Discovery of Highly Potent Small Molecule Kallikrein Inhibitors

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Abstract: Uncontrolled kallikrein activation is involved in diseases such as hereditary angioedema, bacterial septic shock and procedures such as cardiopulmonary bypass. Here we report a series of small molecule compounds that potently inhibit kallikrein activity *in vitro*. Kinetic studies indicate that some of these compounds are slow binding inhibitors of kallikrein with Ki final less than a nanomolar. The ability of these compounds to inhibit the activity of kallikrein was further confirmed in a plasma model by quantitating the release of bradykinin, an endogenous cleavage product of plasma kallikrein. To understand the inhibitory mechanism of the selected compounds toward kallikrein, the interactions between the selected compounds and kallikrein was explored using molecular modeling based on the information of crystal structures of TF/FVIIa and kallikrein. The information presented in the current study provides an initial approach to develop more selective and therapeutically useful small molecule inhibitors.

Key Words: Kallikrein, contact activation, bradykinin, inhibitor, modeling.

INTRODUCTION

The contact system, also referred to as the intrinsic pathway of coagulation consists of three enzymatic factors: factor XI (FXI), coagulation factor XII (FXII), and plasma prekallikrein (PK), as well as the nonenzymatic cofactor H-kininogen (HK) [1]. Kallikrein is converted from PK by FXIIa after the interaction of FXII with a negatively charged surface during contact system activation [2]. Bradykinin (BK), a nonapeptide, is derived from HK by kallikrein-dependent hydrolysis [3]. Activation of PK results in a massive reduction of HK deposition and the liberation of BK into plasma. HK is one of the major factors in blood hemostasis, maintaining vessel patency and functions as an inhibitor of thrombin and other serum proteases [4]. In addition, kallikrein can directly catalyze the conversion of FXII to FXIIa, and thus further amplifies the activation of the contact system. [5]. BK is a potent inducer of vasodilation and increases microvascular permeability [6, 7]. The two effects are partly mediated by the secondary release of other signaling molecules including nitric oxide and platelet activating factor through the activation of vascular endothelium BK receptors [8, 9].

Accumulating data suggests that uncontrolled kallikrein activity is involved in the pathogenesis of hereditary angioedema (HAE) [10, 11], sepsis [12], and the inflammatory response seen in the conditions such as cardiopulmonary bypass (CPB) [13]. In addition, a consistent but more modest activation has been observed in rat models for inflammatory arthritis and inflammatory bowel disease, indicating the involvement of contact activation in these conditions [14]. Thus the inhibition of kallikrein in these diseases or surgical applications would likely have significant benefits.

In the present study we report a group of newly developed anti-inflammatory compounds, primarily designed for tissue factor (TF) / factor VIIa (FVIIa), also potently inhibit kallikrein activity. Using structure based drug design a series of potent small molecule inhibitors toward TF/FVIIa were designed and synthesized at BioCryst Pharmaceuticals (BCX) (Kotian, P. L; Pooran, K.; Bantia, S.; Arnold, S., Krishnan, R.; Gupta, S. N.; Upshaw, R.; Dehghani, A.; Boudreaux, B.; El-Kattan, Y.; Lin, T.-H.; Saini, S.; Zhang, Q.; Rowland, S.; Babu, Y. S.; Abstr for XVIIIth International Symposium on Medicinal Chemistry, Copenhagen August 16, 2004). The inhibitory actions of these compounds towards FVIIa have been thoroughly characterized using a purified enzyme assay and relevant clotting-based assays (Krishnan, R.; Rowland, S.; Kotian, P. L.; Chand, P.; Bantia, S.; Gupta, S. N.; Arnold, S.; Saini, S.; Zhang, Q.; Abstr for American Crystallographic Association, Orlando, FL, May 13, 2005). In addition to FVIIa the inhibitory effects of these compounds against various serum proteases, including C1s, thrombin, plasmin, FXa, activated Protein C (APC), tissue plasminogen activator (tPA) and kallikrein, were also studied. The data from these assays indicate that kallikrein is the only enzyme that was also potently inhibited by some of these compounds. In order to understand their inhibitory mechanism towards kallikrein, binding properties were extensively explored in an enzyme model. The data from binding kinetics studies demonstrate that all five compounds reported in this article are potent kallikrein inhibitors, and some of them showed exceptional binding affinity toward kallikrein with binding constants (*Ki* final) less than a nanomolar, comparable to P8720, which is a peptide based-kallikrein inhibitor [15]. Further studies us-

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ing on-site dissociation and *Ki* determination in extending binding conditions suggest that these compounds belong to slow binding inhibitors, which takes two steps to form stable inhibitor/enzyme complexes. One of the most important indications of contact activation is massive production of BK, a cleavage product of HK by proteolytic action of kallikrein [3]. Thus the ability of selected compounds to inhibit BK generation was examined using a plasma based-assay. The data of BK release assays are consistent with the findings observed in enzyme kinetic assays. In order to understand the mechanism of inhibition, the interaction between these BCX compounds and kallikrein was evaluated using molecular modeling based on coordinates obtained from crystal structures of TF/FVIIa and kallikrein enzyme-inhibitor complexes.

