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Anti-platelet activity of a three-finger toxin (3FTx) from Indian monocled cobra (*Naja kaouthia*) venom

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ABSTRACT

A low molecular weight anti-platelet peptide (6.9 kDa) has been purified from *Naja kaouthia* venom and was named KT-6.9. MALDI-TOF/TOF mass spectrometry analysis revealed the homology of KT-6.9 peptide sequence with many three finger toxin family members. KT-6.9 inhibited human platelet aggregation process in a dose dependent manner. It has inhibited ADP, thrombin and arachidonic acid induced platelet aggregation process in dose dependent manner, but did not inhibit collagen and ristocetin induced platelet aggregation. Strong inhibition (70%) of the ADP induced platelet aggregation by KT-6.9 suggests competition with ADP for its receptors on platelet surface. Anti-platelet activity of KT-6.9 was found to be 25 times stronger than that of anti-platelet drug clopidogrel. Binding of KT-6.9 to platelet surface was confirmed by surface plasma resonance analysis using BIAcore X100. Binding was also observed by a modified sandwich ELISA method using anti-KT-6.9 antibodies. KT-6.9 is probably the first 3FTx from Indian monocled cobra venom reported as a platelet aggregation inhibitor.

1. Introduction

Three finger toxin (3FTxs) family members are low molecular weight polypeptides with 60–80 amino acid residues found mostly in venoms of elapid snakes [1,2]. These molecules have three finger like loops emerging from a globular core stabilized by four disulfide bridges [3,4]. 3FTxs possess various biological properties which include effects on various neuronal receptors, blocking integrins, affecting K^+ channels, affecting platelet aggregation process, etc. [5–8]. Three dimensional structure of 3FTx is very important for its target recognition and binding. It has been observed that even minor structural differences in the folding of three fingers cause recognition of varied molecular targets and affect diverse biological properties [9–11]. We report here the anti-platelet activity of a 3FTx designated KT-6.9 purified from Indian cobra (*Naja kaouthia*) venom.

Platelet aggregation is a crucial step in normal hemostasis. Injury to the endothelial lining causes rapid adhesion of platelets, degranulation followed by further activation of platelets. Platelet surface receptors play a key role in the regulation of platelet aggregation. There are numerous intrinsic glycoprotein receptors on the membrane of platelets which bind to a distinctive set of substrate ligands [12,13]. vWF receptors (GPIb/V/IX) and collagen receptors (GPIa/IIa) are the major platelet surface receptors which are required for the platelet-platelet adhesion and the adhesion of platelets to the extra-cellular matrix during initial stages of platelet aggregation [14]. ADP receptors P_2Y_1/P_2Y_{12}, thrombin receptors PAR-1/PAR-4 and thromboxane receptors TP_α/TP_β are the major G protein coupled receptors (GPCRs) which are involved in the second phase of platelet dependent white thrombus formation [15]. KT-6.9 purified from *N. kaouthia* venom was found to inhibit platelet aggregation process mediated by GPCRs.

2. Materials and methods

2.1. Materials

Dry pooled venom of *N. kaouthia* was purchased from Calcutta Snake Park, Kolkata, India. CM Sephadex C-50, adenosine di-phosphate (ADP), fibrinogen, thrombin, FITC conjugation kit and all other fine chemicals were purchased from Sigma chemicals, USA. Platelet aggregation agonists collagen, ristocetin and arachidonic acid were purchased from Chronolog Corporation, USA. Anti-KT-6.9 polyclonal antibodies were raised in rabbits by Abexome Biosciences, India. Molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. Anti-platelet drug clopidogrel was purchased from Cipla pharmaceuticals, India. Fresh blood

Abbreviations: ADP, adenosine di-phosphate; FITC, fluorescein isothiocyanate; GPCR, G-protein coupled receptor; MW, molecular weight; RP-HPLC, reversed phase high performance liquid chromatography; SPR, surface plasma resonance.

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samples were aseptically collected from healthy 'O' positive volunteers, under medical supervision. It was ensured that none of the volunteers were under any medication for at least 3 weeks. Required consent was taken from all volunteers prior to drawing of blood. All other chemicals used were of analytical grade.

