions. Because of its biocompatibility, "click" chemistry has recently emerged as an efficient strategy for synthesizing labeled-carbohydrate ONs,[9] peptides and protein conjugates,[10,11] fluorescent ONs,[12] self-assembled monolayers ON probes,[13] circular ONs,[14] and multimodified ONs.[15]

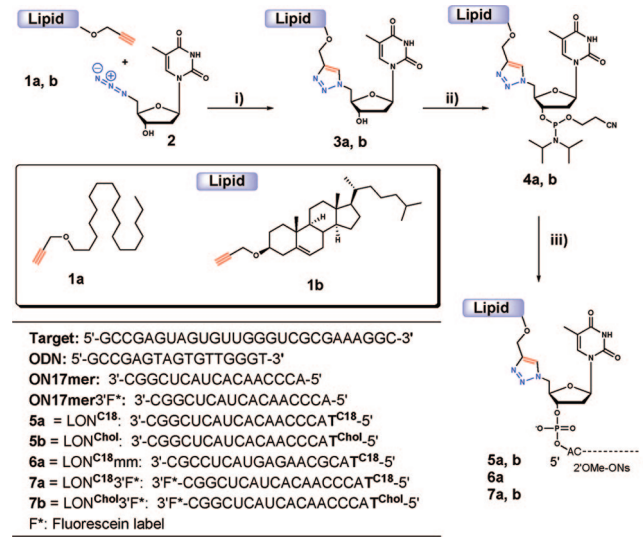

Figure 1. Synthesis of lipid−oligonucleotide conjugates (LONs): (i) 10 mol % $CuSO_4$, 20 mol % sodium ascorbate, THF/H_2O (50/50), 65 °C; (ii) 1.5 equiv of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, 2 equiv of DIEA, DCM, room temp; (iii) solid phase synthesis with 1*H*-tetrazole as activator. Oligonucleotides sequences of ONs (ON17mer and ON17mer3′F*) and LONs (**5a**−**7b**) have been selected to specifically target the subdomain IIId (target) of the hepatitis C virus RNA.

Herein, we covalently conjugated lipids moieties to an ON by using a click chemistry reaction and evaluated the biological activity of the conjugates on cultured human cells (Figure 1). The ON sequence we chose is a 17-mer 2′-*O*-methylribonucleotide antisense (ON17mer, Figure 1) of the hepatitis C virus (HCV) RNA, specifically targeting the subdomain IIId of the internal ribosome entry site (IRES) at the 5′ end of the viral RNA. The antisense efficiency of this ON was correlated to its affinity for the IIId subdomain and to its ability to displace the 40S ribosomal subunit, leading to the inhibition of HCV-IRES-dependent translation in cultured cells.[16]

We hypothesized that a lipid modification of a 2′-OMe oligonucleotide, in particular the ON17mer (Figure 1), could lead to an improvement of its bioavailability and, consequently, enhancement of the antisense activity compared to the nonconjugated ON. To validate our approach, a family of LON conjugates was investigated (**5a**−**7b**, Figure 1). Interestingly, the LON conjugates were prepared in three steps starting from alkyne-modified lipids derived from cholesterol **1a** and octadecanol **1b** (Figure 1). Briefly, 5′-azido-5′-deoxythymidine was refluxed with propargyl lipids (**1a** and **1b**) in water/THF in the presence of sodium ascorbate and a catalytic amount of copper sulfate. The resulting 1,3-dipolar cycloaddition reaction provided the expected 1,2,3-triazole intermediates **3a**, **3b**, which were first converted into the phosphoramidites in one step. The phosphoramidites **4a**, **4b** were further coupled to the ON chain using a classical solid support synthesis, in which the ON is elongated in the 3′−5′ direction. This lipid modification noticeably increased the lipophilicity of the oligonucleotides, as judged by the retention times in reverse-phase (RP C4) HPLC analysis, which were 9 and 23 min for the parent (ON17mer) and the conjugated **5b** oligonucleotides, respectively, under the same experimental conditions (Supporting Information, Figure SI6).