Most studies involving treatment of contact activation-mediated conditions were largely limited to high molecular weight inhibitors, such as C1 INH, an endogenous kallikrein inhibitory protein [11], monoclonal antibodies that target the contact system [16], and peptide based-inhibitors which showed either less potency [17] or high hepatic toxicity [18]. To our knowledge, the present study is the first report using small molecule compounds to selectively block kallikrein activity *in vitro*, though their *in vivo* inhibitory actions towards contact system still need to be determined. However the information from the present study provides structural base and relevant kinetic analysis, which would be of crucial use for developing more specific small molecule compounds that could be applied in conditions associated with uncontrolled contact pathway activations

MATERIALS AND METHODS

Inhibitors

All of the BCX compounds used in the current study were synthesized as previously described at BioCryst pharmaceuticals (Kotian, P. L; Pooran, K.; Bantia, S.; Arnold, S., Krishnan, R.; Gupta, S. N.; Upshaw, R.; Dehghani, A.; Boudreaux, B.; El-Kattan, Y.; Lin, T.-H.; Saini, S.; Zhang, Q.; Rowland, S.; Babu, Y. S.; Abstr for XVIIIth International Symposium on Medicinal Chemistry, Copenhagen, August 16, 2004). The chemical structures of the compounds are shown in Fig. (**1**). All compounds were initially dissolved in DMSO and further diluted to desired concentrations in PBS (Phosphate Buffer Saline, pH 7.4, Invitrogen, Frederick, MD) prior to assay.

Binding Kinetics Studies

Inhibition Constant Determination

The assay reactions to determine *Ki* of the selected compounds were performed in a 96-well microplate (Corning) with a final volume 200 µl for each reaction. The reaction contained 50 mM Tris, pH 7.8, 60 mM NaCl, 200 µM substrate S-3202 (H-D-Pro-Phe-Arg-pNA, Diapharma, West Chester, OH), 76 ng/ml kallikrein (enzyme Research laboratories, South Bend, IN), and inhibitor concentrations cover-

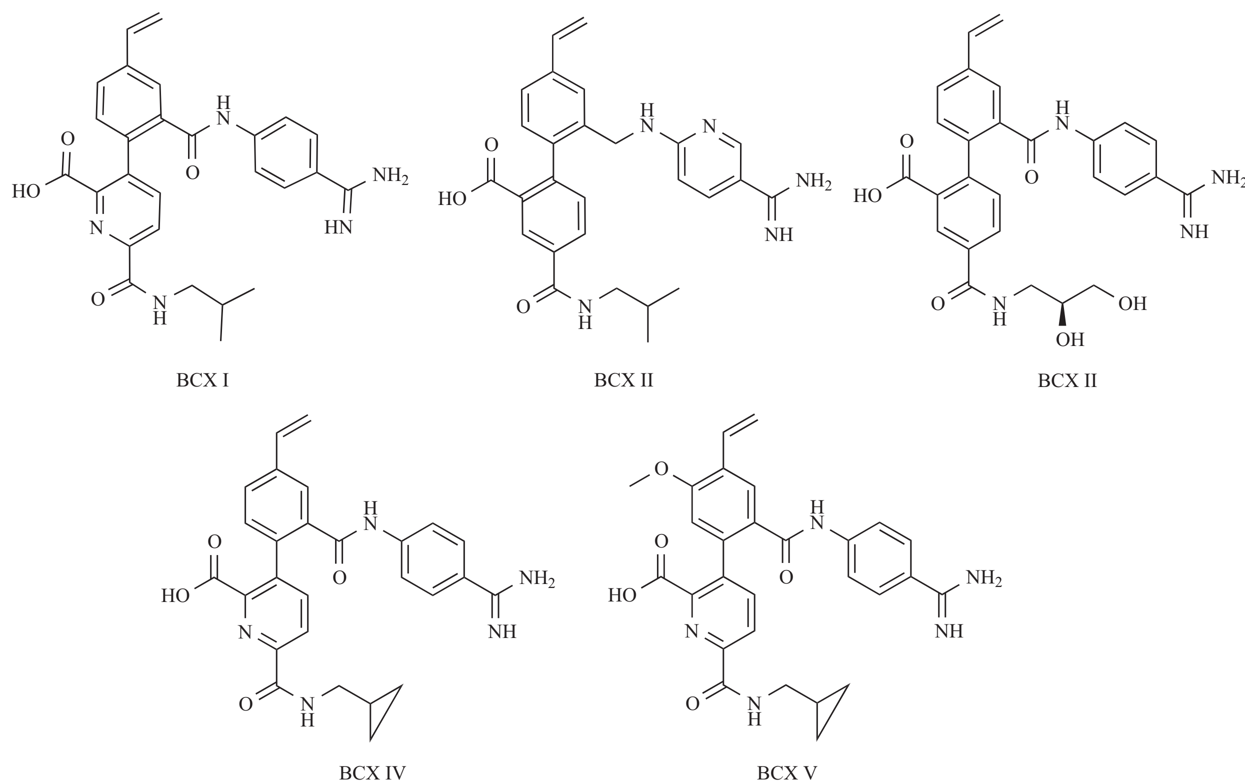

Fig. (1). Chemical structure of the BCX compounds.

ing 0.0025 to 0.1 μM for BCX I, IV and V, and 0.2 to 1.0 μM for BCX II and III. Assay reactions were initiated by either adding enzyme into pre-mixed solution of inhibitors and substrate (enzyme initiated reaction) or adding substrate at final step after pre-incubating kallikrein and inhibitor (substrate initiated reaction). The absorbance changes at 405 nm were recorded for 5 min at room temperature using a PowerWave-340 microplate reader (Bio-Tek Instruments), and the rate of kallikrein activity was expressed as Vmean. The assays for each individual inhibitor concentration were carried out in triplicates, and the average values of Vmean were used to process kinetic data. The *Ki* for each selected compound was calculated by fitting the ratio of rate (*V0'/V0*) between the reaction in presence and absence of inhibitor, and corresponding concentrations of inhibitor to the equation expressing for competition inhibition as described by Singh *et al.* [19].

$$\frac{V0'}{V0} = \frac{Km + [S]}{Km + [S] + Km\,[I]\,/Ki}$$

Where V0' is the rate of Vmean in the presence of inhibitor, V0 is the rate of Vmean in the absence of inhibitor, [I] is the inhibitor concentration, and [S] is the substrate concentration (200 μM), Km is the Michaelis constant (Km = 74 μM) as determined by Kettner *et al.* [20].

