Bioorganic & Medicinal Chemistry Letters 23 (2013) 4157–4161

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of oligonucleotides with glucosamine at the 3′-position and evaluation of their biological activity ☆

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ARTICLE INFO

Article history:
Received 27 March 2013
Revised 1 May 2013
Accepted 10 May 2013
Available online 20 May 2013

Keywords:
RNA
siRNA
RNAi
Glucosamine analogues
Nuclease resistance

ABSTRACT

Short interfering RNA (siRNA) has been proven to be an utilizable tool for post-transcriptional gene silencing research. In this study, we designed and synthesized two glucosamine analogues and tried to modify the siRNA using these two glucosamine analogues at the 3′-overhang region of siRNAs to improve the nuclease resistance and to overcome some other weak points. The siRNAs modified with glucosamine analogues had almost no effect of the thermal stability and showed strong resistance to nuclease degradation. Some of them kept the same gene silencing activity level as unmodified siRNA.

RNA interference (RNAi) has been a vital area of post-transcriptional gene silencing research because of its high efficiency and sequence specificity in plants[1] and mammalian cells.[2] Moreover, a chemically synthesized short interfering RNA (siRNA) duplex was reported to induce an RNAi effect.[3] RNAi technology, as a breakthrough of gene therapy, benefits many incurable diseases;[4–8] however, there are still a number of obstacles for *in vivo* experiments and further clinical testing, such as low nuclease resistance, poor membrane permeability, and off-target effects. In the wake of the elucidation of the RNAi mechanism, chemical modification[9–12] of siRNA is considered a potential approach to overcome these siRNA-related problems.

Until now, many chemical modifications of siRNAs at the 3′-overhang region or 5′-end have been designed and synthesized,[13–16] most of which showed better thermal stability, nuclease resistance, and other biological properties than the natural molecules. In our laboratory, aromatic compounds or abasic nucleosides were introduced at the 3′-overhang region in previous studies (Fig. 1).[14–16] However, none of these modifications were aimed at improving their affinity for the cell membrane or charge neutralization. Therefore, we searched for a residue with high membrane affinity and a positive charge that could be introduced into the 3′-overhang region of siRNA, which was expected to improve cell membrane penetration, nuclease resistance, and other properties.

The amino sugar glucosamine, which is a structural element of the polysaccharides chitosan and chitin, is considered to be a good candidate. It is known that glucosamine shows high membrane affinity.[17] As a positively charged molecule, it can also neutralize the negatively charged phosphates of siRNA. Furthermore, since chitosan exists widely in organisms, such as the exoskeleton of crustaceans and other arthropods and the cell wall of fungi,[18] glucosamine shows low toxicity to the human body.

Synthesis of modified glucosamine monomer units. To introduce glucosamine into the 3′-end of both sense and antisense siRNA strands, we prepared glucosamine monomer unit **12**, with a 1′-C_2 linker and 6′-controlled pore glass (CPG) resin, as the solid support for the DNA/RNA synthesizer from the starting material glucosamine hydrochloride **1** (Scheme 1). Since directly introducing a thiophenyl group into the 1′-position of nonprotected glucosamine would result in an α/β mixture, it was necessary to protect the amino and hydroxyl groups using phthalic anhydride and acetic anhydride, respectively, to generate fully protected **2** in yield of 68%. Treatment of thiophenol in the presence of a Lewis acid ($BF_3{\cdot}OEt_2$) produced only β type **3** in 93% yield. Because of the failure to remove the phthalyl group during the post-treatment step of oligonucleotide (ON) synthesis, we changed the protecting group to a trifluoroacetyl group, which could be removed easily by aqueous ammonia. Deprotection of the acetyl and phthalyl groups of **3** followed by silylation of 6′-OH, trifluoroacetylation of 2′-NH_2, and

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http://dx.doi.org/10.1016/j.bmcl.2013.05.036

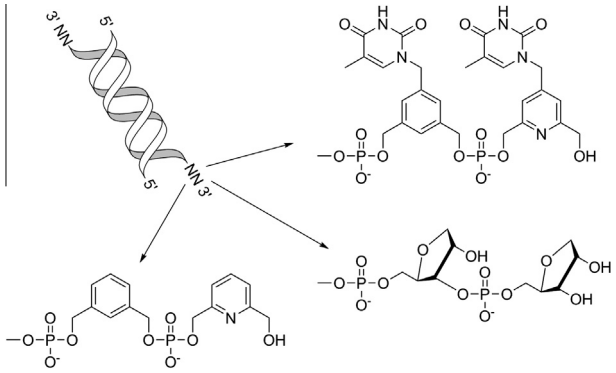


Figure 1. Structures of the modified siRNAs.

