

# Improving the bioactivity of rHirudin with boronophenylalanine site-specific modification

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Received September 6, 2013; Accepted May 2, 2014

DOI: 10.3892/mmr.2015.3194

**Abstract.** To improve the bioactivity of recombinant (r) Hirudin, the orthogonal pair MjB<sup>Tyr</sup>RS/tRNA<sup>Tyr</sup> CUA (made up of the boronophenylalanine, tRNA and tRNA synthetase), was selected to incorporate boronophenylalanine site-specifically into rHirudin at the 63 sites in an *Escherichia coli* system in response to the TAG codon. Following fusion with the gIII signal peptide and a hexahistidine tag, the modified protein was secreted into Luria-Bertani culture medium and purified by nickel-nitrilotriacetic acid affinity chromatography following a gel filtration column. In a 200 ml flask, the yield of boronophenylalanine-modified hirudin was 10 mg l<sup>-1</sup> and that of rHirudin was 19 mg l<sup>-1</sup>. The authenticity of the purified proteins was verified using matrix-assisted laser desorption ionization time of flight mass spectroscopy and antithrombin activity assays. The results revealed that the antithrombin activity of the boronophenylalanine-modified hirudin to human thrombin was more enhanced than that of rHirudin. The modified hirudin demonstrated stronger proliferation inhibiting ability on fibroblast L929 cells compared with that of rHirudin.

## Introduction

Hirudin is a human thrombin-specific inhibitor, the natural form of which was extracted from the salivary gland of medicinal leech *Hirudo medicinalis*. It is composed of 65-66 amino acid residues with a molecular weight of ~6,800 Da. Hirudin has three intramolecular disulfide bonds and a tyrosine sulfation at the 63 site, to acquire a native conformation and antithrombin activity (1). As a human thrombin-specific

inhibitor, hirudin has an increasing number of applications in the clinic (2,3). As the production of hirudin from *Hirudo* is relatively inefficacious, at present, almost all of the therapeutic hirudin is generated by recombinant bacteria.

Though recombinant hirudin (rHirudin) has antithrombin activity and has been expressed in several expression systems, including *Escherichia (E.) coli*, *Saccharomyces* and *Hansenula polymorpha* (4), the antithrombin activity of rHirudin appears to be lower than that of the native form, which is extracted from *Hirudo*. This may be because rHirudin does not bear any sulfate on the 63 tyrosine amino acid residue, as the expression systems used recently appear to lack the sulfotransferase accessory, particularly in the *E. coli* expression system (2).

In order to improve the bioactivity of rHirudin, several modification methods have been applied and site-specifically substituted hirudins have been produced (5). In these studies, L-phenylalanine-modified dodecapeptide was synthesized at position 63 using an Fmoc solid-phase synthesis strategy. The dodecapeptide prolonged the thrombin time to two times more than the recombinant ones *in vitro* (6). Liu *et al* (5) incorporated the unnatural amino acid of sulfo-tyrosine into hirudin on 63 sites using the MjS<sup>Tyr</sup>RS/tRNA<sup>Tyr</sup> CUA orthogonal pair in an *E. coli* expression system. The site-specifically modified hirudin was purified and functional analysis demonstrated that the sulfation at site 63 of hirudin was a crucial factor in the formation of the hirudin/thrombin complex. It interacted directly with Lys 81 and Tyr 76 of human thrombin, formed a strong salt bridge and a new hydrogen bond network. This was a marked difference from that of the rHirudin/thrombin complex. The sulfate group was able to augment the affinity of sulfahirudin with thrombin and gave a lower inhibition constant K<sub>i</sub> of 25 fM than the rHirudin/thrombin complex with a K<sub>i</sub> of 307 fM (5). The prolonged thrombin time of the carboxymethyl-phenylalanine-modified peptide and the enhanced affinity of sulfated hirudin for human thrombin suggested that the different group residues that existed at the 63 site appeared to be important factors affecting hirudin biofunction.

