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Design, synthesis and anti-influenza virus activities of terminal modified antisense oligonucleotides

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ABSTRACT

Four novel terminal modified antisense oligonucleotides (ODNs) were designed, synthesized and tested for their anti-influenza virus activity. Initial biological studies indicated that lipophilic and rimantadin emodificated Flutide exhibited more potent anti-H1N1 activity than Flutide. Among them, lipophilic modificated ODN (Flutide-**I**) showed the most antiviral activity. The EC_{50} value of Flutide-**I** for inhibiting H1N1 induced cytopathic effect (CPE) and H1N1 RNA were respectively (0.26 ± 0.16) μM and (0.11 ± 0.03) μM. The cytotoxicity of these compounds has also been assessed. No significant cytotoxicities were found for any of these compounds with the concentrations up to 20 μM.

Introduction

In 1978, Paul Zamecnik and Mary Stephenson reported the first experiments on antisense mechanisms of gene silencing, using short synthetic antisense oligonucleotides (ODNs) to inhibit replication of the Rous sarcoma virus by binding and blocking the action of 35s RNA, then increasing attention turned to the possible therapeutic applications of antisense technology.[1,2] In 1998, the first antisense drug, Vitravene (ISIS Pharmaceuticals Inc.), was approved for retinitis induced by cytomegalovirus.

In recent years, antisense ODNs have been applied to the treatment of a variety of diseases including viral infection,[3] tumor,[4] vessel restenosis,[5] fulminant septic shock,[6] asthma, and allergies.[7] In Jan 29th, 2013, the U.S. FDA approved the second antisense drug, Kynamro (mipomersen sodium, ISIS Pharmaceuticals Inc. and Genzyme Corp.) to treat inherited cholesterol disorder, which is an oligonucleotide inhibitor for homozygous familial hypercholesterolemia (HoFH).[8] Antisense ODN is designed to hybridize to a complementary target sequence of corresponding mRNA, which inhibits protein expression. Therefore antisense ODNs may display increase in affinity and selectivity for their nucleic acid targets compared with traditional drugs.[9,10]

Flutide (Scheme 1) is a 13-mer antisense phosphorothionate oligonucleotide (PS-ODNs), which is complementary to the 5′ terminal conserved regions of viral RNA found in almost all of the influenza A virus.[11] All of the nonbridging oxygen atoms in the phophodiester bond are replaced by sulfur in its structure. The introduction of phosphorothioate linkages into ODNs is primarily intended to enhance their nuclease resistance.[12] Previous studies have showed that Flutide exhibited the most potent antiviral activity in vitro and in vivo. It inhibited influenza virus A induced cytopathic effects in MDCK cells with the EC_{50} ranging from 2.2 to 4.4 μM. In the infected mouse model, prolonged mean survival days and declined virus titres in lung in the Flutide treatment groups compared with the infected control group, with a dose-dependent manner.[11]

Some reports have showed that terminal modification of ODNs facilitates the cellular import and increases the antiviral activity.[13,14] Therefore, as a continuous research program of our laboratory to improve the drugability of Flutide, we now report the design and synthesis of terminal modificated Flutide (Scheme 2) and their anti-influenza virus activity.

Results and discussion

Flutide and modified ODNs are solid phase synthesized with the phosphoramidite approach. The whole synthetic process has been completely automated with DNA synthesizer (ABI3900).

As shown in Scheme 3, 2-(4-aminobutyl)propane-1,3-diol **3** was prepared from 4-bromobutyronitrile **1** and diethyl malonate