2.2. Purification of KT-6.9

Dry cobra venom (50 mg) was reconstituted in 20 mM phosphate buffer, pH 7.4 (running buffer) overnight at 4 °C. The solution was then centrifuged at 5000 rpm for 5 min at 4 °C to discard cell debris and other particulate matters. The clear solution was then subjected to cation exchange chromatography on a column of CM-Sephadex C-50 (1.5 cm × 10.5 cm) with a flow rate of 0.5 ml/min. The adsorbed fractions were then eluted with a linear gradient of NaCl (0–0.5 M) in 3 column volumes of running buffer. Fraction size was restricted to 1 ml.

Fractions with anti-platelet activity from purification I were pooled and subjected to further fractionation on a shallow linear gradient (0.2–0.5 M) of NaCl on a column of CM-Sephadex C-50 (1.5 cm × 10.5 cm). One milliliter fractions were collected in each tube at a flow rate of 0.5 ml/min. Further purification of the active fraction from purification II was performed using RP-HPLC. One milligram of semi-purified fraction was dissolved in 200 μl of water containing 0.1% trifluoroacetic acid (TFA; solution A). The solution is applied to a Shimadzu C18 reverse phase column. Elution was performed at a flow rate of 1 ml/min. Gradient was maintained with solution B (acetonitrile containing 0.1% TFA). A linear gradient of 0–80% acetonitrile was maintained. Elution of proteins was monitored at 220 nm. Purified proteins were immediately concentrated by removing acetonitrile using Genevac centrifugal vacuum concentrator.

2.3. Platelet aggregation assay

Platelet aggregation assays were performed in whole human blood as follows. Nine parts of whole human blood was mixed with 1 part of 3.8% (w/v) sodium citrate. A Chrono-Log Whole Blood aggregometer was used to monitor platelet aggregation. Five hundred microliters of 0.85% saline was incubated at 37 °C for 5 min and mixed with equal volumes of citrated whole blood for each assay. Blood samples were then treated with different concentrations of KT-6.9 for 2 min. Agonists of platelet aggregation in appropriate concentrations were added immediately to the above treated blood sample and impedance patterns were monitored. Blood samples treated with platelet aggregation agonist alone were considered as positive control. Blood samples without any treatment (i.e., agonists or KT-6.9) were considered as negative controls.

2.4. Preparation of washed platelet suspension

Washed platelet suspension was prepared by the method of Born and Cross [15]. Blood was collected from the healthy human volunteers who had not taken any medicines for at least 3 weeks and immediately mixed with Acid Citrate Dextrose (2.5 g sodium citrate, 1.4 g citric acid, 2 g anhydrous glucose pH 4.5). Blood was then immediately centrifuged at 90*g* for 10 min at room temperature. The supernatant platelet rich plasma (PRP) was retained. PRP was incubated at 37 °C for 15 min and centrifuged at 4500*g* for 20 min. The pellet obtained was suspended in Tyrode-albumin buffer (pH 6.5) mixed well and centrifuged again for 20 min at 4500*g*. Platelets were washed by repeating the above step two times. Washed platelets obtained were then suspended in Tyrode buffer (pH 7.35) containing 2 mM $CaCl_2{\cdot}2H_2O$. This suspension was used for the platelet aggregation studies.

2.5. Washed platelet aggregation studies using photo-optical method

ADP induced platelets aggregation studies were performed using the washed platelets. Platelet count was adjusted to $4.5 \times 10^8 ml^{-1}$. Platelet suspension (350 μl) was incubated with KT-6.9 (40 μg) at 37 °C for 5 min under constant stirring. ADP (10 μl) was added to the platelet suspension and incubated for different time intervals 2 min., 5 min. and 10 min. Absorbance was checked immediately at 697 nm using Shimadzu UV/VIS Spectrophotometer (Born and Cross, 1963).