Boronic acids are known to form complexes with amino acids or hydroxamic acids and have been employed as moieties in ligands for the selective recognition of sugars and have also been used as potent serine protease inhibitors (7). In addition, boronates have clinical utility as boron neutron capture agents for selectively targeting tumor cells (8). The novel chemical

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**Key words:** antithrombin activity, boronophenylalanine, proliferation inhibition, recombinant Hirudin, site-specific modification

properties of boronic acids as components of proteins allows for selective chemistry on the protein surface, including oxidation, reduction, Suzuki coupling reactions, as well as the formation of covalent boronic esters with polyhydroxylated compounds. Proteins modified by addition of boronic acid moieties may be used in combination with a polyhydroxylated solid support to purify native protein sequences in a one-step affinity purification procedure. Furthermore, the ability of boronic acids to bind diols and reactive serine residues allows for the development of boronate-containing antibodies that specifically recognize and covalently bind to various glycoproteins or proteases. It may also be possible to form intramolecular serine-boronate crosslinks in proteins to enhance stability (9,10). The unique chemical characteristics of boronates may allow for the *in vivo* labeling of boronate-containing proteins with polyhydroxylated reporter molecules.

In the present study, using an codon-expanding method, the boronic-amino acid of boronophenylalanine was site-specifically incorporated into target proteins. Previously, Liu and Schultz (11) have successfully used and evolved  $MjS^{Tyr}RS/tRNA^{Tyr}_{CUA}$  orthogonal pairs to substitute the tyrosine at site 63 with sulfotyrosine. The affinity of sulfohirudin was evidently enhanced as compared with that of rHirudin. The orthogonal pair  $MjB^{Tyr}RS/tRNA^{Tyr}_{CUA}$  is able to recognize and incorporate boronophenylalanine efficiently into proteins of interest in the *E. coli* expression system (12) and is not recognized by endogenous tRNAs and aminoacyl-tRNA synthetases. Using this orthogonal pair, the 63 tyrosine of rHirudin was substituted with unnatural amino acids (NAAs) in order to give the protein specific new biofunctions.

Furthermore, it has been reported that hirudin may inhibit the proliferation of fibroblasts and have an important role in wound healing (13). The fibroblast cell line L929 was considered as a primary source of extracellular matrix components. L020 cells have an important function in wound healing, as they were demonstrated to have a critical role in regulating the turnover of the extracellular matrix and have been widely used in cell functional studies (14). In the present study, the effects of the boronophenylalanine-modified hirudin on the proliferation of fibroblasts cells was examined and compared with that of rHirudin. The enhanced bioactivity and convenient synthetic procedure suggested a feasible method for the site-specific modification of hirudin.

## Materials and methods

**Plasmid construction and strains.** The plasmid pEVOL- $MjB^{Tyr}RS/tRNA^{Tyr}_{CUA}$  was used as the boronophenylalanine incorporation vector. The plasmid pBAD-gIII-hirudin was used as a template to construct pBAD-gIII-hirudin-TAG-6xhis tag. All of these plasmids were generously donated by Professor W Liu group of the A&M University (College Station, TX, USA). The gene sequence of hirudin was in accordance with the National Center for Biotechnology Information GenBank entry (accession no., GI: 208479; <https://www.ncbi.nlm.nih.gov/genbank/>). Using a modified Quickchange strategy (15) to construct the new expression plasmid, a TAG sequence at the 5'-end was designed to substitute the original tyrosine codon TAC. The sequence of the forward primer was 5'-TAGCTGCAATGACTCGAG

ATCTG-3' and the reverse primer sequence was 5'-TTCTTC CGGAATTTCTTCAAAATCGCC-3'. To fuse a 6xhis sequence at the 3'-end, the forward primer was 5'-ATGGTG ATGTTGCAGCTAATTCTTCCGGAATTTTC TTC-3' and the reverse primer was 5'-CACCATCACTGACTCGAGATC TGCAGCTGGTAC-3', there were three histidine codons (underlined) in each primer. The constructed plasmids were confirmed by DNA sequencing.

Top10 Electrocomp *E. coli* cells Novagen (Madison, WI, USA) were used to express hirudins with site-specifically incorporated NNAs. The cells transformed with plasmids pBAD-gIII-hirudin-6xhis and pEVOL were used as controls to express rHirudin.

