The design, synthesis and evaluation of hypoxia-activated pro-oligonucleotides†

Nan Zhang,[ab] Chunyan Tan,[a] Puqin Cai,[a] Peizhuo Zhang,[c] Yufen Zhao[b] and Yuyang Jiang*[ad]

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Hypoxia-activated pro-oligonucleotides were synthesized through the commercial phosphoramidite method, and could be readily cleaved to form normal oligos with good hypoxia selectivity *in vitro* under the effect of reductases, as well as in tumor cell extract.

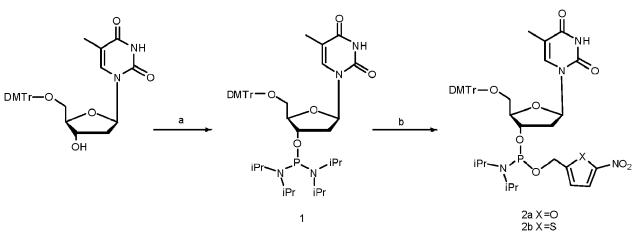
Antisense oligonucleotides (oligos) are important tools for regulating specific gene expression because of their simplified design and synthesis, along with availability of the rapidly expanding genomics. However, their broad applications in biological research and gene therapy has been limited by their low stability against nucleases and poor cellular uptake. The pro-oligos approach has emerged as one of the potential methods to overcome the above limitations by masking the negative charges of the phosphate backbone with biodegradable protection groups.[1–5] However, special non-basic and non-nucleophilic solid supports, and nucleobase protecting groups have to be applied in the solid-phase synthesis, because such protecting groups are sensitive to base and/or nucleophilic treatment.[5–9] More recently, the development of hypoxia-activated prodrugs that preferentially 'release' a therapeutic entity under hypoxic conditions has attracted more and more attention.[10–14] Being intrigued by the hypoxia-activated modification, we designed pro-oligos containing nitroheterocycle-modified phosphate internucleoside linkages in order to not only make the synthesis of pro-oligos commercially applicable, but also achieve better tumor selectivity. Herein, we report our design, synthesis and bio-evaluation of hypoxia-activated pro-oligos.

As shown in Scheme 1, the two thymidine phosphoramidite monomer building blocks, 5′-O-dimethoxytrityl thymidine-3′-O-[(5-nitro-2-furyl)methyl *N*,*N*-diisopropylphosphoramidite] (**2a**) and 5′-O-dimethoxytrityl thymidine-3′-O-[(5-nitro-2-thienyl)methyl *N*,*N*-diisopropylphosphoramidite] (**2b**) were synthesized from 5′-O-dimethoxytrityl protected thymidine in two steps with overall yields at 68% and 85%, respectively.

Oligo **5**, and pro-oligos **3** and **4**, which contain different numbers of modified monomer units in a T_{12}-sequence, were synthesized by the standard phosphoramidite protocol, except that a prolonged coupling time was used to couple the nitroheterocyle-modified monomer units. In contrast to base-sensitive acylthioalkyl and acyloxyalkyl protecting groups,[5–9] both 5-nitro-2-furylmethyl and 5-nitro-2-thienylmethyl groups are quite stable under solid synthesis conditions. After being removed from the solid support, the crude oligos were purified by HPLC. The isolated yield was approximately 30%. The sequences and the structures of the synthesized pro-oligos are shown in Scheme 2.

The mechanistic rationale for the proposed activation of pro-oligos is shown in Scheme 3. Pro-oligos with nitroheterocycle modifications can be bioreduced by reductase *via* a series of one-electron reduction processes to form the hydroxylamino-heterocycle- or aminoheterocycle-modified oligos, followed by cleavage of the heterocycle groups and the release of the desired T-sequence.[14,15]

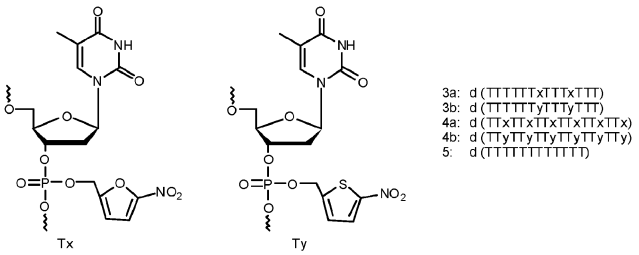
The hydrolysis of pro-oligos by nitroreductase was monitored by reversed-phase HPLC. **3a** and **3b** were incubated with *E. coli* nitroreductase either in aerobic (air) or hypoxic (nitrogen) conditions. As we expected, both pro-oligos were hydrolyzed more readily in N_2 than in air, as suggested by shorter lifetimes of 3–4 h in N_2 compared to those of 8–9 h in air (Fig. 1(a) and Table S1†). The difference between the nitroheterocyclic pro-oligos in N_2 and in air is mainly due to the nitro anion radical, which is the first intermediate during the multi-step