2.6. Surface plasma resonance (SPR) analysis

Platelet protein binding was studied by the surface plasma resonance (SPR) measurement using BIAcore X100. SPR detects the change in the refractive index at the surface of a sensor. KT-6.9 was dissolved in 10 mM sodium acetate buffer, pH 4.0 to a final concentration of 50 μg/ml and immobilized on CM5 chip (GE Healthcare) using standard amine coupling procedure. KT-6.9 flow rate was maintained at 10 μl/min during immobilization. Ten millimolar PBS, pH 7.4 with 0.005% surfactant P20 was used as running buffer. Different dilutions of purified human blood platelets were passed onto the CM5 chip at a flow rate of 30 μl/min. Binding of platelets to the KT-6.9 was analyzed by the BIAcore evaluation software.

2.7. Modified sandwich ELISA method

Washed platelets (4.5×10^8/ml) were allowed to bind to 96 well plates using carbonate-bicarbonate coating buffer by incubating overnight at 4 °C. All the platelet dilutions were made in coating buffer. Wells were then washed three times with washing buffer (PBS with 0.05% tween20) to remove the unbound platelets, followed by blocking with 5% skimmed milk to prevent unwanted binding. Wells were then washed three times with washing buffer. Fifteen micrograms of KT-6.9 was added to the wells and incubated for 10 min at room temperature followed by washing with washing buffer three times. FITC labeled anti-KT-6.9 antibody (2:1 M ratio) was then incubated in the wells for 10 min at room temperature. Wells were then washed five times with washing buffer to remove the unbound labeled antibody. Intensity of the fluorescence was measured using ELISA reader (VICTOR3 Multi-label plate reader). Fluorescence emitted by the empty wells, wells with bound platelets without KT-6.9 treatment and the empty wells with 15 μg KT-6.9 treatment were taken as negative controls. All other treatments given to the negative control wells were similar to that of the platelets coated wells.

3. Results

3.1. Protein purification and identification

KT-6.9 was purified form the venom of *N. kaouthia* using cation exchange chromatography, and RP-HPLC (Fig. 1(A–C)), MALDI TOF–TOF mass spectrometer analysis revealed homology of KT-6.9 with many three finger toxin family proteins. This has been confirmed by the protein BLAST analysis of the query strings (i.e. Trypsin digested KT-6.9 fragments) using NCBI server which showed many hits with the sequences of 3FTx family members (Result in supplementary section).

3.2. Dose dependent inhibition of agonist induced platelet aggregation

KT-6.9 inhibited ADP induced platelet aggregation in human whole blood in a concentration dependent manner (Fig. 2(A)).