On-Site Dissociation Assay

Complex Preparation

To carry out on-site dissociation experiments, the complexes of kallikrein and inhibitor with a 1:1 stoichiometric ratio were prepared as following. The binding reaction contained 350 ng of kallikrein, 1 μM BCX-V or 10 μM BCX-II, 60 mM NaCl, 50 mM Tris HCl, pH 7.8 in a final volume 200 μl, and the reactions were incubated in 4 ºC for 10 min. As assay controls, kallikrein was incubated in the absence of inhibitors in similar conditions. At the end of the binding incubation, unbound inhibitors were removed by using Micro-Bio-Spin@30 Chromatography column (Bio-Rad). The prepared complexes were immediately subjected to dissociation reaction.

On-Site Dissociation Assay

To initiate on-site dissociation reaction, 80 μl of prepared complexes was added into 920 μl of substrate solution (220 μM S3202, 66 mM NaCl, 55 mM Tris HCl, pH 7.8), and immediately transferred to a 1-ml Quartz cuvette (0.2 CM light path) after mixing. The increase in absorbance at 405 nm was recorded over 100 min in a UV/VIS Spectrophotometer (Varian).

Amidase Assay

The *K*m value of FXIIa was first determined using purified human plasma FXIIa (Calbiochem, La Jolla, CA) and the synthetic substrate, D-proly-L-phenylalany-L-arginin-*p*-nitroaniline (Calbiochem, La Jolla, CA). The reactions were carried out in a 96-well microplate, in which each reaction contained 0.8 mU XIIa, 50 mM potassium phosphate, pH 7.5, and different concentrations of substrate (0.05 – 2.5 mM) in a final volume of 200 μl. The reactions were initiated by adding FXIIa, followed by 10 min incubation at 30 °C. The reactions were terminated by adding 50 μl of 20% acetic acid. The changes at 405 nm were measured using a PowerWave X-340 microplate reader. To evaluate the inhibitory efficiency of the selected compounds toward XIIa the concentration of substrate was chosen based upon the determined *Km* (*Km* = 0.5 mM). The reactions were carried out in a 200 μl mix containing 0.8 mU FXIIa, 50 mM phosphate buffer, pH 7.5, and 0.5 mM D-proly-L-phenylalany-L-arginin-*p*-nitroaniline with varying concentrations of inhibitors. The reactions were initiated by adding substrate solution, and terminated by the addition of 50 μl of 20% acetic acid, and changes at 405 nm were measured.

Plasma BK Release Studies

Bacteria

A strain of bacteria *Staphylococcus aureus* (strain 5120) was originally derived from a blood culture from a patient with septic shock [17]. The bacteria were grown in BHI Broth with Fildes Enrichment (BD, Sparks, Maryland) at 37 °C overnight. Prior to plasma incubation bacteria were harvested and washed four times with PBS. Washed bacteria were resuspended in HEPES buffer (15 mM HEPES, pH 7.4, 135 mM NaCl, 50 mM ZnC_{l2}), and further diluted to the desired concentrations prior to assays.

Plasma Source

Blood was collected from randomly chosen employees of BioCryst Pharmaceuticals in sterile tubes (BD, Vacutainer TM) containing sodium citrate. Plasma was obtained after centrifugation of citrated blood at 2000 rpm for 15 min (Beckman, GS-6R), and stored at – 80 ˚C until use.

Bradykinin Release Inhibition Assay

To assess inhibitory properties of BCX compounds toward kallikrein in plasma as well as kallikrein that assemble on the surface of bacteria, the assays were conducted under three different reaction conditions.

I: 500 μl of human plasma was pre-incubated with designated concentrations of C1 INH or the selected compounds in room temperature for 10 min. 500 μl of bacterial suspension was then added into the plasma ($2.5x10^{10}$ CFU/ml final), and incubated for 30 min in room temperature. At the end of the incubation, the inhibitor-plasma-bacteria suspensions were centrifuged at 13,000 rpm for 10 min. The supernatants were collected for BK quantitation, which represents the level of BK released into the plasma during 30 min incubation.

II: The bacterial pellets from the above mentioned step were resuspended in 1 ml of PBS followed by two washes using the same buffer. The resulting bacterial pellets were resuspended in 300 μl HEPES buffer and incubated at room temperature for 15 min. At the end of the incubation, the suspension was centrifuged at 13,000 rpm for 10 min. The supernatants were collected for BK quantitation, which rep-

resented the remaining activity of kallikrein that assembled on surface of bacteria after removing the free inhibitors.

III: To directly determine inhibitory effects of C1 INH or the selected compounds towards kallikrein that assembly on surface of bacteria, kallikrein preabsorbed-bacteria pellets were first prepared. 500 µl human plasma was pre-incubated with same volume of bacterial suspension (2.5 x 10^{10} CFU/ ml final) in room temperature for 30 min, then bacteria were collected and washed twice with PBS. The resulting bacterial pellets were resuspended in 300 µl HEPES buffer containing designated concentrations of C1 INH or the selected compounds and followed by additional 15 min incubation. At the end of the incubation, the suspension was spun down, and the supernatants were collected for BK quantitation.

BK content quantitation was performed by using an Enzyme Immunoassay (EIA) kit for human BK (Phoenix Pharmaceuticals, Inc. Belmont, California). All testing samples were further diluted 4 to 8-fold prior to EIA. The samples from the plasma bacteria suspension (incubation of plasma + bacteria) were diluted 8-fold with EIA assay buffer prior to assay. While the samples from the bacterial suspension (incubation of bacteria in HEPES buffer) were diluted 4-fold with EIA assay buffer prior to assay. A standard curve was created for each measurement by using standard synthetic BK with known concentrations (250, 125, 25, 5, 2.5, and 1.25 pg/ml) provided by the manufacturer. The colorimetric EIA results were recorded using a PowerWave X340 (Bio-Tek Instruments) microplate reader. All other EIA operations followed the manufacture's instructions.