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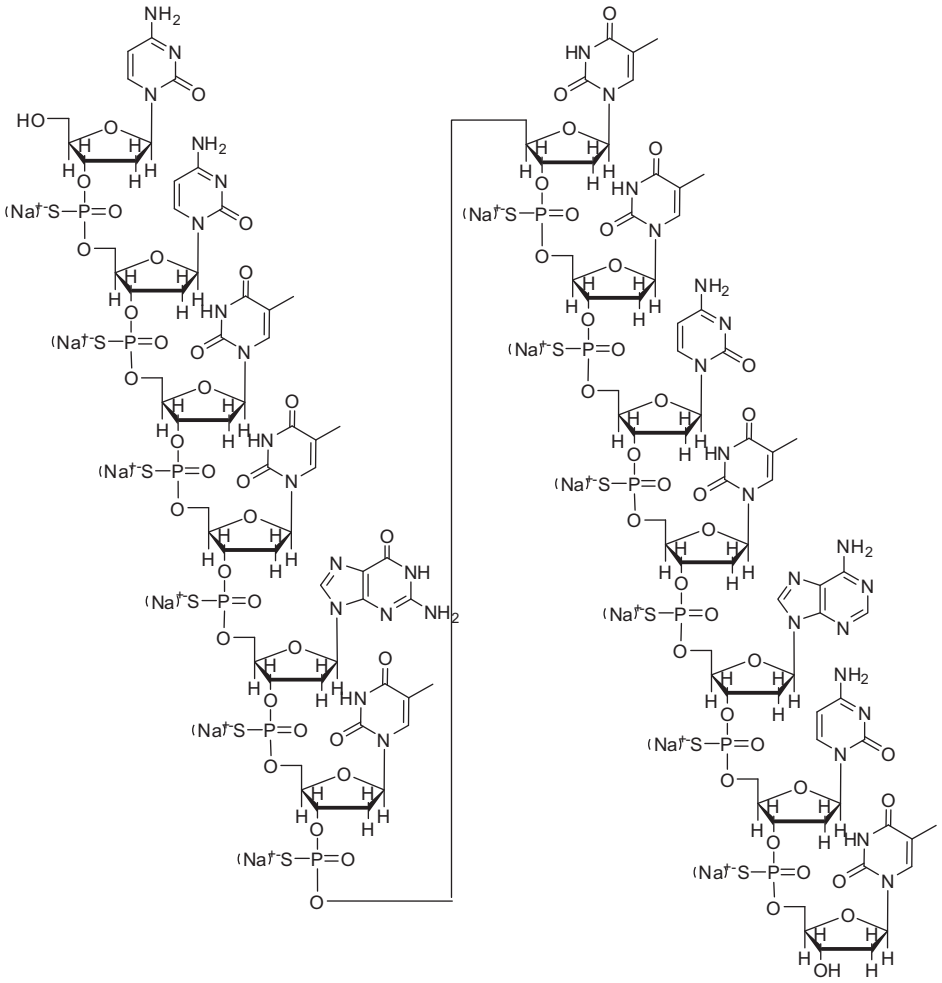