**Expression and purification of the proteins.** A single colony of the recombinant *E. coli* Top 10 cells harboring the pBAD-gIII-hirudin-TAG-6xhis tag and pEVOL was collected and cultured at 37°C overnight in 5 ml Luria-Bertani (LB) medium containing ampicillin (100  $\mu$ g ml<sup>-1</sup>) and chloramphenicol (34  $\mu$ g ml<sup>-1</sup>) (Takara Biotechnology Co., Ltd., Dalian, China). The cultures were diluted 1:100 in a 250-ml flask with 50 ml LB and grown at 37°C with agitation at 4.3 x g until the optical density at 600 nm (OD<sub>600</sub>) reached 0.6. Then, L-arabinose was added to a final concentration of 0.2%, followed by a further 16 h incubation at 37°C to induce rHirudin expression.

To express the site-specifically modified hirudins, a single colony of the recombinant *E. coli* Top 10 cells harboring the pEVOL- $MjB^{Tyr}RS/tRNA^{Tyr}_{CUA}$  mutant and pBAD-gIII-hirudin-TAG-6xhis tag plasmids were cultured at 37°C overnight in 5 ml LB containing ampicillin (100  $\mu$ g ml<sup>-1</sup>) and chloramphenicol (34  $\mu$ g ml<sup>-1</sup>). The culture was diluted 1:100 in a 250-ml flask with 50 ml LB. Boronophenylalanine (1 mM; Sigma-Aldrich, St. Louis, MO, USA) was added to the medium and grown at 37°C, 4.3 x g until OD<sub>600</sub> reached 0.6. L-arabinose was added to the medium to a final concentration of 0.2%, following another 16-h induction at 37°C.

Next, the medium was collected and concentrated through the ultra-filter apparatus with 3 kD cut-off membranes. After filtering through a 0.45  $\mu$ m filter, the samples were loaded on an AKTA-purifier system (Amersham Biosciences, Uppsala, Sweden). It was equipped with a nickel nitrilotriacetic acid (Ni-NTA) affinity column which was pre-equilibrated with buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0). Proteins of interest were eluted using elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 300 mM imidazole, pH 8.0) at an elution rate of 0.4 ml/min. The fractions with anticoagulant activity were collected, concentrated and dialyzed against 25 mM Tris-HCl, 10 mM NaCl and 5% glycerol (pH 7.6) for 3 h at 4°C. The dialysis buffer was changed three times prior to sample concentration. Each of the concentrated samples were loaded on a gel filtration column (Superdex 200 10/300 GL; GE Healthcare, Pittsburgh, PA, USA) for further purification. The proteins of interest were eluted with 25 mM Tris-HCl, 125 mM NaCl and 2 mM KCl (pH 7.6) at an elution rate of 0.2 ml/min. The collected samples were stored at 4°C for further detection.

**Tricine/SDS-PAGE and matrix-assisted laser desorption time-of-flight (MALDI-TOF) analysis.** Tricine/SDS-PAGE analysis of the purified protein samples was performed according to Schägger (16). The low-molecular weight marker

was obtained from Fermentas (Vilnius, Lithuania). The gels were stained with Coomassie Brilliant R-250. Mass analysis of the purified proteins was performed using MALDI-TOF mass spectrometry on an ABI 4800 MALDI/TOF analyzer (Applied Biosystems, Foster City, CA, USA).