All EIA samples were analyzed in triplicates for at least three repeats, and the results were expressed either as pg / ml or converted to relative BK release (% initial). The IC50 of BK release were determined by fitting the relative BK releases (%initial) with corresponding compound concentrations using a SigmaPlot kinetics calculation Module (SigmaPlot 8.0).

Molecular Modeling

A model structure of kallikrein in complex with BCX V was generated by docking BCX V into the kallikrein active site using atomic coordinates determined from single crystal X-ray diffraction studies of TF/FVIIa-BCX V and human kallikrein 6-benzamidine (PDB entry code 1L2E) complexes. [20]. In addition to the main-chain atoms of 209 structurally equivalent (1.08 Å, root mean square deviation) amino acid residues of the catalytic domains, it was also possible to superpose the benzamidine moieties in both these complexes, based on the sequence alignment of human kallikrein 6 and the heavy chain of the catalytic domain of human FVIIa. The remarkable sequence similarity between these two proteins (40% identical residues) (Table **4**) translates into a very similar structural element in and around the active site, justifying the docking studies. All the modeling studies were performed on Dell Personal computer running windows XP using the DS Modeling 1.1 (Discovery Studio suite of programs) [22].

RESULTS

Binding Constants of the Selected BCX Compounds for Kallikrein

The chemical structures and binding constants for all the five selected compounds are shown in Fig. (**1**) and Table **1**. All the BCX compounds in the present study are reversible

Table 1. Binding Constants of the BCX Compounds to Kallikrein

Compound ID	*Ki* initial (nM)	*Ki* final (nM)
BCX I	3.71 ± 0.1	0.99 ± 0.03
BCX II	154 ± 24	139 ± 23
BCX III	35.5 ± 3.51	42.9 ± 5.5
BCX IV	1.94 ± 0.4	0.52 ± 0.17
BCX V	1.31 ± 0.15	0.26 ± 0.05

Ki for each compound toward kallikrein was determined either by pre-incubating inhibitor with kallikrein for 2 hours in 25 ºC prior to initiate reaction (*Ki* final), or directly initiating reaction without a pre-incubation step (*Ki* initial), reported values are mean ± S.D. from 6 individual determinations.

competitive inhibitors to kallikrein in spite of their wide range of differences in *Ki* up to 500-fold. Since the binding of BCX I, IV and V to kallikrein were so strong, it was speculated that these three compounds might interact with kallikrein in a pattern of slow binding inhibitor like P8720: which forms an initial complex first, EI, and then in a slower step forms a stable complex, EI* [15].

Thus the values of *Ki* for each compound toward kallikrein were determined in conditions with or without a pre-incubation step prior to assays and their corresponding *Ki* were defined as either *Ki* initial or *Ki* final respectively [20]. In five selected compounds, BCX I, IV and V showed robust binding affinity toward human plasma kallikrein with nanomolar range in their *Ki* initial and picomolar range for their *Ki* final. In contrast, the binding affinities of BCX II and III to kallikrein were relatively weak though their *Ki* both initial and final were still under micromolar range (Table **1**). In addition, there was a 3 to 5-fold difference between *Ki* final and *Ki* initial for Compound I, IV and V, whereas there was no substantial different between *Ki* initial and *Ki* final for Compound II and III (Table **1**), indicating that BCX I, IV and V are slow binding inhibitors, and BCX II and III are not.

Fig. (**2**) demonstrates the slow binding property of the selected compounds by plotting the *Ki* of each compound *vs*. the corresponding incubation time from 0 to 480 min in 4 ºC. The variations of *Ki* were represented as fold of initial. There were significant decreases in *Ki* for BCX I, IV and V in the first 2 hour pre-incubation (about 3-5 folds), however further extending pre-incubation did resulted in an additional decrease of *Ki*, indicating that the process of formation of final complex from initial complex was achieved within 2 hours.

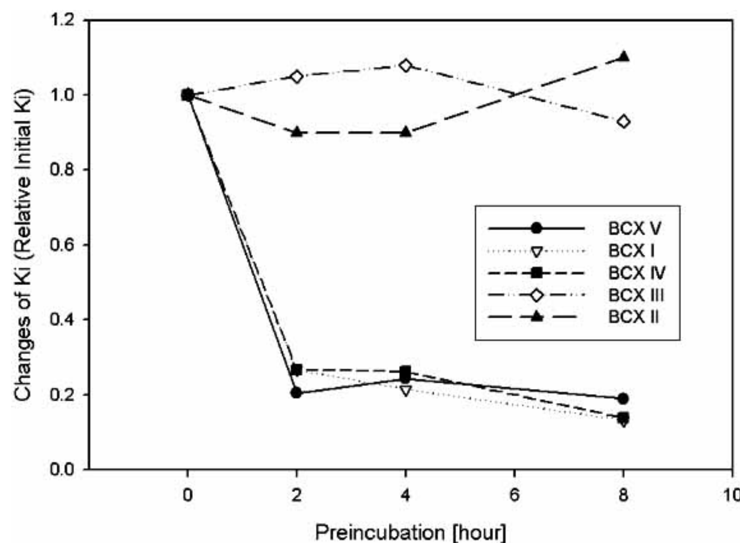

Fig. (2). Decreases in *Ki* with extension of pre-incubation. The values of *Ki* for each compound were determined after pre-incubating kallikrein with inhibitor for indicated period in 4 ºC (0, 2, 4, 8 hours). The changes of *Ki* for each compound were expressed as fold decrease of initial *Ki*. With prolonging time of incubation, the *Ki* of BCX I, IV and V was significantly decreased to a lower level compared to their initial *Ki.*

Therefore the interaction of theses compounds to kallikrein follows a pattern of slow binding process [20]. In contrast, there was no such pre-incubation-dependent *Ki* changes in BCX II and III although pre-incubation was extended up to 480 min, suggesting that compound II and III are not slow binding inhibitors to kallikrein.

