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OLIGONUCLEOTIDE-HIRULOG18 CONJUGATED BY CLICK CHEMISTRY AND ITS INHIBITION ON THROMBIN

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Abstract- We have shown previously that oligonucleotide conjugated hirulog (bivalirudin) has a higher inhibitory activity against thrombin compared with hirulog. The negative charged oligonucleotide was considered to be responsible for the additive activity. To further investigate the related effect of oligonucleotide, the oligonucleotide conjugated hirulog18 was prepared in the present work and the activity of the conjugate against thrombin was measured. Hirulog18, a peptide lack of two N-terminal amino acid residues of hirulog, has little inhibitory activity against thrombin compared with hirulog. The conjugate oligonucleotide-hiruglog18 was successfully synthesized using click chemistry and validated by MALDI-MS and gel electrophoresis. The activity of the conjugate against thrombin was measured by the chromogenic assay using Chromozym TH as substrate. It was found surprisingly that oligonucleotide-hirulog18 had an inhibitory effect better than hirulog18 and hirulog. Strong negative charged heparin was used to study the binding mode between oligonucleotide-hirulog18 and thrombin. The results suggested that the negative charged oligonucleotide could be helpful for the conjugate's binding to the anion-binding exosite of thrombin via the Coulomb force. The highly inhibitory effect of the oligonucleotide-hirulog18 conjugate against thrombin may present a new strategy to generate a novel class of direct thrombin inhibitors.

Key words- oligonucleotide, hirulog, conjugated, thrombin, heparin.

Introduction

DNA is selected as the genetic material in nature because of its unique structure for base-pairing and extraordinary chemical properties. In the research field, scientists focus their attention on taking use of DNA properties for the development of its applications. The major and minor grooves of DNA duplex is able to intercalate small molecules which could be used as therapeutic agents [1]; DNA duplex were employed as template to direct chemical reaction [2, 3]; DNA single strands were designed to create 2D lattices [4], nano devices [5] and even 3D crystals [6].

Recently, we succeeded in taking use of 2-D DNA lattice to investigate the lattice morphologies of the combination of hirulog (also called bivalirudin or angiomax) and thrombin by AFM. Hirulog is a peptide that derived from hirudin. Hirudin is an excellent reversible thrombin inhibitor binding to both the anion binding exosite and active site of thrombin with its C terminus and N terminus, respectively [7]. To find out whether conjugated hirulog is still active, we also measured the thrombin inhibitory activity of oligonucleotide-hirulog DNA lattice and oligonucleotide-hirulog. The results showed that both of them had higher inhibitory activities than hirulog alone. We preliminarily owed the reason to the negative charge of

DNA strand.

In this paper, we chose hirulog18, another thrombin inhibitor, to further investigate the effect of DNA strand on the inhibitory activity of oligonucleotide-peptide against thrombin. Hirulog18 was firstly synthesized as a competitor or antidote for hirulog [8]. Compared with hirulog, hirulog18 (molecular structure see Scheme S2 in the Supporting Information) is only two amino acids shorter, without D-phenylalanine and proline at the N terminus, while its activity against thrombin is much lower than hirulog. To observe the effect of the DNA strand on the inhibitory activity, the conjugate oligonucleotide-hirulog18 was then synthesized by click chemistry and its activity was measured by using the chromogenic substrate method. In the end, strong negative charged heparin was used to study the binding mode between oligonucleotide-hirulog18 and thrombin.

Click chemistry has been recently recognized as the most practical chemical transformations to make molecular connections with excellent fidelity in biomaterials and nanostructures [9]. It is a 1,3-dipolar cycloaddition of alkynes and azides to give substituted 1,2,3-triazoles [10]. As shown in Scheme 1, firstly, the two esterized azide alkyne, succinimidyl-11-azidoundecanoate and succinimidyl-3-propiolate were prepared. Then the two products reacted with the amino-terminated oligonucleotide **1** and hirulog18 **2** to produce oligonucleotide-azide **3** and hirulog18-alkyne **4** respectively. Finally, the oligonucleotide-azide was coupled with hirulog18-alkyne to form the target conjugate product **5**, oligonucleotide-hirulog18, using the Cu^{I}-catalyzed [3+2] Huisgen cycloaddition ("click") reaction (Scheme 1c). Similarly, peptide-azide and oligonucleotide-alkyne could also be synthesized and then coupled to make the oligonucleotide-peptide conjugate.