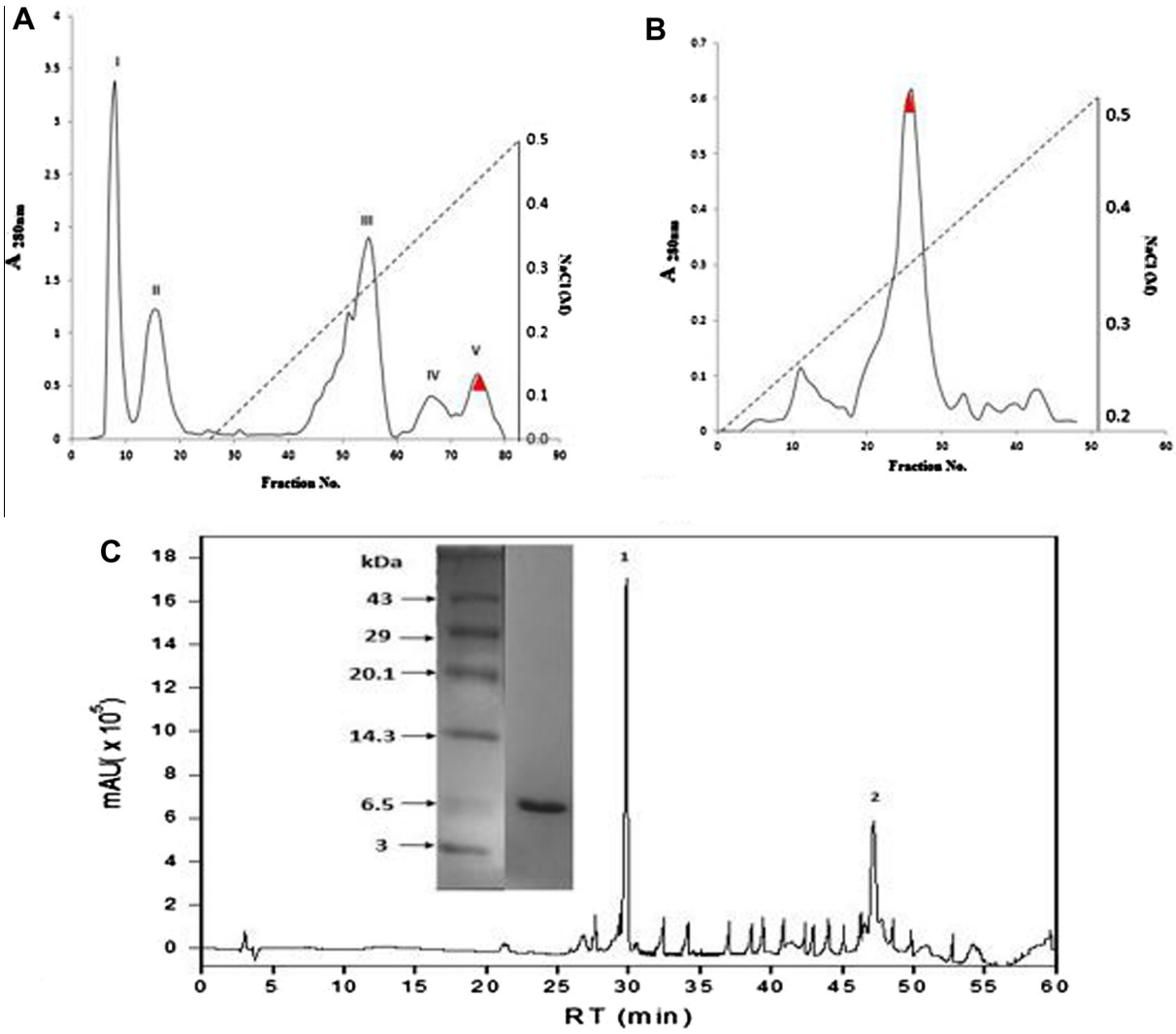

Fig. 1. (A) Cation exchange chromatography of Indian cobra venom on CM-Sephadex C-50. The dotted line represents linear gradient of NaCl. Elution peaks are numbered with roman numerals as mentioned in *Results*. (B) Rechromatography of Peak V on CM-Sephadex C-50. The fractions under Peak V (Fig. 1(A)) were pooled, concentrated and fractionated. Fractions were eluted with a linear gradient of NaCl (0.2 to 0.5 M). The dark tip of the major peak denotes presence of anti-platelet activity. (C) RP-HPLC of peak from purification II using C 18 column. KT-6.9 and NKV 66 peaks were represented as 1 and 2 respectively. Inset. 15% SDS–PAGE of KT-6.9 with Molecular weight markers.

35% inhibition of platelet aggregation was observed with 20 μg of KT-6.9 and about 70% inhibition with 40 μg of KT-6.9. Half maximum inhibitory concentration (IC_{50}) of KT-6.9 during ADP induced platelet aggregation was found to be 5.82 μg/ml. Cheng-Prusoff equation [16] for competitive inhibition was used to find the inhibitor constant (K_i) at cellular receptors. K_i for KT-6.9 during ADP induced platelet aggregation was found to be 1.1×10^{-5} M.

Inhibition of aggregation was also observed with purified platelets by photo-optical method using UV/VIS Spectrophotometer at 697 nm (Born and Cross, 1963) (Fig. 2B). Two fold increase in absorbance was observed when purified platelets were treated with 40 μg of KT-6.9, indicating inhibition of aggregation.

A significant 17% decrease in platelet aggregation was observed in arachidonic acid induced platelet aggregation when treated with 40 μg of KT-6.9 (Fig. 2C).