To further confirm the slow binding properties of the selected BCX compounds to kallikrein, on-site dissociation assays were performed. In the experiments, the complex forms of kallikrein and inhibitor with a 1:1 stoichiometric ratio were prepared by pre-incubating kallikrein with excess molar concentration of inhibitors, and the unbound inhibitors were quickly removed by using size exclusion chromatography column. The on-site dissociation reactions were then initiated by adding an excess molar concentration of substrate (200 µM) into complex preparation. As expected, in such conditions the inhibitor with low binding affinity (BCX II) was quickly dissociated from kallikrein complex by substrate, and the activity of the enzyme was recovered immediately (Fig. (**3**), red triangle dot line). However it took much longer to compete off the tightly bound inhibitor (BCX V)

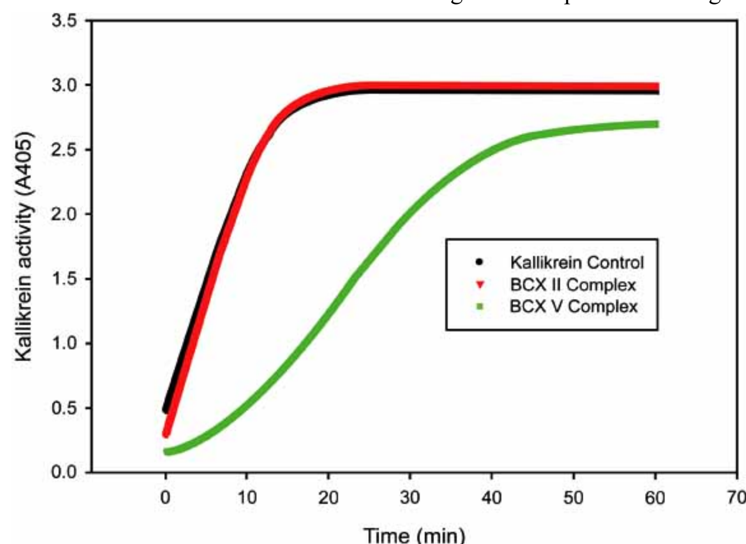

Fig. (3). On-site dissociation assays of kallikrein in complex with BCX compounds. To initiate on-site dissociation reaction, 80 µl of prepared complexes (kallikrein-BCX II and kallikrein-BCX V) was added into 920 µl of substrate solution (220 µM S3202, 66 mM NaCl, 55 mM Tris, HCl, pH, 7.8). The increase in absorbance at 405 nm was recorded over 60 min in a UV/VIS Spectrophotometer. The kinetic profile reveals that the inhibitor with low binding affinity (BCX II) was quickly displaced from kallikrein complex by substrate, and the activity of the enzyme was recovered immediately (red triangle dot line), but it took much longer to compete off the tightly bound inhibitor (BCX V) from the complex (green square dot line). Black dotted line is kallikrein control.

from the complex (Fig. (**3**), green square dot line). Thus on-site dissociation data further confirms the slow-binding properties of BCX I, IV and V with kallikrein.

The Selected Compound BCX IV Potently Inhibits Bacterial Mediated-Plasma BK Production

To confirm that the inhibition not only occurs in a reconstituted enzyme system but also takes place in kallikrein-containing plasma, the bacterial mediated-plasma BK release assay was performed in the presence or absence of inhibitors at early or late incubation steps, and the content of BK present in plasma or washed bacteria suspension was examined. C1 INH is a major kallikrein inhibitor present in plasma, which forms a 1:1 stoichiometric complex with kallikrein, resulting in the loss of kallikrein's proteolytic and amidolytic activity [13, 23, and 24]. Therefore, C1 INH was used as a reference kallikrein inhibitor in the current study. Fig. (**4a**) shows that BK, which was released into the reaction media during the 1st incubation (inhibitor + plasma + bacteria), was substantially attenuated by C1 INH (about 55% reduction, Fig. (**4a**), column 2), but was inhibited to a greater extend by Compound IV (95% reduction in BK release, Fig. (**4a**), column 3). Fig. (**4b**) shows the differential inhibitory effects of C1 INH and BCVX IV on bacteria surface assembled-kallikrein after the free inhibitors were removed. The BK release was significantly recovered once the free C1 INH was removed from the bacteria containing suspension (Fig. (**4b**), column 2). However the BK release was still potently suppressed by Compound IV (80% inhibition) though the free compounds were removed by bacteria washing steps (Fig. (**4b**), column 3). The inhibitory effect of the inhibitors towards kallikrein assembled on the surface of bacteria was also confirmed by including the inhibitors into the reaction at the last incubation step (2nd incubation). Fig. (**4c**) shows that