**Antithrombin activity.** Antithrombin activity was assessed by titration of a thrombin solution and the thrombin neutralizing activity of hirudins was determined in antithrombin units (ATU) (17). Briefly, 200  $\mu$ l standard solution of 0.05% bovine fibrinogen (Ameresco, Inc., Columbus, OH, USA) was mixed with 10  $\mu$ l hirudin samples and 5  $\mu$ l thrombin [47 National Institute of Health (NIH) units; Ameresco, Inc.], mixed gently and left to stand for 1 min at 37°C. If the fibrin did not clot, the hirudin samples had 47 ATU. The hirudin samples were diluted to 1 mg/ml in the working buffer (50 mM Tris, pH 7.5, 100 mM NaCl; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) in a series of gradient as 1:100, 1:200, 1:500, 1:1,000, 1:3,000 and 1:6,000. A total of 10  $\mu$ l diluted solution each were incubated with 200  $\mu$ l 0.05% bovine fibrinogen in Tris-HCl buffer (pH 7.4) at 37°C, 5  $\mu$ l (47 NIH units) of standard thrombin solution was added progressively at intervals of 1 min and mixed gently. The end point of the titration was considered reached when a fibrin clot formed within 1 min.

**Protein analysis.** Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Columbia, SC, USA) according to the manufacturer's instruction.

**Fibroblast proliferation test.** The fibroblast cell line L929 (#CCL-1; American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI-1640 supplemented with 100 mg l<sup>-1</sup> benzylpenicillin, 100 mg l<sup>-1</sup> streptomycin and 10% fetal calf serum (Gibco Life Technologies, Grand Island, NY, USA). Cells were seeded in 96-well dishes (5x10<sup>3</sup> cells/well) and incubated under sterile conditions at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. A total of 16 h later, 1, 0.5, 0.25, 0.125 and 0 ATU  $\mu$ l<sup>-1</sup> hirudin solutions, respectively, were added to the medium. The solution concentration was 1.158  $\mu$ g ml<sup>-1</sup> for the boronophenylalanine-modified hirudin and 1.475  $\mu$ g ml<sup>-1</sup> for rHirudin. Cell proliferation was assessed using an MTT assay following 24 h (18).

**Statistical analysis.** SPSS software, version 20 (IBM SPSS, Armonk, NY, USA) was used for all statistical analyses. Data are expressed as the mean  $\pm$  standard deviation. P<0.05 was considered to indicate a statistically significant difference compared with the hirudin-untreated sample.

## Results

**Plasmid construction.** The gIII signal peptide nucleotide sequence and hirudin gene hv II were fused with 6xhis codons at the 3'-end of the hirudin gene on the expression vector of pBAD (Fig. 1). Next, a TAG mutation was introduced into the fusion gene at position 63 of the hirudin gene to substitute the original TAC codon (Fig. 1B). The constructed plasmids were confirmed by DNA sequencing.

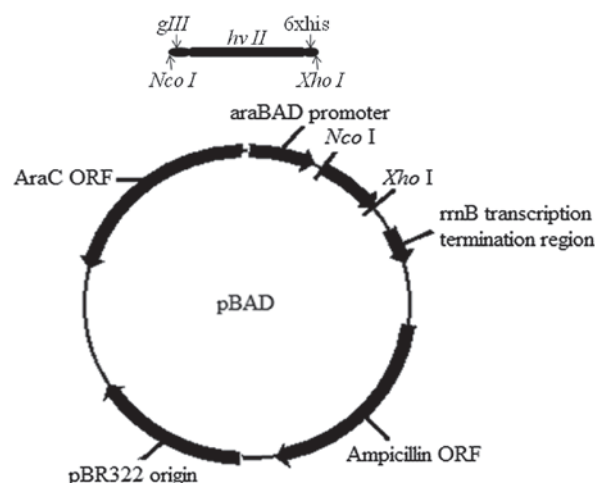


Figure 1. Plasmid construction of pBAD-g III-hv II-TAG-6xhis vector. gIII, signal peptide gene at the 5'-terminal; hv II, hirudin gene; 6xhis, 6 histidine tag; hv II-TAG, hirudin gene mutant with a TAG codon introduced at position 63.