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[a] Abbreviations: DCM, methylene chloride; DIEA, diisopropylethylamine; IRES, internal ribosome entry site; HCV, herpes virus C; Huh-7, human hepatoma-7; LON, lipid conjugated oligonucleotide; ODN, oligodeoxynucleotide; ON, oligonucleotide; THF, tetrahydrofurane; T_m, melting temperature.

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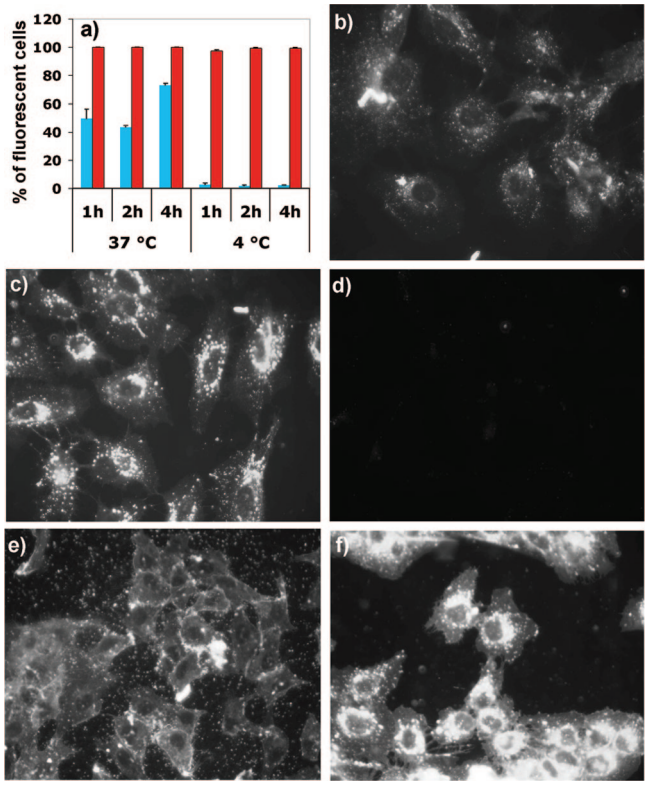

Figure 2. (a) Percentage of fluorescent cells (Huh7) incubated in the presence of 0.5 μM LON^{C18}3′F* (**7a**, blue) and LON^{Chol}3′F* (**7b**, red) at 37 and 4 °C. (b−f) Fluorescence microscopy of Huh7 cells co-incubated at 37 °C with 0.5 μM LONs or ONs. Cells co-incubated with (i) **7a** at 37 °C for 2 h (b) and 4 h (c), (ii) **7b** for 4 h at 4 °C (e) and 37 °C (f), (iii) ON17mer for 24 h at 37 °C (d).

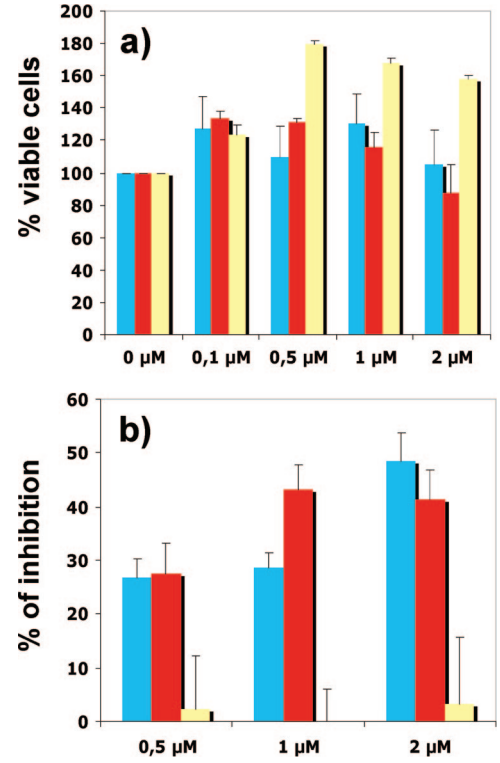

Figure 3. (a) Cell viability at different concentrations of LONs **5a** (blue), **5b** (red), and **6a** (yellow). (b) Inhibition (in %) of the HCV IRES-dependent translation in the presence of different concentrations of **5a** (blue), **5b** (red), and mismatch **6a** (yellow).