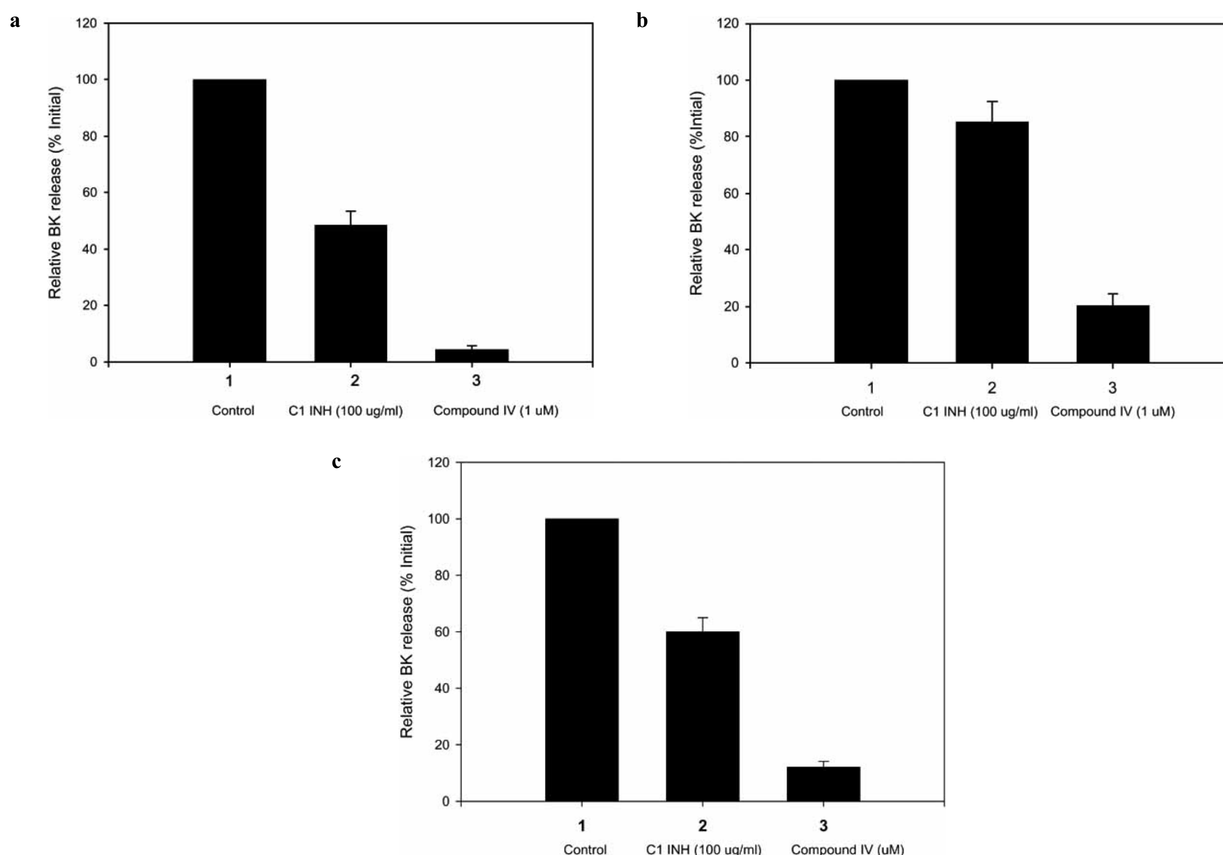

Fig. (4). C1 INH and BCX IV effectively inhibit bacteria mediated-BK release. C1 INH (100 µg/ml final) and Compound IV (1 µM final) were pre-incubated with 500 µl of plasma for 10 min, then mixed with 500 µl of bacterial suspension (2.5 x 10^{10} CFU/ml final) for a 30 min incubation. At the end of the incubation the bacteria were spun down, and the supernatant for this incubation (1st incubation) was collected for BK EIA (4**a**). The resulting bacterial pellets from the above mentioned step were washed twice with PBS and resuspended in 300 µl of HEPES buffer for additional 15 min incubation. The supernatant for this incubation (2nd incubation) was collected for BK EIA (4**b**). 500 µl of plasma were incubated with 500 µl of bacterial suspension (2.5 x 10^{10} CFU/ml final) for 30 min. At the end of incubation the bacteria were spun down, and washed twice with PBS. The resulting bacterial pellets were resuspended in a HEPES buffer containing C1 INH (100 µg/ml final) or Compound IV (1 µM final) followed by a 15 min incubation. The supernatant of the incubation was collected for BK EIA (4**c**). Values are mean ± SEM (n = 3).

both C1 INH and BCX IV effectively inhibits the activity of bacterial assembled-kallikrein in a similar extent as observed in kallikrein that was present in plasma (Fig. (**4a**)).

Inhibitory Properties of the Selected Compounds on Bacterial Mediated-Plasma BK Release

Table **2** shows the list of IC50 values of all five compounds toward BK release in different reaction conditions: in the plasma (1st incubation of inhibitors + plasma + bacteria, IC50 I), in the bacterial suspension after removing free inhibitors (2nd incubation, IC50 II), and in the kallikrein-preabsorbed bacterial suspension in presence of the selected compounds (2nd incubation, IC50 III). Generally the values of IC50 I and IC50 III are very similar for each designated compound; in contrast the values of IC50 II for compounds I, IV and V were about 10 to 25 folds higher than the values of IC50 I and IC50 III. Notably the values of IC50 II for BCX II and III can not be achieved at the highest compound concentration used in the assay (over 10 µM), indicating the high dissociation rate of BCX II and III toward plasma kallikrein. Thus the results from these BK release inhibition studies using plasma were consistent with the *Ki* determination using a pure enzyme analysis system (Table **1**).

The Selected Compounds are Poor Inhibitors of FXIIa

Although it is known that plasma BK is normally generated by proteolytic action of kallikrein [3], there is also evidence that FXIIa may cleave HK and release BK [25]. Thus the effect of these compounds toward factor FXIIa was evaluated using a reconstituted enzyme assay. The data (Table **3**) shows that all selected compounds were poor inhibitors of FXIIa with IC50 values from 1 to 9 µM for compound I, IV and V, and the inhibitions of compound II and III toward FXIIa were much worse (over 10 µM), suggesting that the inhibiting action of the selected compounds toward plasma BK release was primary due to the inhibition of kallikrein itself.