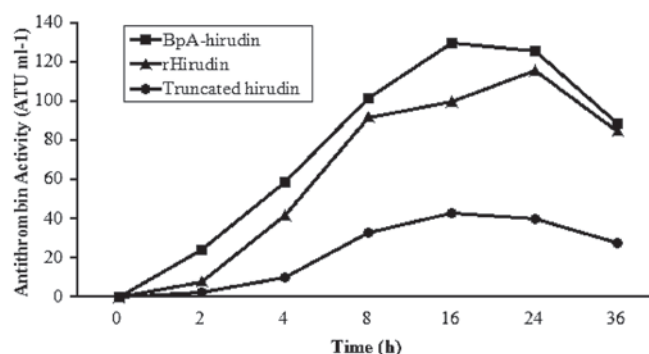


Figure 2. Time course of the antithrombin activity in culture medium of BpA-hirudin, rHirudin and the truncated hirudin peptides. rHirudin, recombinant hirudin; BpA-hirudin, boronophenylalanine-modified hirudin; ATU, antithrombin units.

**Expression of boronophenylalanine-modified hirudin and rHirudin.** The plasmids pEVOL-M/JB<sup>Tyr</sup>RS/tRNA<sup>Tyr</sup> CUA and pBAD-gIII-hv II-TAG-6xhis were co-transformed into *E. coli* Top 10 and named as Top 10(pBAD-hv II-TAG-pEVOL-B). The translation of the fusion protein was started at the start codon of the gIII signal peptide at the 3'-terminal and followed by the hirudin gene and a 6xhis-hirudin, which was controlled by the araB promoter.

The expression of the recombinant vectors was induced by 0.2% arabinose and 1 mM boronophenylalanine. The time course of hirudin expression was examined using a thrombin inhibitory assay. The medium exhibited antithrombin activity following 2 h. The boronophenylalanine-modified hirudin had an antithrombin activity of ~130 ATU ml<sup>-1</sup> culture medium following 16 h, and that of rHirudin was ~100 ATU ml<sup>-1</sup> after 16 h. The truncated derivative was about 40 ATU ml<sup>-1</sup> at 16 h. After this, the antithrombin activity of the three types of hirudins decreased with time, particularly after 24 h (Fig. 2).

**Purification of hirudin products.** The fractions which exhibited antithrombin inhibitor activity were collected and eluted with 300 mM NaCl-buffer B from the Ni-NTA

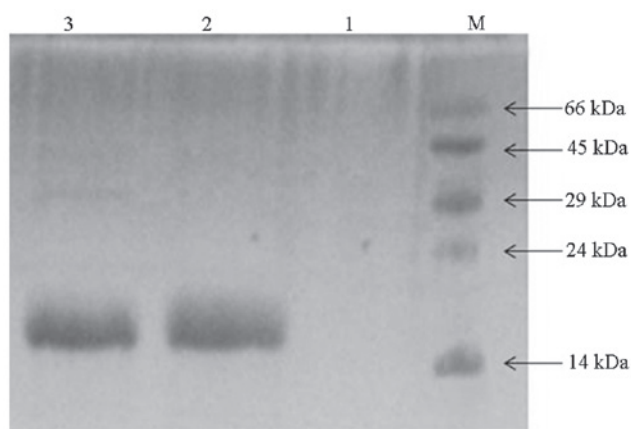


Figure 3. Tricine/SDS-PAGE analysis of three types of hirudins on 18% (w/v) resolving gel. Lane M, low molecular protein marker (kDa); lane 1, negative control, no unnatural amino acids were in the medium, no His-tag at the C-terminal of the truncated rHirudin and no protein was detected on the gel; lane 2, the purified rHirudin; lane 3, the purified boronylphenylalanine site special modified hirudin.