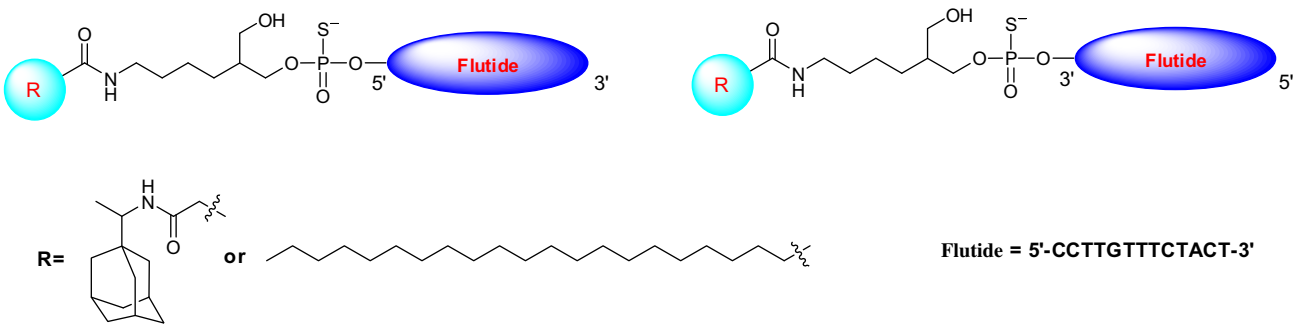
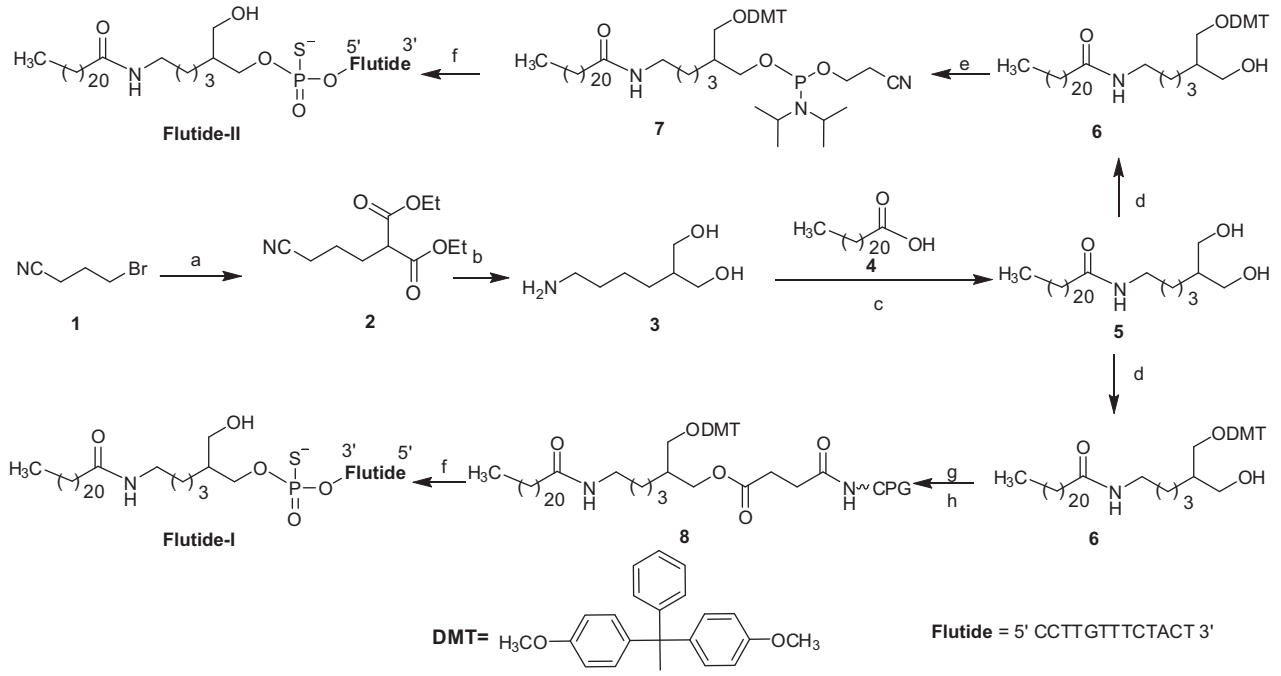