We first studied the thermal denaturation of the complementary duplexes ON17mer/ODN, **5a**/ODN, and **5b**/ODN by measuring the absorbance at 260 nm in a cacodylate aqueous buffers. Melting temperatures of roughly 70 °C were observed for any complementary duplex (Supporting Information, Figure SI9). As expected, with a T_m of 30 °C the mismatch sequence LON^{C18}mm (**6a**/ODN) induced a destabilization of the double helix. The lipid modification appeared to have little or no impact on the duplex stability and melting temperature values (T_m).

We next used fluorescence microscopy and flow cytometry to determine whether the LON conjugates penetrated cultured eukaryotic cells. Human hepatic Huh7 cells were incubated with 0.5 mM of 3′-fluorescein-labeled LONs or unconjugated ONs for increasing periods of times at 37 and 4 °C (Figure 2). At 37 °C, fluorescein-labeled LON^{C18}3′F* (**7a**) was internalized within 30 min and accumulated in the cytoplasm as a function of the incubation time (Figure 2b,c). Cholesterol derivative LON^{Chol}3′F* (**7b**) was quickly taken up by the cells at 37 °C (Figure 2f and Figure SI10). By contrast, the nonlipidic labeled ON17mer3′F* did not penetrate cells (Figure 2d) after 24 h of incubation in the same conditions, indicating that efficient uptake requires lipid modification of the ONs. Flow cytometry studies confirmed the time-dependent uptake of the C18 lipid conjugate and indicated that **7a** (LON^{C18}3′F*) penetrated in 80% of cells after 4 h (Figure 2a and Figure SI11). The mechanism by which the LON conjugates enter cells is unknown. Recent results obtained with cholesterol polyanionic derivatives[17] suggest that endocytosis is a possible pathway[18] for LON conjugates. Interestingly, the cellular uptake of the C18 lipid conjugate **7a** was dramatically decreased after incubation at 4 °C as shown by flow cytometry (Figure 2a). Surprisingly, at 4 °C the cholesterol derivative **7b** was not internalized to the cytoplasm and was limited to the cellular membrane (Figure 2e), indicating a strong affinity of the cholesterol moiety for the bilayers. These results suggest that LON conjugates enter the cells via an energy-dependent mechanism rather than by passive diffusion.

Next, we evaluated the cytotoxicity of the LON, assessing the cell viability after a 4-day incubation of growing Huh7 cells with increasing concentrations of LONs. Figure 3a indicates that the LONs were neither toxic nor cytostatic for these cells at any of the tested LON concentrations. Huh7 cell uptake of the unconjugated ON17mer required transfecting reagents. However, these formulations led to toxic effects (data not shown). This observation highlights the interest of lipid conjugation for achieving cell permeability.

Finally, the antisense activity of LON^{C18} (**5a**) and LON^{Chol} (**5b**) was evaluated after transient transfection of a bicistronic luciferase reporter mRNA, in which the second cistron translation depends on the HCV IRES,[16] in Huh7 cells preincubated with ONs or LONs in the presence of serum for 4 or 24 h. Whereas the unconjugated antisense ON17mer was ineffective, both lipid-conjugated **5a** and **5b** induced a dose-dependent reduction of HCV IRES-dependent translation: 50% inhibition (plateau) was reached with about 1 μM **5b** and 2 μM **5a** preincubated for 4 h before the RNA target (Figure 3b). A 24 h preincubation did not improve the antisense activity of the LONs or the unconjugated ON (data not shown). This inhibition was selective, as the mismatched **6a** induced only a 5% decrease at 2 μM, and related to the lipid modification of the ON, since the unconjugated ON (ON17mer) and the lipid (**3a**) added simultaneously also were inactive (data not shown).