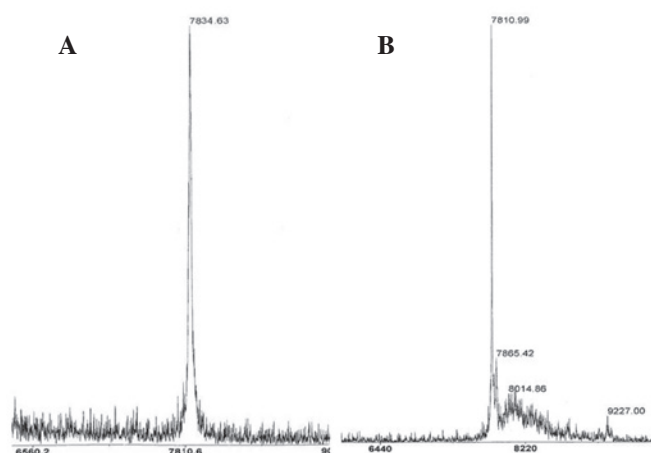


Figure 4. MALDI-TOF mass spectrometry. (A) MALDI-TOF mass spectrometry of purified boronylphenylalanine-modified hirudin. The predominant [M+H] peak is 7,834.63 Da and its theoretical molecular weight is 7,834.5 Da. (B) MALDI-TOF mass spectrometry of the purified rHirudin. The predominant [M+H] peak is 7,810.99 Da and its theoretical molecular weight is 7,810.5 Da. MALDI-TOF, matrix assisted laser-desorption time-of-flight.

affinity column. Following concentration, a further purification step was performed with an Superdex-G250 filtration column (Superdex 200 10/300 GL; GE Healthcare). The proteins of interest were eluted out with 25 mM Tris-HCl, 125 mM NaCl and 2 mM KCl (pH 7.6), with an elution rate of 0.2 ml/min. The collected samples demonstrated single bands of ~14 kDa on the coomassie brilliant blue stained tricine-PAGE (Fig. 3 lane 2 and 3), implying the formation of homodimers as reported by previous studies (19). Purification was performed on Ni-NTA, which only has affinity for positively charged compounds. The hirudins fused with a 6xhis tag at the C-terminus bound to the solid phase of the column and were then eluted, as were the protein samples of rHirudin (lane 2) and the full length boronophenylalanine-incorporated hirudin (lane 3). The negative control confirmed that there appeared to be no protein from the PAGE-gel, as revealed in lane 1. No boronophenylalanine was added to the negative

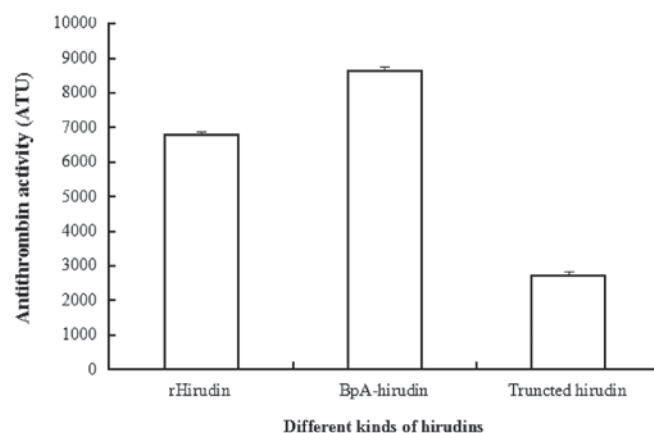


Figure 5. The antithrombin activity analysis of different types of hirudins. The samples were assessed by titrated 10  $\mu$ l hirudin and 5  $\mu$ l thrombin (47 NIH units), mixed gently with 200  $\mu$ l 0.05% bovine fibrinogen and left for 1 min at 37°C until a fibrin clot formed. NIH, National Institute of Health; rHirudin, recombinant hirudin; BPA-hirudin, boronophenylalanine modified hirudin.

control culture medium, so the introduced TAG codon may not be translated into the NAA of boronophenylalanine and a truncated polypeptide was expressed in this case. The expressed truncated hirudin peptide was composed of 62 amino acid residues counted from the N-terminus, but lacked the hexapeptides at the C-terminus, and was not able to bind to the Ni-NTA column, so there was no protein sample eluted (lane 1).

The purity was ~93% for the purified hirudins detected by tricine-PAGE. The yield of the boronophenylalanine site-specifically modified hirudin was 10 mg l<sup>-1</sup> and that of the rHirudin was 19 mg l<sup>-1</sup>.

**Authenticity analysis using MALDI-TOF.** The detected molecular weight of boronophenylalanine-incorporated hirudin was 7,834.6 kDa as determined by MALDI-TOF mass spectrometry. The calculated theoretical molecular weight was 7,834.5 Da, which matched with the experimental result. As for the rHirudin, the MALDI-TOF peak indicated a molecular weight of ~7,810.9 Da (Fig. 4A and B) and the theoretical molecular weight was 7810.5 Da, which confirmed the identity of the proteins.