The whole reaction process was detected by MALDI-TOF-MS which is the best way to characterize the intermediates and the oligonucleotide-conjugated species with the measurement of their molecular mass. Oligonucleotide-azide **3**a, prepared by azidization of 5'-amino modified 25-mer oligonucleotide **1,** was purified by electrophoresis, and the other part of the conjugate, hirulog18-alkyne **4**, was purified by HPLC (the HPLC trace is in Figure S1 of Supporting Information). Both products were confirmed by MALDI-TOF-MS in Figure 1. The molecular weight (MW) of 5'-amino oligonucleotide **1** is 7811.8 Da in Figure 1a. After tagging an 11-azidoundecanoate, the MW of the product **3** should be increased theoretically by 209 to 8020.8 Da, our experimental value is 8020.1 Da (Figure 1c). MW of hirulog18 is 1935.8 Da (Figure 1b), while MW of the alkyne-modified product **4** is 1987.8 Da (Figure 1d), 52 Da over hirulog18 corresponds to the propionate group. After click chemistry, the theoretical MW of oligonucleotide-hirulog18 **5** is 10007.9 Da, sum of oligonucleotide-azide and hirulog18-alkyne. Practically we got a main peak at 10007.1 Da (Figure 1e).

Gel electrophoresis images in Figure 2 also validated the conjugation of oligonucleotide-azide **3** and hirulog18-alkyne **4**. 5'-Amino oligonucleotide **1** in the left lane was used as a control. The central lane was loaded with oligonucleotide-azide **3**, which was a little bit higher than **1**, because **3** was only 209 Da larger than **1** and the azido species carries no charges. The oligonucleotide-hirulog18 **5**, shown in the last lane, ran slowly and stayed on the upper position of the gel because compared to **1** and **3**, oligonucleotide-hirulog18 carries an additional 18 amino acid peptide with net negative charges. The reaction yield was estimated at over 90% from the gray scale of **5** and **3**.

Typically, the inhibition of thrombin activity is used to evaluate its inhibitors [11]. Thrombin could bind to the substrate Chromozym TH, and the substrate was then cleaved into two parts, a residual peptide and a yellow substance 4-nitraniline which could be measured by UV-vis. If the substrate has no competitor, the OD will be high. However, if there is a competitor such as hirulog, the binding between thrombin and the substrate will be prohibited as OD decreases. In this study we followed the chromogenic substrate method to assay the activity of hirulog18, oligonucleotide-hirulog18 and hirulog against thrombin and the result was listed in Figure 3. In the experiment, thrombin, hirulog18, oligonucleotide-hirulog18 and hirulog were all dissolved in PBS buffer. To make the samples comparable to each other, they were made in the same concentration and volume. Experimental details were in supporting information. From the data, we could get three pieces of information: 1) hirulog18 had a weak

inhibitory activity against thrombin because its absorbance was just a little bit lower than that of positive control; 2) for contrast, we also measured the OD value of hirulog, its average was 0.944 which showed higher activity against thrombin than that of hirulog18; 3) the inhibition of oligonucleotide-hirulog18 against thrombin seemed to be much better than that of hirulog18, because its average OD value at 405 nm was only 0.45 which meant the inhibitory activity of oligonucleotide-hirulog18 was even better than that of hirulog.

Why the difference of anti-thrombin activity between hirulog18 and oligonucleotide-hirulog18 was so big? Obviously, the different structure was the point. Oligonucleotide-hirulog18 was a 25-base oligonucleotide and a linker with carbon chain and triazoles longer than hirulog18. To know which part effected the different activity, we also did another experiment by linking the hirulog18-alkyne with 5-azidopentanoic acid and testing its activity against thrombin, the result of which was almost the same as hirulog18 itself (see Supporting Information). This indicated that oligonucleotide was critical for improving the activity of oligonucleotide-hirulog18 against thrombin.

To further confirm the role of oligonucleotide in thrombin inhibition, negatively charged heparin was used. Heparin is often used as an anti-coagulation drug. It enhances the affinity of thrombin with anti-thrombin III [12]. Heparin was added to hirulog18, oligonucleotide-hirulog18 and hirulog in the thrombin activity assay as shown in Figure 4. Compared to hirulog18, the average OD value of hirulog18 & heparin was 0.018 higher, which seemed that heparin had little effect on the interaction between hirulog18 and thrombin. However, the difference of OD value between oligonucleotide-hirulog18 and oligonucleotide-hirulog18 & heparin was 0.835, and that of hirulog with hirulog & heparin was 0.29, which indicated heparin affected the interaction of oligonucleotide-hirulog18 and hirulog with thrombin indeed. The average OD value of hirulog18 & heparin, oligonucleotide-hirulog18 & heparin and hirulog & heparin was 1.244, 1.239 and 1.252, respectively. It seemed that heparin could not distinguish their different activity against thrombin. It is most likely explained as negative charged heparin blocked the binding of hirulog18, oligonucleotide-hirulog18 and hirulog to the anion-binding exosite of thrombin at first, effected their binding to the active site further, then disabled these inhibitors against thrombin. In other words, the charge recognition was important for the binding of inhibitors with thrombin and the improved inhibition of oligonucleotide-hirulog18 against thrombin was due to the negative charged DNA strand.