Molecular Modeling

The numbering of kallikrein and FVIIa residues is based upon topological equivalences with Chymotrypsin [26]. A model structure of the catalytic domain of kallikrein in complex with BCX V generated by docking the inhibitor into the kallikrein-substrate binding active site using atomic coordinates determined from single crystal X-ray diffraction studies is shown in Fig. (**5**). The modeled structure revealed that BCX V binds to the active site of kallikrein without any unfavorable steric contacts with the protein. The salient features of this model complex between kallikrein and BCX V can be highlighted as follows. In addition to the two hydrogen bonded salt bridge interactions with the acidic Asp189 at the bottom of the S1 site [26], the amino groups of the benzamidine also interact with the residue of Ser190 to form hydrogen bonds [21]. The central aromatic ring of BCX V stacks on top the His57 of the catalytic triad of kallikrein [21], and the vinyl group occupies the hydrophobic S2 site. The carboxylate group interacts with Gly193 in the oxyanion hole and His57 residues to form hydrogen bonds [21]. The terminal cycloisopropyl group in BCX V group nestles in hydrophobic cavity lined by Leu40 and Phe151 residues.

Table 3. Inhibitory Effects of Selected Compounds Toward FXIIa

Compound ID	*IC*50 Inhibitions of FXIIa Activity (µM)
BCX I	9.37
BCX II	>10
BCX III	>10
BCX IV	6.33
BCX V	0.84

The value of IC50 were means from three individual measurements (n = 3) with SEM less than 10 %

DISCUSSION

Many diseases are associated with the activation of the contact system. This activation triggers the massive release of potent biologically active peptides in conjunction with switching the storage equilibrium of HK in plasma [27]. HAE is a congenital condition associated with a deficiency or defect in C1 INH [11], and acute attacks of HAE are re-

Table 2 IC50 of BCX Compounds Toward Human Plasma BK Release Induced by *S. aureus*

Compound ID	*IC*50 I *of KB Release* (µM)	*IC*50 II *of KB Release* (µM)	*Release IC*50 III *of KB* (µM)
BCX I	0.5 ± 0.06	2.3 ± 0.29	0.2 ± 0.02
BCX II	>1	>10	0.3 ± 0.06
BCX III	0.36 ± 0.17	>10	0.6 ± 0.23
BCX IV	0.04 ± 0.012	0.43 ± 0.06	0.05 ± 0.002
BCX V	0.03 ± 0.005	0.5 ± 0.17	0.02 ± 0.003

IC50 I was determined using the supernatant of 1st incubation of suspension reaction in presence of varying concentrations of the compounds as described in method I; **IC50 II** was determined using the supernatant of 2nd incubation of bacteria suspension reaction after removing free compounds as described in method II; **IC50 III** was determined using the supernatant of 2nd incubation of bacteria suspension reaction in presence varying concentrations of compounds as described in method III. The values of IC50 were means ± SEM (n = 3).

Table 4. Amino Acid Sequence Homology of the Catalytic Domains of Human Kallikrein-6 and FVIIa # (Heavy Chain) with Sequences Derived from PDB Entries 1L2E and 1DAN Respectively

	Sequence
	10 · 20 · 30 · 40 · 50 · 60
1dan	I V G G K V C P K G E C P W Q V L L L V N G A Q L C G G T L I N T I W V V S A A H C F D K I K N W R N L I A V L G E H D L S E H D G
1l2e	L V H G G P C D K T S H F Y Q A A L Y T S G H L L C G G V L I H P L W V L T A A H C K - - - K - - P N L Q V F L G K H N L R Q R E S
	70 · 80 · 90 · 100 · 110 · 120 · 130
1dan	D E Q S R R V A Q V I I P S T Y V P G T T N H D I A L L R L H Q P V V L T D H V V P L C L P E R T F S E R T L A F V R F S L V S G W
1l2e	S Q E Q S S V V R A V I H P D Y D A A S H D Q D I M L L R L A R P A K L S E L I Q P L P L - E R D C S A N T T S - - - - C H I L G W
	140 · 150 · 160 · 170 · 180 · 190
1dan	G Q L L D R G A T A L E L M V L N V P R L M T Q D C L Q Q S R K V G D S P N I T E Y M F C A G Y S D G S K D S C K G D S G G P H A T
1l2e	G K T A D - G D F P D T I Q C A Y I H L V S R E E C E H A Y P - - - - - G Q I T Q N M L C A G D E K Y G K D S C Q G D S G G P L V C
	200 · 210 · 220 · 230 · 240 · 250 · 260
1dan	H Y R G T W Y L T G I V S W G - Q G C A T V G H F G V Y T R V S Q Y I E W L Q K L M R S E P R P G V L L R A P F P X F R X
1l2e	G D - - - - H L R G L V S W G N I P C G S K E K P G V Y T N V C R Y T N W I Q K T I Q - - - - - - - - - - - - - - - - - -

lated to the activation of the contact pathway [10]. Also, patients with severe gram-negative sepsis or septic shock show an activation of the contact system as evidenced by the depletion of FXII and prekallikrein, and an elevation of BK [12, 28]. Additionally, CPB is performed on more than 350,000 Americans each year. During CPB, there is extensive contact between blood and the synthetic surfaces of the extracorporeal circuit, and both contact system and the classic complement pathway are markedly activated [13]. Recently, a consistent but more modest activation has been observed in rat models of inflammatory arthritis and inflammatory bowel disease, indicating the implication of contact activation in these conditions [14]. Together, these indications illustrate the involvements of the activation of the contact pathway in many disease cases and clinical procedures, and indicate that kallikrein could be a potential therapeutic target.