**Antithrombin activity assay.** The antithrombin activity of rHirudin following purification in the present study was 6,778 ATU mg<sup>-1</sup> protein, the antithrombin activity of the boronophenylalanine-modified hirudin was 8,639 ATU mg<sup>-1</sup> protein following purification. The antithrombin activity of the site 63 boronophenylalanine-modified hirudin was enhanced compared with that of rHirudin (Fig. 5).

**Fibroblast cell proliferation.** Both hirudin solutions were demonstrated to have an inhibitory effect on the proliferation of L929 cells when the concentration was 0.5 U  $\mu$ l<sup>-1</sup>, and this proliferation inhibition of L929 cells was dose-dependent. The proliferation rate of boronophenylalanine-modified hirudin-treated L929 cells at 0.75 and 1 U  $\mu$ l<sup>-1</sup> was significantly lower than that of rHirudin-treated L929 cells (Fig. 6). The inhibition rate of the fibroblasts was increased by 38.4 and 40.9% following treatment with boronophenylalanine-modified



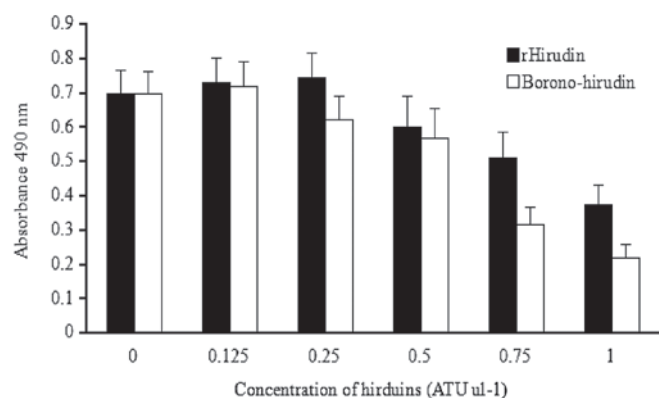


Figure 6. Proliferation inhibition of rHirudin and BPA-hirudin on L929 cells. L929 cells were treated with various concentrations of the two types of hirudin solution (0 U  $\mu\text{l}^{-1}$  was the vehicle in 0.9% NaCl) and cultured under the same conditions. Cell proliferation was assessed using a colorimetric MTT assay. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , BPA-hirudin-treated cells vs. the rHirudin solution-treated cells in each group. rHirudin, recombinant hirudin; BPA-hirudin, boronophenylalanine modified hirudin; ATU, antithrombin units.

hirudin solution at 0.75 and 1 U  $\mu\text{l}^{-1}$ , respectively, compared with the inhibition by rHirudin. At the lower concentration of the hirudin solution ( $<0.5$  U  $\mu\text{l}^{-1}$ ), the growth of the fibroblasts was marginally stimulated, possibly due to the increased nitrogen source from the low-dose hirudin peptides. However, the borono-hirudin solution demonstrated a higher proliferation inhibition effect on the L929 cells than rHirudin at any of the concentrations.

## Discussion

Using the evolved tRNA/aminoacyl-tRNA synthetase (aaRS)/tRNA orthogonal pairs, which are able to recognize and carry boronophenylalanine to the amber stop codon TAG, rHirudin was successfully modified at the 63 site in the *E. coli* expression system and its antithrombin activity was improved.

To avoid the degradation of the cytoplasm and improve the production of hirudin, the gIII signal peptide was fused to the N-terminus of hirudin, which led to the secretion of the proteins of interest into the culture medium efficiently and successfully. A 6x histidine sequence at the C-terminus of the hirudin simplified the purification procedure, as only the full-length proteins carried this histidine tag and bound to the Ni-NTA column. Besides, the hexapeptides at the C-terminus had no evident effect on its anticoagulant activity (6,778 ATU  $\text{mg}^{-1}$  protein) compared with previous studies, in which no His-tag was used (7, 229.13 ATU  $\text{mg}^{-1}$  protein) (18). Following Ni-NTA affinity chromatography and gel filtration, the purity was up to 95%, as detected by SDS-PAGE.