Thrombin induced platelet aggregation was not irreversibly inhibited but aggregation was drastically slowed down with two different doses of KT-6.9 treatment (Fig. 2D). In contrast, ADP and arachidonic acid induced platelet aggregations were irreversibly inhibited by KT-6.9.

Interestingly, collagen (Fig. 2E) and ristocetin (Fig. 2F) induced platelet aggregations were not inhibited by KT-6.9. A minor change of about 7% and 5% decrease in platelet aggregation, respectively, were observed with 40 μg KT-6.9 treatment.

3.3. Comparison of clopidogrel with KT-6.9

Anti-platelet activity of KT-6.9 was compared with P2Y receptor blocking drug clopidogrel. In a separate experiment, anti-platelet activity of KT-6.9 was found to be 25 times stronger than that of the clopidogrel at the same concentration (Result in supplementary section).

3.4. Platelet protein binding studies

Surface Plasma Resonance analysis using BIAcore X100 confirmed the binding of platelets to the immobilized KT-6.9. However, mode of binding with platelets was found to be non specific. Sensogram showing a gradual increase in the resonance units with increase in the number of platelets (Fig. 3a).

Binding studies were performed with purified platelets coated on the ELISA plate. KT-6.9 binding on platelets was assessed as a measure of fluorescence emitted by the anti-KT-6.9 antibody (Fig. 3B). Fluorescence emitted by the empty wells, wells with