The therapeutic application of anti-contact pathway drugs in these conditions has been explored extensively in recent years. Since a deficiency or defect of C1 INH is associated with the progress of HAE, supplements of C1 INH are expected to provide a therapeutic benefit in HAE [11, 29]. An anti-FXIIa monoclonal antibody has been reported to successfully block contact activation and increase the survival rate in a bacteremia baboon model [1]. Recently, some peptide-based specific kallikrein inhibitors, such as aprotinin and P8720, have been shown therapeutic benefits during CPB [13] and relevant conditions in the Lewis rat [15]. HD-Pro-Phe-Arg- CMK, a FXII and kallikrein inhibiting peptide, has also been suggested to be a potential therapy for the treatment of sepsis [17].

The selected BCX compounds described in the current study were initially designed for FVIIa based on the crystal structure of soluble form of TF/FVIIa, and their inhibitory properties to TF/FVIIa were extensively investigated previously. However, preliminary studies indicate that kallikrein could be another target for this group of novel compounds. The kinetics study from enzyme analysis demonstrates that the selected compounds are potent kallikrein inhibitors with binding constants (*Ki* final) ranging from 139 to 0.26 nM. Further kinetic studies by quantitating the *Ki* in extended

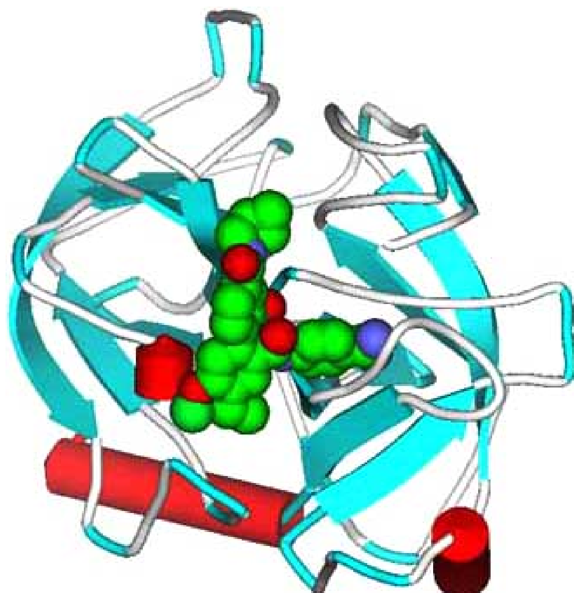

Fig. (5). Schematic representation of the model structure of the Catalytic Domain of Kallikrein in Complex with BCX V (cpk).

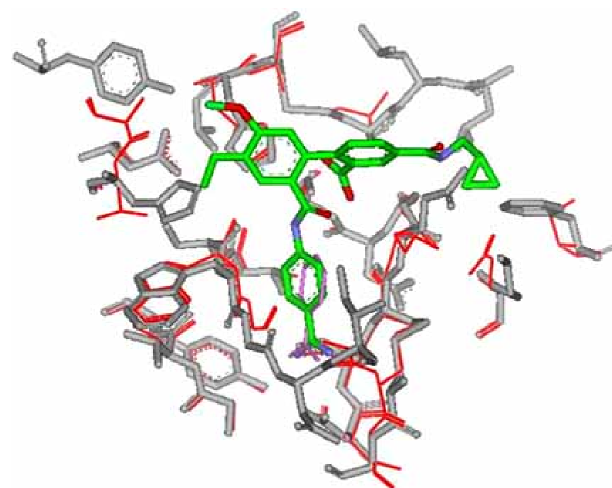

Fig. (6). Superposed Model of the catalytic domains of both complexes in the vicinity of their active sites Kallikrein (grey stick) and FVIIa (thin red sticks) .BCX V shown in atom colors and Benzamidine in magenta.

binding reactions and determining the dissociation rate using on-site dissociation assays revealed that selected compounds BCX I, BCX IV and BCX V are slow binding inhibitors for kallikrein with a value of *Ki* final less than nanomole. Plasma based-BK release experiments confirm that the selected compounds strongly interact to plasma kallikrein and result in potent inhibition on bacterial mediated-BK production in various incubation conditions. Thus, data from BK production assays using human plasma is fully consistent with the results from the kinetic studies. It is common practice to model enzymes, enzyme-inhibitor complexes based on the structural similarities and sequence homologies when the crystal structure of the complex cannot be determined [30-34]. The modeling studies indicate that BCX V binds to the active site of kallikrein without any unfavorable steric contacts; an obvious explanation as to why these FVIIa inhibiting compounds show such potent inhibition towards kallikrein. In addition, these simple modeling studies also help us rationalize the drastic variation in binding constants observed for the five BCX compounds in Table **1** and indicate the chemical groups necessary for optimal interactions. The worst binding compound BCX II has a carbonyl group missing from the linker peptide group between the core and the P1 benzamidine. BCX III has a charged group directed towards the hydrophobic P' sites, which results in loss of binding relative to the BCX I, IV and V. A further comparison of the binding constants of each compounds (Table **1**) and corresponding chemical structures (Fig. (**1**)) reveal that a cycloisopropyl is the most preferred moiety in the P' sites followed by an isopropyl group. BCX V, the best compound in these series, has in addition to all the better binding compounds, a methoxy group which fills up the P2 cavity quite optimally. The S2 site of kallikrein is relatively smaller compared to FVIIa, as a histidine (His99, Thr in FVIIa) side chain sticks into this cavity. Therefore adding a chemical moiety that can interact with this Histidine will probably result in a more specific inhibitor for kallikrein.

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ABBREVIATIONS

TF	=	Tissue factor
FVIIa	=	Coagulation factor VIIa
XII	=	Coagulation factor XII
PK	=	Prekallikrein
BK	=	Bradykinin
HK	=	H-kininogen
HAE	=	Hereditary angioedema
CPB	=	Cardiopulmonary bypass

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