The boronophenylalanine-modified hirudin had an antithrombin activity of 8,639 ATU  $\text{mg}^{-1}$  protein, which was higher than that of rHirudin (6,778 ATU  $\text{mg}^{-1}$  protein). A number of studies demonstrated that boronophenylalanine is able to be used as a negatively charged amino acid, and the boronic acids of it may form reversible covalent complexes with peptides or proteins (20). The side chain had a good spatial overlap with the positively charged arginine residues of the interactive proteins and afforded effective activity to the modified proteins. The

boronic acids have the ability of binding with diols or reactive serine residues, and it was suggested that this unique feature of boronate-containing proteins may specifically recognize and covalently bind various glycoproteins or proteases (21). It may also be possible to form intramolecular serine-boronate crosslinks in proteins and enhance the combining stability between the complexes as previously described (21). Therefore, the boronophenylalanine side chain may be one of the possible reasons for the enhanced antithrombin activity of the modified hirudins, in a similar fashion to the effect of the sulfotyrosine-modified hirudin. Sulfotyrosine was recognized and incorporated specifically into target proteins when an amber nonsense codon with an orthogonal aminoacyl-tRNA synthetase/tRNA pair was employed in the *E. coli* expression system, and the affinity of the resulting sulfotyrosinyl hirudin for human thrombin was enhanced by 10-fold (9). According to a crystal structure study of a sulfo-hirudin - thrombin complex, it was demonstrated that the side chain of the sulfate group on sulfotyrosine at the 63 site formed a strong salt bridge with the positively charged Lys 81 of thrombin, and thus improved the affinity of the interaction between sulfo-hirudin and human thrombin (22).

As described in previous studies, the repressed expression of plasminogen activator inhibitor-1 (PAI-1), the type I collagen  $\alpha$ -chain (COL1A1) and a member the of interleukin family all resulted in the inhibition of proliferation of fibroblasts (23). The modified hirudin may have had a stronger effect on the expression of one or all of the proliferation factors mentioned above. Further studies are required to determine the mechanisms underlying this effect.

In the present study, it was identified that the incorporation efficiency of the boronophenylalanine amber suppressor MjB<sup>Tyr</sup>RS/tRNA<sup>Tyr</sup> CUA pair was higher than that of the reported sulfotyrosine incorporation amber suppressor aminoacyl-tRNA synthetase/tRNA orthogonal pair. A total of ~52.6% full length proteins was produced and the yield was 10 mg  $\text{l}^{-1}$  in a 500-ml flask containing 200 ml culture medium, while only 25% full length protein was generated in the sulfotyrosine incorporation system, due to the low permeability of the anionic sulfotyrosine into *E. coli* cells as described previously (11).

The improved bioactivity and protein yield of the boronophenylalanine-modified hirudin suggested a possible clinical application of the boronophenylalanine recognition and incorporation of orthogonal pairs for producing site-specifically modified hirudins. The enhanced bioactivity of the site-specifically modified hirudin suggested a higher potential for therapeutic application over the prevailing rHirudin. Furthermore, boronic acids do not naturally occur in polypeptides and are introduced either as posttranslational modifications or as co-factors. Therefore, the successful incorporation of this amino acid into proteins allowed for a notably more selective chemical reaction on the modified protein surface, including oxidation, reduction, Suzuki coupling reactions or the forming of covalent boronic esters with other polyhydroxylated compounds, and may be used to purify native protein sequences in a one-step affinity purification procedure.

In conclusion, the present study reported a feasible method to improve the biological activity of hirudin and provided a

new way to access novel hirudin derivatives that may have marked clinical utility.

## Acknowledgements

The authors are grateful to Dr K.Odoi of A&M University for reviewing the manuscript. This study was supported by the Fundamental Research Funds for the Central Universities of China (nos. WF1114045 and 222201314027) and the Open Project for the State Key Laboratory of Bioreactor Engineering (no. 2060204).

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