Scheme 1. Structure of Flutide.

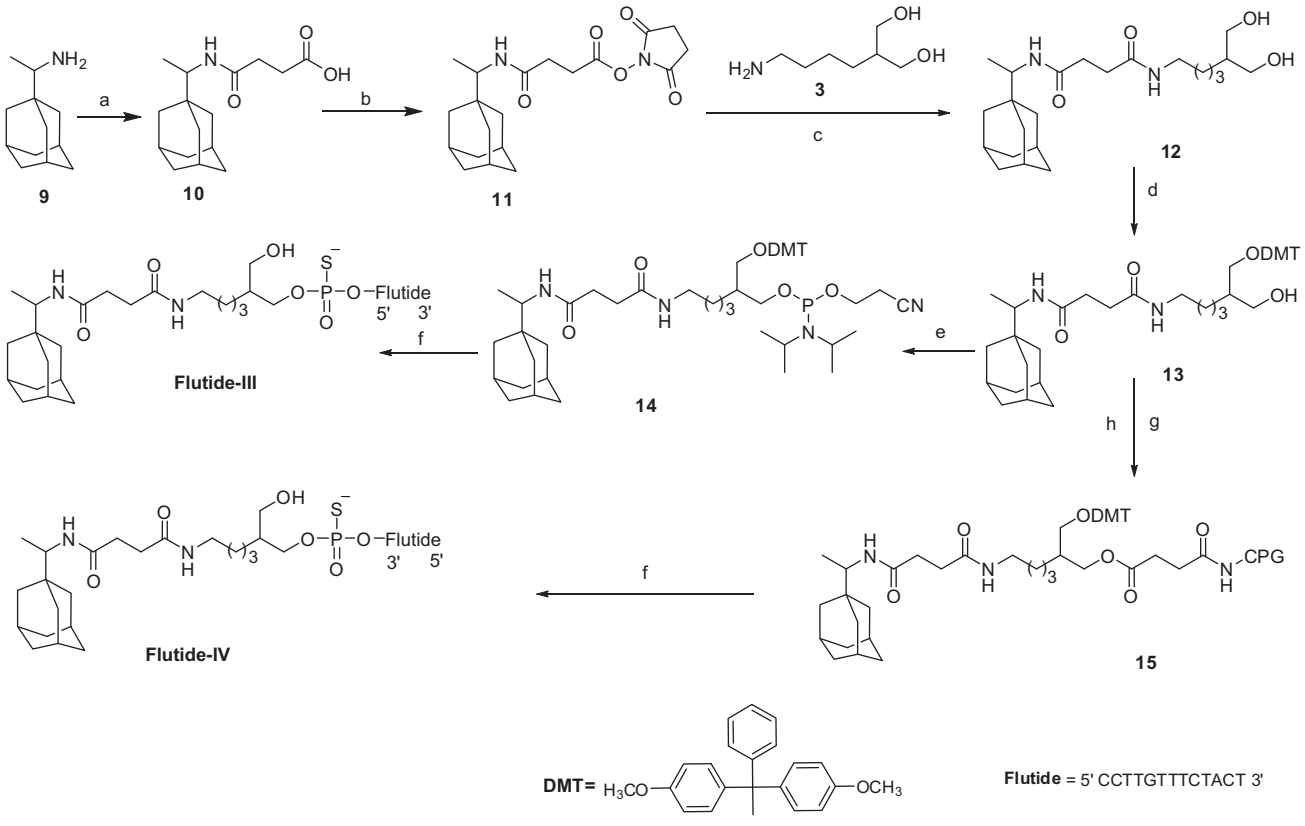


Scheme 2.

2 via condensation reaction and reduction reaction successively.[15] Treatment of behenic acid **4** and **3** with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride in acetonitrile gave *N*-(6-hydroxy-5-(hydroxymethyl)hexyl)docosanamide **5** in 88% isolated yield. Subsequent selective protection of one primary hydroxy group of **5** with 4,4′-dimethoxytrityl group (DMT) provided **6** in 37% yield. Next, **6** was treated with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite ($((iPr_2N)_2POCH_2CH_2CN$) together with diisopropylammonium and tetrazolide in dry acetonitrile to afford 2-((bis(4-methoxyphenyl)(phenyl) methoxy)methyl)-6-docosanamidohexyl(2-cyanoethyl) diisopropylphos-phoramidite **7** in 78% isolated yield. Then, the terminal modified Flutide-**II** was synthesized on a 1.0 μmol scale by using **7**, as a kind of modified phosphoramidite, which coupled to the 5′ termini of Flutide following the standard PS-ODN synthesis process with the commercially DNA phosphoramidites (Sigma–Aldrich) and Unylinker™ 200 solid support (Nitto Denko Cor.). On the other hand, **6** was treated with butanedioic anhydride in tetrahydrofuran at ambient temperature for 12 h. Then NH_2 group of the Controlled Pore Glass (CPG) was added to the reaction system to afforded **8**, which was used as the solid support for the synthesis of Flutide-**I**. Rimantadine terminal modified



Scheme 3. Reagents and conditions: (a) Na, diethyl malonate, CH_3CH_2OH, 85%; (b) $LiAlH_4$, THF, 93%; (c) CH_3CN, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, rt to 50 °C, 88%; (d) 4,4′-dimethoxytrityl chloride, dimethylaminopyridine, pyridine, 0 °C, 37%; (e) $(iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, CH_3CN, THF, 78%; (f) DNA synthesizer for Flutide-**I** and Flutide-**II**; (g) butanedioic anhydride, THF, 0 °C to rt; (h) 2,3,4,5,6-pentafluorophenol, NH_2-CPG, THF, rt.



Scheme 4. Reagents and conditions: (a) CH_3CN, butanedioic anhydride, reflux, 99%; (b) THF, HoSu, DCC, rt, 97%; (c) CH_3CN, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, rt to 50 °C, 83%; (d) 4,4′-dimethoxytrityl chloride, dimethylaminopyridine, pyridine, 0 °C, 63%; (e) $(iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, CH_3CN, THF, 78%; (f) DNA synthesizer for Flutide-**III** and Flutide-**IV**; (g) butanedioic anhydride, THF, 0 °C to rt; (h) 2,3,4,5,6-pentafluorophenol, NH_2-CPG, THF, rt.

Flutide (Flutide-**III** and Flutide-**IV**) was prepared employed the similar strategy as shown in Scheme 4.

