Toxicon 152 (2018) 9–15

Contents lists available at ScienceDirect

Toxicon

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

The Kv1.3 channel-inhibitory toxin BF9 also displays anticoagulant activity via inhibition of factor XIa

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ARTICLE INFO

Keywords:
Kv1.3 channel
Factor XIa
Structural fold
Kunitz-type
Anticoagulation

ABSTRACT

The Kv1.3 channel plays potential roles in immune, inflammation and coagulation system. Many studies showed that Kv1.3 channel inhibitors have immunosuppressive and anti-inflammatory activities, but no Kv1.3 channel inhibitors have been found to have anticoagulation activities. Here, based on our previous work about Kv1.3 channel toxin peptide inhibitors, we first attempt to test anticoagulation activities of four known venom-derived Kv1.3 channel inhibitors with different structural folds: BmKTX with CSα/β structural fold, OmTx3 with CSα/α structural fold, BF9 with Kuntz-type structural fold, and SjAPI-2 with Ascaris-type structural fold. Our results showed that BmKTX and OmTx3 have no activities towards both intrinsic and extrinsic coagulation pathway, SjAPI-2 just has weak activity towards intrinsic coagulation pathway, and BF9 has potent activity towards intrinsic coagulation pathway with no apparent effect on extrinsic coagulation pathway. Enzyme and inhibitor reaction kinetics experiments further showed that BF9 inhibited intrinsic coagulation pathway-associated coagulation factor XIa, but have no apparent effects on common coagulation pathway coagulation factor IIa. Structure-activity relationship showed that Gly14, Asn17, Ala18 and Ile20 of BF9 are main residues involved in the inhibiting effect on factor XIa. To the best of our knowledge, BF9 is the first anticoagulant with Kv1.3 channel inhibitory activity. Together, our present studies found the first dual functional peptides with Kv1.3 channel and coagulation factor XIa inhibitory activities, and provided a new molecular template for the lead drug discovery towards immune and thrombosis-associated human diseases.

1. Introduction

The Kv1.3 channel plays important roles in the immune, inflammation and coagulation system (Beeton et al., 2006; Emerson, 2010; Kazama, 2015; Nguyen et al., 2010; Peng and Huss, 2010). In the immune system, the Kv1.3 channel modulates the activation and proliferation of the immune response of leukocytes. The Kv1.3 channel is the primary expressed potassium channel in lymphocytes, and is a therapeutic target for many autoimmune diseases (Gilhar et al., 2011; Hansen, 2014; Rangaraju et al., 2009; Upadhyay et al., 2013). The role of the Kv1.3 channel in the chronic inflammatory pathogenesis is mediated by an overexpression not only in T lymphocytes, but also other immune cells, such as B lymphocytes, microglia, macrophages and dendritic cells (DC) (Zhang et al., 2016; Zhu et al., 2017). Besides this, the Kv1.3 channel has also been identified in platelets and their precursor megakaryocytes, suggesting its potential role in the coagulation system (Emerson, 2010; McCloskey et al., 2010). Many studies have showed the immunosuppressive and anti-inflammatory activities of Kv1.3 channel inhibitors with different structural folds, such as sea anemone toxins ShK, BgK with ShK-structural fold (Dauplais et al., 1997; Krishnarjuna et al., 2018; Tudor et al., 1996), scorpion toxins AOSK1, ADWX-1, HsTx1 and Vm24 with CSα/β structural fold (McCloskey et al., 2010; Peng and Huss, 2010; Rangaraju et al., 2009; Yang et al., 2014). However, no Kv1.3 channel inhibitors have been found to have anticoagulation activities.

In the previous work of our group, we reported a series of Kv1.3 channel inhibitors with different structural folds and different biological activities, including BmKTX family peptides that adopts the classical CSα/β structural fold with diverse binding interfaces and ion channel inhibitory activities (Chen et al., 2014, 2015c; Han et al., 2008;

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https://doi.org/10.1016/j.toxicon.2018.07.014
Received 19 April 2018; Received in revised form 10 July 2018; Accepted 11 July 2018

Tanner et al., 2017), the first Kv1.3 inhibitory family with Kunitz-type structural fold and protease inhibitory activities (Chen et al., 2012; Schweitz et al., 1995), and a unique Kv1.3 inhibitor SjAPI-2 with Ascaris-type structural fold and KCNQ1 channel inhibitory activity (Chen et al., 2013b, 2015a). These studies suggested that Kv1.3 channel inhibitory peptides with different structural folds might have its unique and novel biological activities.

Here, based on our previous work about Kv1.3 channel inhibitors, we first attempted to test anticoagulation activities of four known Kv1.3 channel inhibitors with different structural folds: BmKTX with CSα/β structural fold (Chen et al., 2014), OmTx3 with CSα/α structural fold (Hu et al., 2015), BF9 with Kuntz-type structural fold (Yang et al., 2014), and SjAPI-2 with Ascaris-type structural fold (Chen et al., 2015a), and found a unique anticoagulant BF9 from venom-derived Kv1.3 channel inhibitory peptides. Enzyme and inhibitor reaction kinetics experiments further showed that BF9 inhibited intrinsic coagulation pathway-associated coagulation factor XIa, but have on apparent effects on common coagulation pathway coagulation factor IIa. To the best of our knowledge, BF9 is the first anticoagulant with Kv1.3 channel inhibitory activity.

2. Material and methods

2.1. Recombinant plasmids and site-directed mutagensis

Wild-type recombinant plasmid pGEX-6P-1-BmKTX, pGEX-6P-1-OmTx3, pET-28a-BF9 and pET-28a-SjAPI-2 were conserved in our group based on our previous work (Chen et al., 2014, 2015a; Hu et al., 2015; Yang et al., 2014). QuikChangeH Site-Directed Mutagenesis Kit (Stratagene, USA.) was used for generating these mutants based on the wild-type plasmid pET-28a-BF9 (Yang et al., 2014). All plasmids of mutants were verified by DNA sequencing before expression.