In conclusion, we have developed a straightforward procedure for creating lipid−oligonucleotide conjugates. The incorporation of a lipid moiety via a nonscissile triazole linker potentiates

the cellular uptake of the oligonucleotides and affords an increase in ON delivery as measured by fluorescence microscopy and flow cytometry. Both lipid-conjugated LON^{C18} (**5a**) and LON^{Chol} (**5b**) induced a dose-dependent reduction of HCV IRES-dependent translation in the Huh7 cell line. More importantly, toxicity of the lipid−oligonucleotide conjugates was negligible and biological activity of the LONs was not affected by the presence of serum. The results reported in this study have important implications for the future design of cell-permeable oligonucleotides, including antisenses, antagomirs, siRNA, or aptamers.

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Supporting Information Available: Experimental procedures (**1**−**7**), cell culture methods, and complementary fluorescence microscopy images. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Verma, I. M.; Somia, N. Gene therapy—promises, problems and prospects. *Nature* **1997**, *389*, 239–242. (b) Soutschek, J.; Akinc, A.; Bramlage, B.; Charisse, K.; Rainer, C.; Donoghue, M.; Elbashir, S.; Geick, A.; Hadwiger, P.; Harborth, J.; John, M.; Kesavan, V.; Lavine, G.; Pandey, R. K.; Racie, T.; Rajeev, K. G.; Röhl, I.; Toudjarska, I.; Wang, G.; Wuschko, S.; Bumcrot, D.; Koteliansky, V.; Limmer, S.; Manoharan, M.; Vornlocher, H.-P. Therapeutic silencing of an endogenous gene by systemic administration of modified si RNAs. *Nature* **2004**, *432*, 173–178.

(2) (a) Prakash, T. P.; Kawasaki, A. M.; Wancewicz, E. V.; Shen, L.; Monia, B. P.; Ross, B. S.; Baht, B.; Manoharan, M. Comparing in vitro and in vivo activity of 2′-*O*-[2-(methylamino)-2-oxoethyl]- and 2′-*O*-methoxyethyl-modified antisense oligonucleotides. *J. Med. Chem.* **2008**, *51*, 2766–2776. (b) Opalinska, J. B.; Gewirtz, A. M. Nucleic-acid therapeutics: basic principles and recent applications. *Nat. Rev. Drug Discovery* **2002**, *1*, 503–514.

(3) (a) Mukherjee, K.; Bhattacharyya, J.; Sen, J.; Sistla, R.; Chaudhuri, A. Covalent grafting of common trihydroxymethylaminomethane in the headgroup region imparts high serum compatibility and mouse lung transfection property to cationic amphiphile. *J. Med. Chem.* **2008**, *51*, 1967. (b) Li, S. D.; Huang, L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. *Gene Ther.* **2006**, *13*, 1313–1319. (c) Fraley, A. W.; Pons, B.; Dalkara, D.; Nullans, G.; Behr, J.-P.; Zuber, G. Cationic oligonucleotide−peptide conjugates with aggregating properties enter efficiently into cells while maintaining hybridization properties and enzymatic recognition. *J. Am. Chem. Soc.* **2006**, *128*, 10763.

(4) (a) Sundaram, S.; Lee, L. K.; Roth, C. M. Interplay of polyethyleneimine molecular weight and oligonucleotide backbone chemistry in the dynamics of antisense activity. *Nucleic Acids Res.* **2007**, *35*, 4396–4408. (b) Roth, C. M. Quantitative measurements and rational materials design for intracellular delivery of oligonucleotides. *Biotechnol. Prog.* **2008**, *24*, 23–28.

(5) (a) Letsinger, R. L.; Zhang, G.; Sun, D. K.; Ikeuchi, T.; Sarin, P. S. Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6553–6556. (b) Manoharan, M.; Tivel, K. L.; Condon, T. P.; Andrade, L. K.; Barber-Peoch, I.; Inamati, G.; Shah, S.; Mohan, V.; Graham, M. J.; Bennett, F. C.; Crooke, S. T.; Cook, P. D. Conjugated antisense oligonucleotides. *Nucleosides Nucleotides* **1997**, *16*, 1129–1138.