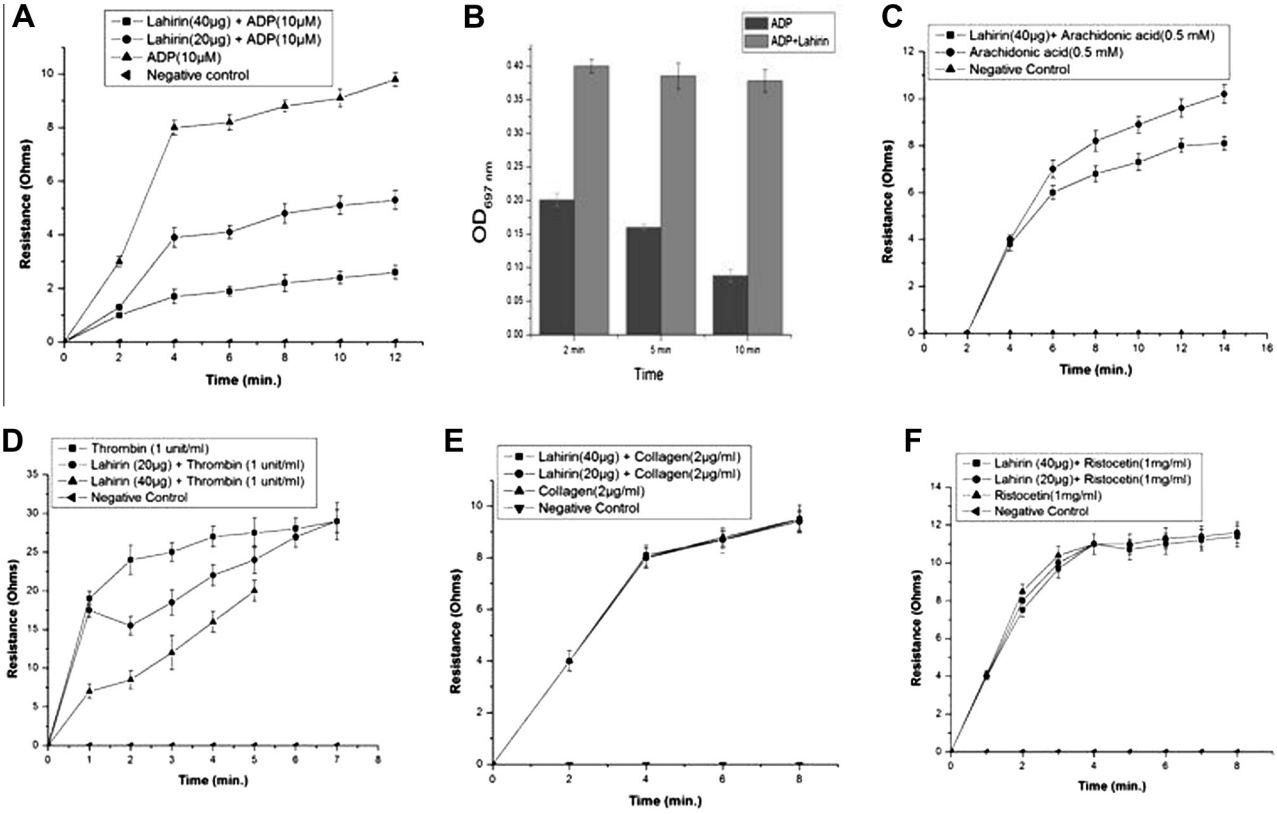

Fig. 2. (A) Effects of KT-6.9 on ADP induced platelet aggregation. Blood was treated with KT-6.9 at two different concentrations (20 and 40 µg) and the platelet aggregation process was induced by ADP ($n = 10$). Aggregation kinetics were observed in terms of resistance (Ohms) measured by the platelet aggregometer. (B) Washed platelet aggregation studies using photo-optical method. Washed blood platelets were incubated with KT-6.9 (40 µg) and aggregation was induced with ADP. OD at 697 nm was noted at different time intervals. Increase in Absorbance values for the KT-6.9 treated samples suggests the inhibition of platelet aggregation. (C) Effects of KT-6.9 on arachidonic acid induced platelet aggregation. Blood was treated with KT-6.9 (40 µg) and the platelet aggregation process was induced by arachidonic acid ($n = 7$). (D). Effects of KT-6.9 on thrombin induced platelet aggregation. Blood was treated with KT-6.9 at two different concentrations (20 and 40 µg) and the platelet aggregation process was induced by thrombin ($n = 6$). (E) Effects of KT-6.9 on collagen induced platelet aggregation. Blood was treated with KT-6.9 at two different concentrations (20 and 40 µg) and the platelet aggregation process was induced by collagen ($n = 8$). (F) Effects of KT-6.9 on ristocetin induced platelet aggregation. Blood was treated with KT-6.9 at two different concentrations (20 and 40 µg) and the platelet aggregation process was induced by ristocetin ($n = 8$).