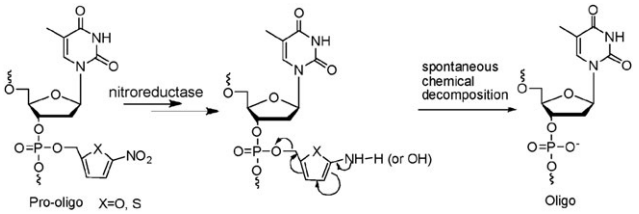
Scheme 1 The synthesis of monomer building blocks with nitroheterocycle-modified phosphoramidites. *Reagents and conditions:* (a) bis(*N*,*N*-diisopropylamino)chlorophosphine, *N*,*N*-diisopropylethylamine, CH_2Cl_2, RT; (b) 5-nitro-2-hydroxymethylfuran or 5-nitro-2-hydroxymethylthiophene, 4,5-dicyanoimidazole (DCI), CH_2Cl_2, RT.

[a] *Key Laboratory of Chemical Biology, Guangdong Province, Graduate School at Shenzhen, Tsinghua University, Shenzhen, 518055, P.R. China. E-mail: jiangyy@sz.tsinghua.edu.cn*
[b] *Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Ministry of Education, Department of Chemistry, Tsinghua University, Beijing, 100084, P.R. China*
[c] *Shanghai GenePharma Co. Ltd, 1011 Halley Road, Z.-J. High Tech Park, Shanghai, 201203, P.R. China*
[d] *School of Medicine, Tsinghua University, Beijing, 100084, P.R. China*

† Electronic supplementary information (ESI) available: Details on experimental procedures of the synthesis of monomer building block **2a**, **2b** and oligonucleotides, hydrolysis with *E. coli* nitroreductase, CEM cell extract preparation, hydrolysis in CEM cell extract, hydrolysis with snake venom phosphodiesterase, DNase I and in 10% fetal bovine serum, and confocal microscopic analysis. See DOI: 10.1039/b903331a



Scheme 2 The structures and sequences of synthesized pro-oligos **3** and **4**, and oligo **5**.



Scheme 3 The mechanism of the hypoxia-activated release of the normal oligo from pro-oligos.

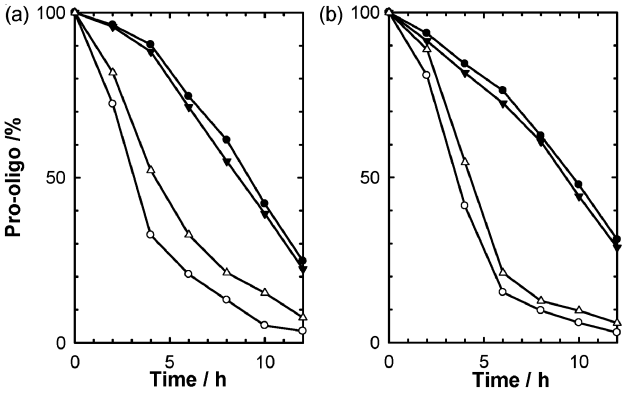


Fig. 1 (a) The bio-degradation of pro-oligos **3a** and **3b** by *E. coli* nitroreductases in N_2 and in air: **3a** in N_2 (○), **3a** in air (●), **3b** in N_2 (△), and **3b** in air (▼). (b) The bio-degradation of pro-oligos **4a** and **4b** in CEM cell extract in N_2 and in air: **4a** in N_2 (○), **4a** in air (●), **4b** in N_2 (△), and **4b** in air (▼).

process of reduction, undergoing oxidation back to the nitro compound under the effect of oxygen.[15]

To reflect the intracellular degradation of pro-oligos, **4a** and **4b** were incubated in human leukemia CEM cell extract. As shown in Fig. 1(b), pro-oligos **4a** and **4b** were hydrolyzed to the model oligo, **5**, with half-lives around 4 h under N_2, whereas the half-lives of **4a** and **4b** were more than 9 h in air. The faster release of the pro-oligos in N_2 than in air by nitroreductases, both *in vitro* and in simulated *in vivo* environment, suggests that such pro-oligo design could be potentially used in the specific targeting of tumor cells which are under the most hypoxic conditions, which in turn can realize the tumor cell recognition and lower the side-effects of antisense oligos to normal cells.