(6) Gissot, A.; Camplo, M.; Grinstaff, M. W.; Barthélémy, P. Nucleoside, nucleotide and oligonucleotide based amphiphiles: a successful marriage of nucleic acids with lipids. *Org. Biomol. Chem* **2008**, *6*, 1324–1333.

(7) Huisgen, R. *1,3-Dipolar Cycloaddition Chemistry*; Wiley: New York, 1984; pp 1−176.

(8) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.

(9) (a) Bouillon, C.; Meyer, A.; Vidal, S.; Jochum, A.; Chevolot, Y.; Cloarec, J.-P.; Praly, J.-P.; Morvan, F. Microwave assisted "click" chemistry for the synthesis of multiple labeled-carbohydrate oligonucleotides on solid support. *J. Org. Chem.* **2006**, *71*, 4700–4702. (b) Chevolot, Y.; Bouillon, C.; Vidal, S.; Morvan, F.; Meyer, A.; Cloarec, J.-P.; Jochum, A.; Souteyrand, E. DNA-based carbohydrate biochips: a platform for surface glyco-engineering. *Angew. Chem., Int. Ed.* **2007**, *46*, 2398–2402.

(10) Gogoi, K.; Mane, M. V.; Kunte, S. S.; Kumar, V. A. A versatile method for the preparation of conjugates of peptides with DNA/PNA/analog by employing chemo-selective click reaction in water. *Nucleic Acids Res.* **2007**, *35*, e139.

(11) Humenik, M.; Huang, Y.; Wang, Y.; Sprinzl, M. C-Terminal incorporation of bio-orthogonal azide groups into a protein and preparation of protein−oligodeoxynucleotide conjugates by CuI-catalyzed cycloaddition. *ChemBioChem* **2007**, *8*, 1103–1106.

(12) Seo, T. S.; Li, Z.; Ruparel, H.; Ju, J. Click chemistry to construct fluorescent oligonucleotides for DNA sequencing. *J. Org. Chem.* **2003**, *68*, 609–612.

(13) Devaraj, N. K.; Miller, G. P.; Ebina, W.; Kakaradov, B.; Collman, J. P.; Kool, E. T.; Christopher, E. D.; Chidsey; C. E, D. Chemoselective covalent coupling of oligonucleotide probes to self-assembled monolayers. *J. Am. Chem. Soc.* **2005**, *127*, 8600–8601.

(14) (a) Kumar, R.; El-Sagheer, A.; Tumpane, J.; Lincoln, P.; Wilhelmsson, L. M.; Brown, T. Template-directed oligonucleotide strand ligation, covalent intramolecular DNA circularization and catenation using click chemistry. *J. Am. Chem. Soc.* **2007**, *129*, 6859–6864. (b) Lietard, J.; Meyer, A.; Vasseur, J.-J.; Morvan, F. New strategies for cyclization and bicyclization of oligonucleotides by click chemistry assisted by microwaves. *J. Org. Chem.* **2008**, *73*, 191–200.

(15) Gramlich, P. M. E.; Warncke, S.; Gierlich, J.; Carell, T. Click−click−click: single to triple modification of DNA. *Angew. Chem., Int. Ed.* **2008**, *47*, 3442–3444.

(16) Tallet-Lopez, B.; Aldaz-Carroll, L.; Chabas, S.; Dausse, E.; Staedel, C.; Toulmé, J. J. Antisense oligonucleotides targeted to the domain IIId of the hepatitis C virus IRES compete with 40S ribosomal subunit binding and prevent in vitro translation. *Nucleic Acids Res.* **2003**, *31*, 734–742.

(17) De Jong, L.; Moreau, X.; Thiéry, A.; Godeau, G.; Grinstaff, M. W.; Barthélémy, P. Amphiphilic copolymer for delivery of xenobiotics: in vivo studies in a freshwater invertebrate, a mesostominae flatworm. *Bioconjugate Chem.* **2008**, *19*, 891–898.

(18) Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **2003**, *278*, 585–590.

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