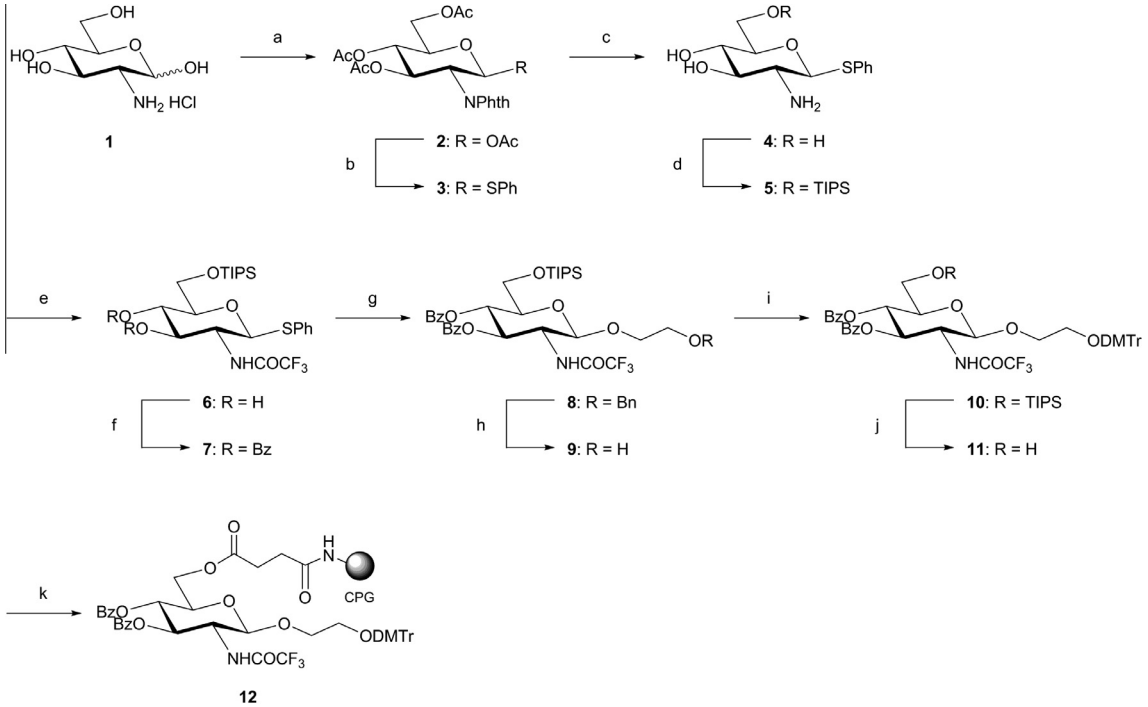
benzoylation of 3′,4′-OH afforded **4**–**7** in yields of 60, 86, 79, and 83%, respectively. In the presence of trifluoromethanesulfonic acid, treatment of 2-benzyloxyethanol, *N*-iodosuccinimide with molecular sieves 4 Å gave **8** in 78% yield. To remove the end of the 2C linker, $Pd(OH)_2$/C-catalyzed hydrogenating debenzylation and 4,4′-dimethoxytritylation were carried out to produce **9** and **10** in respective yields of 89% and 98%. Next, compound **10** was desilylated by treatment with tetrabutylammonium fluoride to afford **11** in yield of 68%. Subsequently, compound **11** was succinated to yield the corresponding succinate linked to the CPG resin to generate solid support **12** (19 μmol/g).

Furthermore, in order to examine the difference of glucosamine-modified siRNAs with/without a base moiety, we also synthesized another type of glucosamine monomer unit that included thymine at the 6′-position by an improved synthesis route (Scheme 2). In the presence of $BF_3{\cdot}OMe_2$, the treatment of 2-benzyloxyethanol to **2** could directly introduce a C_2 linker into the 1′-position of glucosamine to give **13** in 67% yield, thereby skipping the thiophenylation step. On account of the side reaction of the later Mitsunobu reaction, we deprotected the acetyl groups of **13** by treatment with MeONa in MeOH to give **14** in 88% yield. N^3-Benzoylthymine was introduced into the 6′-position of **14** by the Mitsunobu reaction to afford **15** in 84% yield. After removing the phthalyl group of the 2′-position and the benzoyl group of thymine, the synthesis strategy was the same as Scheme 1 to generate **16**–**19** in 87, 90%, quant., and 79% yields, respectively. Subsequently, the CPG resin-linked solid support **20** (37 μmol/g) was synthesized by the succination of **19**.

Synthesis of glucosamine analogue-modified siRNAs. We chose the *Renilla* luciferase gene as the target sequence. The basic siRNA sequences were as follows: *Renilla* sense, 5′-CUUCUUCGUCGAGACCAUG-3′; *Renilla* antisense 5′-CAUGGUCUCGACGAAGAAG-3′. By using **12** and **20**, ONs containing X or Y were synthesized using an automatic DNA/RNA synthesizer (Fig. 2). Each of these siRNA sequences were specified by their 3′-overhang region (0–2 molecules of thymidine and glucosamine analogue X or Y), and fluorescent amidites were also introduced at the 5′-end of ONs **42**–**44** for further assay tests.

The synthesized ONs were post-treated with the conventional process[11] and analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The observed molecular weights of these ONs coincided with their calculated molecular weight.[19]

Thermal stability. The melting temperatures (T_m) of these siRNAs were recorded using a UV–visible spectrophotometer in a buffer of 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) containing 100 mM NaCl. Absorbance at 260 nm was measured as a function of temperature from 20 to 95 °C with a 0.5 °C/min increase. The T_m values of these siRNAs are shown in Table 1. Neither of these 3′-end modified siRNAs showed a significant decrease of T_m compared with the unmodified molecule TT (T_m = 76.1 °C). Thus, we found that introduction of glucosamine analogues at the 3′-end of the ONs had



Scheme 1. Synthesis route of 6′-abasic glucosamine monomer unit. Reagents and conditions: (a) (i) MeONa, MeOH, rt; (ii) phthalic anhydride, MeOH, rt; (iii) Ac_2O, pyridine, rt, 68%; (b) PhSH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt, 93%; (c) ethylenediamine, MeCN, reflux, 60%; (d) TIPSCl, pyridine, rt, 86%; (e) $(CF_3CO)_2O$, pyridine, rt, 79%; (f) BzCl, pyridine, rt, 83%; (g) 2-benzyloxyethanol, NIS, TfOH, CH_2Cl_2, MS4 Å, rt, 78%; (h) H_2, $Pd(OH)_2$/C, THF, rt, 89%; (i) DMTrCl, pyridine, rt, 98%; (j) TBAF, THF, rt, 68%; (k) (i) succinic anhydride, DMAP, pyridine, rt; (ii) CPG, EDC, DMF, rt, 19 μmol/g.