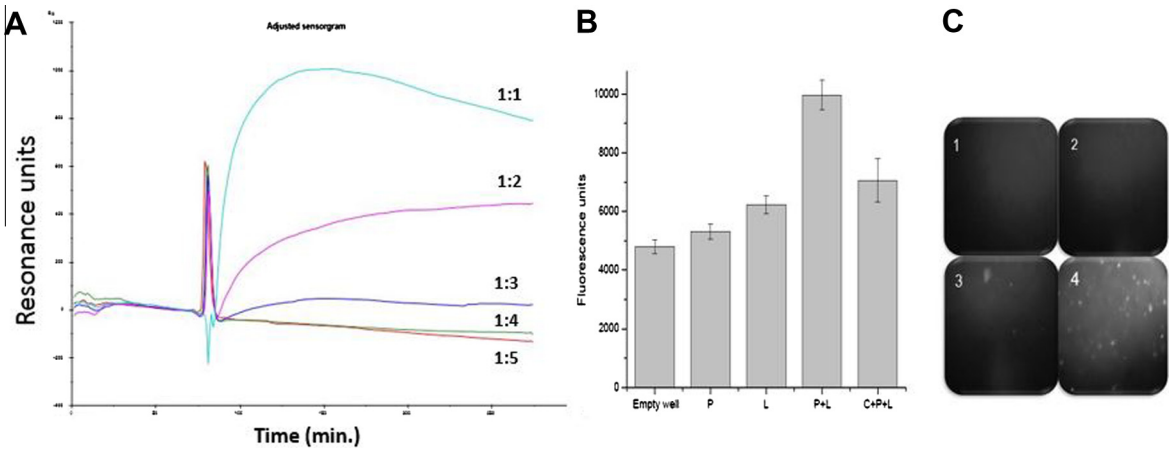

Fig. 3. (A) SPR analysis using BIAcore X100. KT-6.9 was immobilized onto the CM5 chip and binding analysis was carried out with different dilutions (dilutions started with platelet count $4.5 \times 10^8 ml^{-1}$) of platelets passed at a flow rate of 30 µl/ml. Sensogram (Resonance/Responsive units VS Time) showing the binding kinetics of KT-6.9 with blood platelets at different dilutions. (B) Extent of fluorescence emitted by FITC labeled anti-KT-6.9 antibody. *P* = well coated with platelets alone, *L* = uncoated wells treated with KT-6.9, *P* + *L* = platelet coated wells treated with KT-6.9, *C* + *P* + *L* = platelet coated wells treated by clopidogrel followed by KT-6.9 treatment. Values represent mean ± SD of three independent experiments. (C) Immuno-fluorescenceof FITC labeled anti-KT-6.9 antibody. 1) empty wells 2) wells coated with platelets alone 3) uncoated wells treated with KT-6.9 4) platelet coated wells treated with KT-6.9.

bound platelets alone and the empty wells with 15 µg KT-6.9 treatment were taken as negative controls. Increase in fluorescence values compared to negative controls indicating the binding of KT-6.9 to the platelet surface. We further confirmed the above result with fluorescent microscopy (Fig. 3C). Clopidogrel treated platelets showed reduced binding of the anti-KT-6.9 antibody, suggesting both KT-6.9 and clopidogrel might be acting on same receptors.

4. Discussion

KT-6.9, a low molecular weight (6.9 kDa) polypeptide purified from Indian monocled cobra venom was found having anti-platelet activity. MALDI- TOF/TOF analysis of KT-6.9 was carried out using peptide bands separated by SDS–PAGE. KT-6.9 showed strong homology with some 3FTx sequence. Earlier Alexey et. al., 2008 reported 3FTxs from the venom of *N. kaouthia* collected from Thailand [17]. Molecular mass of those 3FTxs varied from 14.5 to 15.6 kDa. However, the authors did not mention platelet inhibiting activity of any of the toxins.

KT-6.9 specifically inhibited ADP, thrombin and arachidonic acid induced platelet aggregation in a dose dependent manner. This emphasizes the possibility of KT-6.9 binding to G protein coupled receptors (GPCRs), as these agonists are specific to GPCRs e.g., ADP on P_2Y receptors, thrombin on PAR, and TXA_2 on TP_α and TP_β of platelets. KT-6.9 could not inhibit collagen and ristocetin induced aggregation (even at a very high dose), which act on GpIa/IIa and GpIb receptors.

Thrombin induced platelet aggregation was not irreversibly inhibited but was drastically slowed down by KT-6.9 treatment, whereas arachidonic acid induced platelet aggregation was irreversibly inhibited by KT-6.9. Reciprocal regulation and activation among the GPCRs is generally observed during the platelet aggregation process [18–20]. This could be one plausible explanation for inhibition of all the GPCRs (P_2Y, PAR and TXA_2) on the surface of platelets by KT-6.9. KT-6.9 might be binding to P_2Y receptors as it inhibits about 70% of ADP induced platelet aggregation.

SPR analysis using BIAcore X100 suggests binding of KT-6.9 to the platelets surface. Fluorescence values of the anti-KT-6.9 antibody and the fluorescence microscopic analysis confirms the KT-6.9 binding to human platelets. Binding of anti-KT-6.9 antibody on platelets was reduced significantly after clopidogrel treatment, suggesting competition between KT-6.9 and clopidogrel for the same receptor. However, exact mechanism of action of KT-6.9 is still unknown. Further studies are required to find out the target receptor(s) of KT-6.9 and to reveal its exact mechanism of action.

KT-6.9 was found to have strong homology with 3FTxs, which are primarily short chain and long chain neurotoxins. KT-6.9 was also found to be acting through GPCRs, which are integral parts of many muscarinic acetyl choline receptor signaling systems. Neurotoxic activities and other biochemical characteristics are required to be studied in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.125.

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