In conclusion, we firstly synthesized oligonucleotide-hirulog18 by click chemistry, and measured its activity against thrombin in comparison with hirulog18 and hirulog. It was discovered that oligonucleotide-hirulog18 had a better inhibitory activity than hirulog18 and even better than hirulog through the negative charged DNA strand. This may present a new strategy to generate a novel class of direct thrombin inhibitors.

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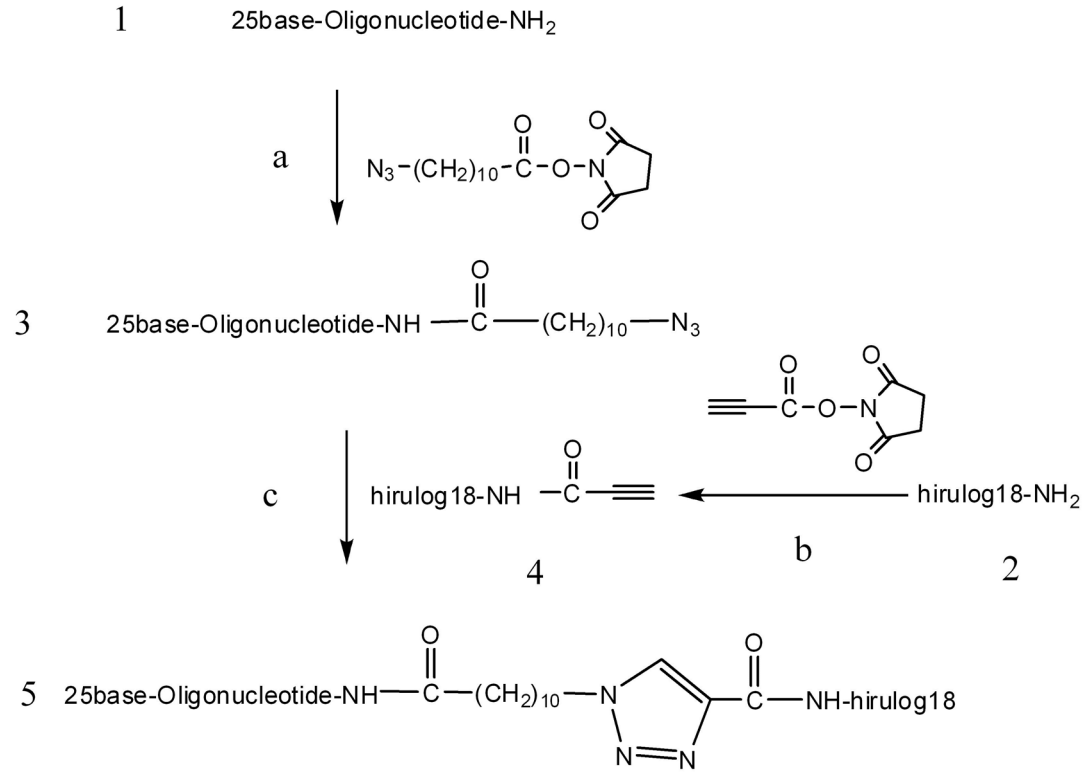

Scheme 1- Preparation of Oligonucleotide-hirulog18 Conjugate [a]

[a]5'-Amino oligonucleotide **1** reacted with succinimidyl-11-azidoundecanoate at pH = 9.0 to yield oligonucleotide-azide **3** (a). N-Terminal hirulog18 **2** reacted with succinimidyl-3-propiolate at pH = 9.0 to obtain hirulog18-alkyne **4** (b). Oligonucleotide-azide **3** reacted with hirulog18-alkyne **4** under the condition of $CuSO_4$, TBTA and TCEP in the phosphate

buffer (pH = 7.3) to yield oligonucleotide-hirulog18 **5** (c). TBTA = tris(benzyltriazolylmethyl)amine; TCEP = tris(2-carboxyethyl)phosphine.