2.2. Recombinant expression and purification of BF9 mutants

Wild-type recombinant proteins BmKTX, OmTx3, BF9 and SjAPI-2 were obtained based our previous work (Chen et al., 2014, 2015a; Hu et al., 2015; Yang et al., 2014). Recombinant BF9 mutants were expressed and purified using similar methods with wild-type BF9 peptide (Yang et al., 2014). For example, the plasmid of a pET-28a-BF9-mutant was transformed into competent *Escherichia coli* BL21 (DE3) cells for expression. The BF9-mutant with enriched disulfide bridges was found to exclusively accumulate in inclusion bodies. It was refolded in vitro as we have described before (Chen et al., 2012; Yang et al., 2014). The insoluble inclusion bodies were washed twice with washing buffer (1%–2% Triton X-100 in phosphate buffered saline), and denatured in 2 ml denaturation solution (6 M guanidinium-HCl, 0.1 M Tris-HCl pH 8.0, 1 mM EDTA, and 30 mM reduced glutathione). Then, recombinant peptide was reactivated by 100-fold dilution in renaturation solutions at about pH8.0 (0.2 M ammonium acetate at pH8.0, containing 0.2 mM oxidized glutathione and 0.5-M arginine) at 16 °C for 24 h. Renatured protein was finally purified by high-performance liquid chromatography (HPLC) on a C18 column (10 × 250 mm^2, 5 μm Dalian Elite, China). Peaks were detected at 230 nm. The fraction containing recombinant peptide was eluted at about 16–18 min and further analyzed by a Jasco-810 spectropolarimeter (Jasco Analytical Instruments, Easton, MD).

2.3. Circular dichroism spectroscopy

The secondary structures of Kunitz-type toxin peptides BF9 (Protein Data Bank (PDB) code: 1JC6) and BF9 mutants were analyzed by circular dichroism (CD) spectroscopy (Chen et al., 2013b). Samples were dissolved in water at a concentration of 0.2 mg/ml. Spectra were recorded at 25 °C from 250 to 190 nM with a scan rate of 50 nM/min, on a Jasco-810 spectropolarimeter (Jasco Analytical Instruments, Easton, MD). The CD spectra were collected from averaging three scans after subtracting the blank spectrum of water.

2.4. Activated partial thromboplastin time (APTT)

Equal volumes (100 μl) of human plasma, APTT regent (mdpacific, Tianjin, China), 25 mM $CaCl_2$ (100 μl), and various concentrations of 50 μl tested peptide diluted with PBS buffer (25 mM, pH 7.4) were prewarmed to 37 °C individually. Peptide or control buffer was added to plasma and incubated for 10 min, followed by the addition of APTT regent and further incubation for 3 min. Clotting was initiated by the addition of 25 mM $CaCl_2$ to the mixture, and the clot formation was measured using an Infinite M200 microplate reader at 650 nm for fibrin polymer formation.

2.5. Prothrombin time (PT)

Plasma, thromboplastin (Pacific Hemostasis), and peptides diluted with PBS buffer were prewarmed to 37 °C individually. peptides (50 μl) was added to plasma (100 μl) and incubated for 10 min. Clotting was initiated by the addition of thromboplastin (200 μl) to the mixture, and the clot formation was measured using an Infinite M200 microplate reader at 650 nm for fibrin polymer formation.

2.6. Coagulation factors inhibitory activity assay

The inhibitory activity of BF9 or BF9 mutant was tested in the presence of serine proteases as described previously (Bajaj et al., 2011; Chen et al., 2015b). Respective serine protease (25 μl) diluted with buffer (50 mM tris, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA) was preincubated with 25 μl of inhibitor for 30 min at 37 °C, followed by the addition of 50 μl of the appropriate chromogenic substrate. In a total volume of 100 μl, the final serine protease/substrate concentrations were as follows: FXIa (0.5 nM)/S-2366 (1.2 mM) thrombin (2 nM)/S-2238 (1.6 mM). The cleavage of substrate was measured at 405 nm using a microplate reader.

3. Results

3.1. Discovery a new anticoagulant BF9 from Kv1.3 channel inhibitory toxin peptides

In our previous work, we reported a series of Kv1.3 channel inhibitors with different structural folds and diverse functional profiles (Chen et al., 2012, 2014, 2015a, 2015c; Yang et al., 2014). Based on these, we first attempted to test the anticoagulation activity of four known Kv1.3 channel inhibitors with different structural folds: BmKTX with CSα/β structural fold, OmTx3 with CSα/α structural fold, BF9 with Kuntz-type structural fold, and SjAPI-2 with Ascaris-type structural fold (Fig. 1A and B). APTT and PT tests were performed for detecting activities of peptides on the intrinsic and extrinsic coagulation pathway, respectively (Fig. 1C and D). Our results showed that BmKTX and OmTx3 have no activities towards both intrinsic and extrinsic coagulation pathway, and SjAPI-2 just has weak activity towards intrinsic coagulation pathway. BF9 prolonged APTT over the normal time period, but clotting times for PT was normal, which was similar with normal time of about 10–12 s. The time taken for clot formation in APTT gradually increased with the concentration of BF9 peptide. There was over 2-fold increase with 93.75 nM BF9 peptide (Fig. 2A and B). Together, these studies indicated BF9 with Kunitz-type structural fold and Kv1.3 channel inhibitory activity also has potent activity towards intrinsic coagulation pathway.