The cleavage of the synthesized terminal modified ODNs (Flutide-**I**–Flutide-**IV**) from the solid support, and the deprotection of the nucleotides were carried out with 28% aqueous ammonia at 60 °C for 8 h. The solution was evaporated under reduced pressure at room temperature to remove ammonia, and the residue was diluted with 0.10 M ammonium acetate (50 mL). The solution was

Table 1
Anti-influenza virus (H1N1) activity and cellular toxicity of terminal modificated ODNs (Flutide-**I**–Flutide-**IV**) and Flutide

Compounds	EC_{50}[a] (μM) H1N1, CPE	EC_{50} (μM) H1N1, RNA	CC_{50}[b] (μM)
Flutide-**I**	0.26 ± 0.16	0.11 ± 0.03	>20
Flutide-**II**	0.72 ± 0.17	0.17 ± 0.04	>20
Flutide-**III**	0.28 ± 0.10	0.26 ± 0.02	>20
Flutide-**IV**	0.54 ± 0.24	0.39 ± 0.06	>20
Flutide	1.74 ± 0.91	2.15 ± 0.87	>20

[a] EC_{50} (μM) is the concentration that inhibits IV by 50%.
[b] CC_{50} (μM) is the concentration of the compound at which 50% of the cells were destroyed.

placed on the C18 cartridge column and the failure sequences were eluted by use of 10% acetonitrile/0.10 M ammonium acetate. After being washed with 0.1 M ammonium acetate and water, the column was treated with aqueous 2% DCA to remove the DMT group, washed with 0.10 M ammonium acetate and water. The target oligonucleotide was eluted by use of 30% acetonitrile/water and the fractions containing the target were lyophilized to give the crude oligonucleotide. Pure material was obtained by being purified on an anion-exchange HPLC by use of 0–50% gradient of 1.0 M NaCl in 25 mM sodium phosphate–10% acetonitrile. The salts were removed by use of the C18 cartridge column to give the pure oligonucleotide after being lyophilized to dryness.

The anti-influenza virus activity of terminal modificated Flutide and the primary ODN Flutide were evaluated by detecting cytopathic effect (CPE) using CCK8 assay and detecting viral replication using qRT-PCR analysis. Influenza virus-infected MDCK cells were treated with series of two fold dilution of terminal modificated Flutide or the primary ODN Flutide started at 0.10–4.0 μM for 48 h. CPE was measured by cell viability with CCK8 assay according to the manufacturer's instructions. The virus RNAs from cell medium were isolated and subjected to qRT-PCR described as previous reports.[16] The EC_{50} values were calculated and listed in Table 1. Flutide showed significant anti-H1N1 (A/Jingfang/01/1986 (H1N1)) activity, which is the same as the previous reports.[11] Lipophilic and rimantadine terminal modification could increase the anti-H1N1 activity. Among them, Flutide-**I** showed the most potent anti-H1N1 activity. Compared with Flutide, the inhibiting activity of H1N1 induced CPE of Flutide-**I** increased 6.69 folds, the inhibiting activity of H1N1 RNA increased 19.55 folds. In addition, the cytotoxicity of terminal modificated Flutide had been assessed by cell proliferation assay. MDCK cells were treated with series dilutions of the compounds started at 1.0–20 μM for 48 h. CCK8 assay were then used. As showed in Table 1, no significant cytotoxicity was found for any of them with concentrations up to 20 μM.

We speculated that the possible mechanism is that the terminal lipophilic and rimantadine modification changed the polarity of Flutide, which is an ASODN. From the structure and many reports, the polarity of ASODN is large, which may result in low cellular uptake.[13,14] In this study, terminal lipophilic and rimantadine modification makes the molecule Flutide less polar and thus the cellular uptake might be improved via better transmembrane ability of antisense ODN–rimantadine conjugate and antisense ODN–lipoid conjugate. Therefore, the dosage was decreased and anti-influenza virus activity was strengthened obviously.

In addition, both 3′ and 5′ terminal modified Flutides showed significant increase of antiviral activity. And we conjecture that the modification on both ends is more conducive to improve the antiviral activity. The above speculation content needs to be further studied.

In summary, four novel terminal modificated PS-ODNs were designed and synthesized and their anti-influenza virus activities were evaluated in vitro. Initial biological studies indicated that lipophilic terminal modification at the 3′ end of Flutide (Flutide-**I**) showed the most promising activity against H1N1 replication. Further investigations of the biological activities are currently underway.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.10.129.

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