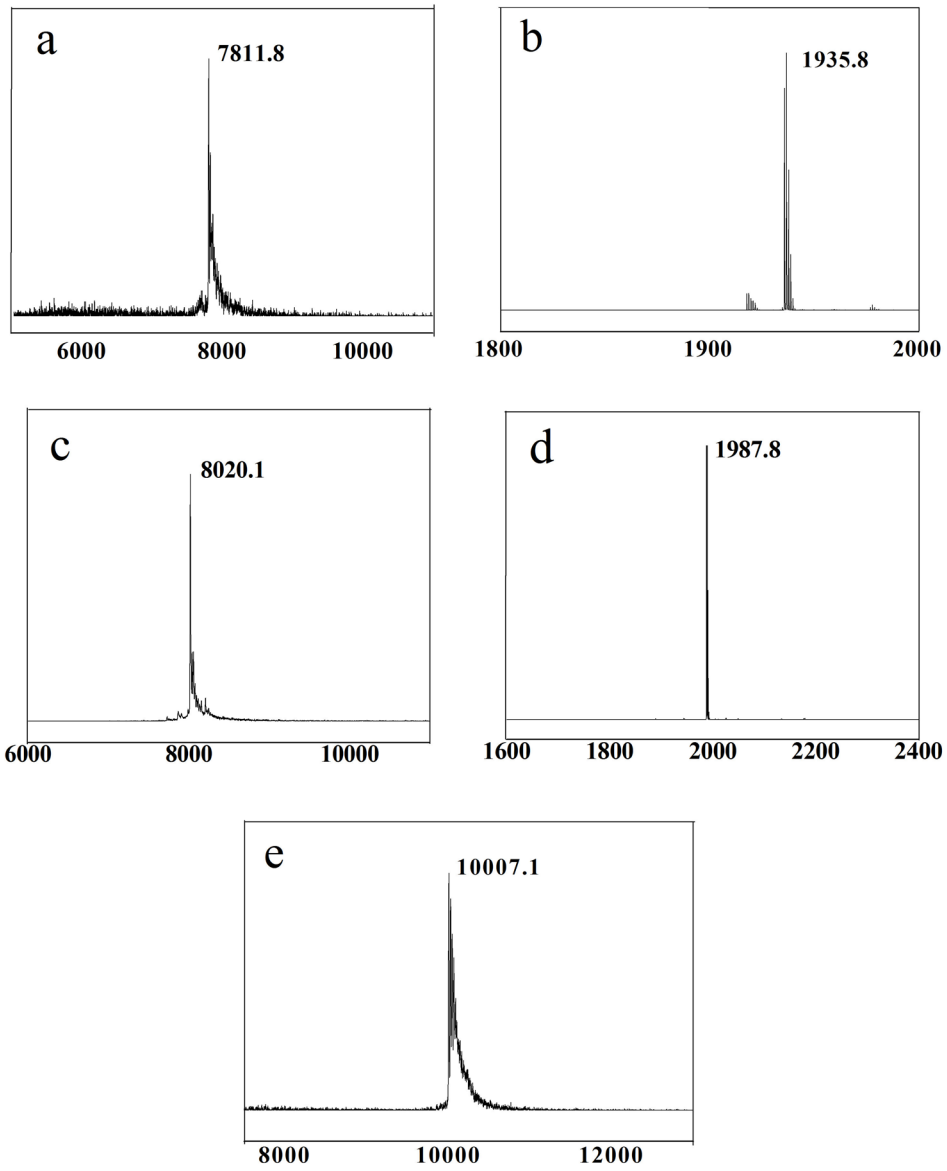

Fig. 1-MALDI-TOF-MS spectra: (a) 5'-amino oligonucleotide **1**; (b) hirulog18 **2**; (c) oligonucleotide-azide **3**; (d) hirulog18-alkyne **4**; (e) oligonucleotide-hirulog18 **5**.

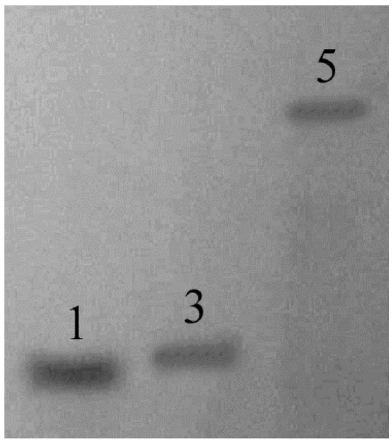

Fig. 2- Gel electrophoresis image: the left band (1) is 5'-amino oligonucleotide **1**; the central band (3) is oligonucleotide-azide **3**; the right band (5) is oligonucleotide-hirulog18 **5**