The stability of pro-oligos **4a** and **4b** against exonuclease (snake venom phosphodiesterase, SVPDE) and endonuclease (deoxyribonuclease I, DNase I) was evaluated and compared to model oligo **5** (shown in Fig. 2 and ESI, Table S2†). Model

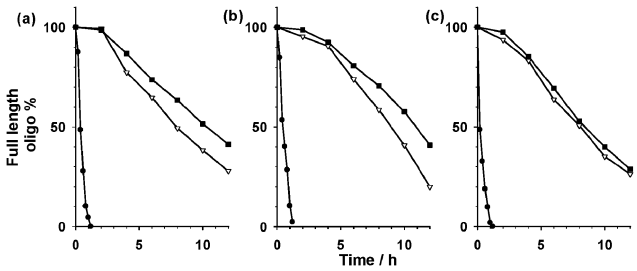
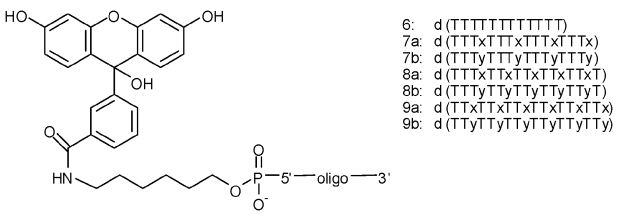


Fig. 2 A comparison of the stability of pro-oligos **4a** (▽) and **4b** (■), and oligo **5** (●) in the presence of SVPDE (a) and DNase I (b), as well as in fetal bovine serum (c).

oligo **5** was rapidly hydrolyzed under the effect of both SVPDE and DNase I with half-lives at 0.30 and 0.35 h, respectively. Both pro-oligos **4a** and **4b** demonstrated significantly improved nuclease resistance compared to oligo **5**, with much longer half-lives in the range of 8–10 h. The stability of these three oligos in 10% fetal bovine serum was also studied. The half-life of oligo **5** was only 0.2 h, whereas the half-lives of **4a** and **4b** were 8.2 h and 8.5 h, respectively. As a comparison, the reported half-lives of a pro-oligo with an *S*-acyl-2-thioethyl group is 3 h in human serum and 19 h against SVPDE[8] Our system has shown a significantly enhanced nuclease resistance in human serum. The stability enhancement of pro-oligos **4a** and **4b** compared to non-modified oligo **5** strongly suggest that pro-oligos with both nitrofuryl and nitrothienyl modifications have good resistance against different nucleases, and thus have potential applications in gene therapy.

A key challenge for the biomedical application of pro-oligos is to improve the cellular uptake. In order to evaluate the *trans*-membrane ability of our pro-oligos, fluorescein-labelled oligo and pro-oligos with 4–6 protecting nitroheterocylcle groups were synthesized (sequences are shown in Scheme 4). After incubation with HeLa cells without any transfecting reagent at 37 °C for 3 h, no fluorescence was detected for the non-modified oligo under a confocal fluorescence microscope, while as a distinctive comparison, all pro-oligos showed fluorescence emission with different intensities, indicating different degrees of cellular uptake (Fig. 3). The cellular uptake was related to the lipophilicity of the oligos. As we expected, the cellular uptake of pro-oligos increased with the number of protecting groups. The most lipophilic pro-oligos, **9a** and **9b**, were more efficiently taken up by cells, whereas the fluorescence signal for the most hydrophobic pro-oligos, **7a** and **7b**, was remarkably reduced. The cellular uptake was similar to that of the *S*-acyl-2-thioethyl protected pro-oligos.[7,16]

In summary, we have demonstrated for the first time the design of a hypoxia-activated pro-oligo with T-sequence



Scheme 4 The structures of the fluorescein-labelled oligo and pro-oligos.

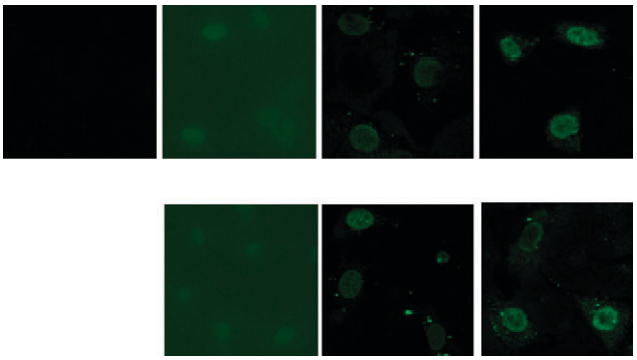


Fig. 3 Incubation of oligo **6** and pro-oligos **7–9** at 10 μM concentration with Hela cells at 37 °C for 3 h. The first row from left to right shows oligo **6**, and pro-oligos **7a**, **8a** and **9a**; the second row from left to right shows pro-oligos **7b**, **8b** and **9b**.

synthesized using simple monomer preparation and the commercial phosphoramidite route. These pro-oligos, which contain mixed normal linkages and nitroheterocyle-protecting phosphate internucleoside linkages, can be readily cleaved to form normal oligos with good hypoxia-selectivity *in vitro* under the effect of reductases, as well as in tumor cell extract by cellular reductases. Moreover, the pro-oligos demonstrated significantly improved nuclease resistance and cellular uptake. Such favorable properties make these hypoxia-activated pro-oligos good candidates for therapeutics to specifically inhibit tumor related gene expression with low toxicity to normal cells.

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