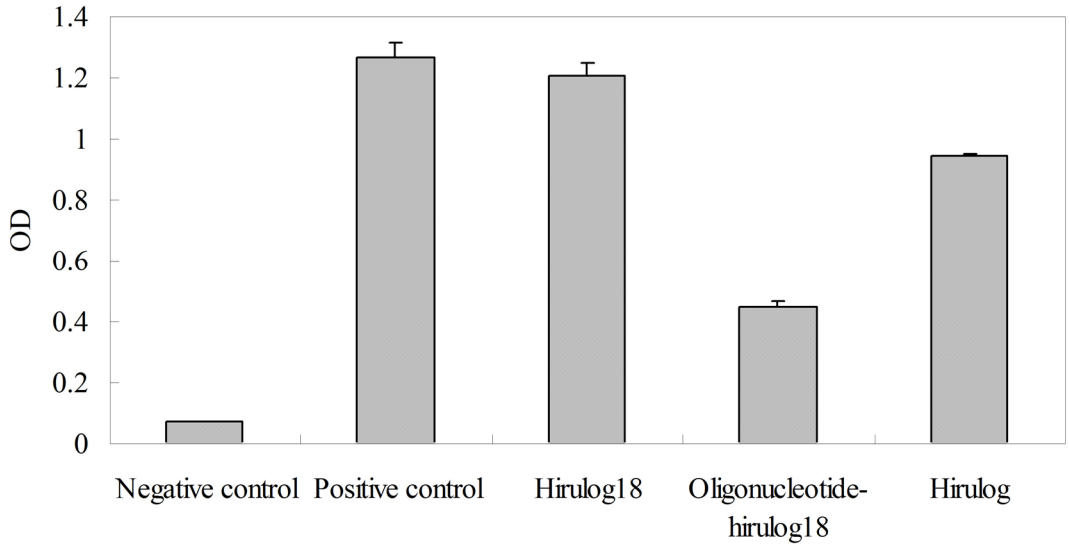

Fig. 3-The activity assay of hirulog18 and oligonucleotide-hirulog18, in which the chromogenic substrate method was used. Negative control was composed of Chromozym TH and buffer; positive control contained thrombin, Chromozym TH and buffer; hirulog18 represented hirulog18, thrombin, Chromozym TH and buffer; oligonucleotide-hirulog18 was for oligonucleotide-hirulog18, thrombin, Chromozym TH and buffer and hirulog for hirulog, thrombin, Chromozym TH and buffer. The total volume of each sample was the same. Data were expressed as Mean ± Standard Deviation (STDEV).

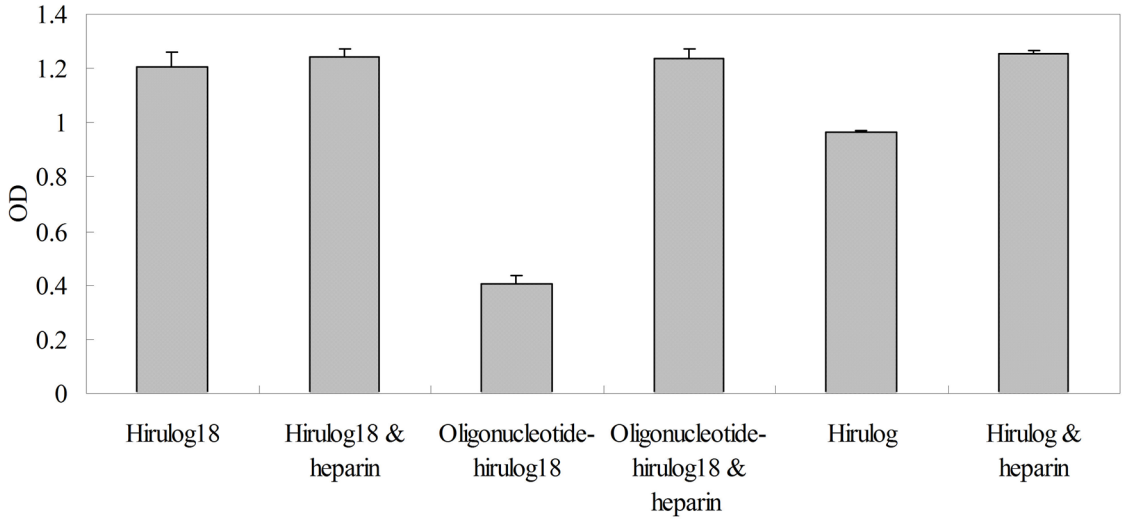

Fig. 4-The activity assay of hirulog18, oligonucleotide-hirulog18, hirulog and their sample with heparin. The total volume of each sample was the same. Data were expressed as Mean ± Standard Deviation (STDEV).