

CALIX[4]ARENES METHYLENE BISPHOSPHONIC ACIDS EFFECT ON PLASMIN ACTIVITY

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The aim of the work was to study plasmin inhibiting properties of calix[4]arenes functionalized by phosphonic acid residues. The following methods were used: turbidimetry, analysis of enzyme activity using chromogenic substrates, evaluation of caseinolytic activity by tyrosine release from casein, Dixon's method for determining the type and inhibition constant. It was found, that calix[4]arenes C 296, C-425, C-427 and C-145 inhibited fibrin clot lysis by plasmin in dose-dependent manner and inhibition rate is proportional to the number of phosphonic acid residues. C-145 were the most effective plasmin inhibitor (competitive mechanism, $K_i = 0,52 \mu\text{M}$). However, C-145, as well as C 296, C-425 and C-427, did not affected amidolytic and caseinolytic plasmin activity but inhibited plasminogen activation by streptokinase. Thus, we assume that the mechanism of calyx[4]arene selectivity to fibrinolysis and its ability to obstruct plasminogen-streptokinase interaction is provided by complex formation between calix[4]arene negatively charged phosphonic groups and positively charged amino acids in substrate recognition exosites of plasmin. Calix[4]arene C-145 is effective plasmin fibrinolytic activity inhibitor and perspective for further investigation as anti-fibrinolytic agent.

Key words: fibrinolysis, plasmin, plasminogen, plasmin inhibitor, plasminogen activation, streptokinase.

Plasminogen overactivation, plasmin hyperactivity and low level of plasmin inhibitors in the circulation lead to wide range of haemorrhagic disorders. Development of effective low molecular weight plasmin inhibitors opens up new prospects in hyperfibrinolysis medication. Calixarenes belong to perspective class of low-toxic compounds due to their ability to form supramolecular complexes with biological molecules.

Fibrinolytic system activation is an appropriate response to blood clotting and the main event that leads to fibrin clot dissolution by proteolysis. Plasminogen and activators from plasma bind to specific sites in fibrin during fibrinogen conversion to fibrin, or to specific receptors on cell surface, and activators cleave proenzyme into plasmin [1].

The key fibrinolytic enzyme plasmin is a trypsin-like serine protease generated from inert precursor plasminogen. Proenzyme molecule consists of N terminal and five kringle domains connected with serine protease domain by activation loop, which is cleaved during plasminogen activation by either tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) or factor XIIa complex with callicreine [2].

As result of high level of the activators release into bloodstream or acute decrease of plasmin inhibitors formation excessive activation of fibrinolytic system activation develops and causes hyperfibrinolysis. Elevation of plasmin content in blood leads to hydrolysis of fibrin, fibrinogen, clotting factors and accumulation of fibrinogen/fibrin degradation products, which

detain blood clotting and platelets aggregation, and finally to hard bleeding and hemorrhages. Hyperfibrinolysis accompanies the range of pathologic states like haemophilia, liver diseases, DIC-syndrome, oncological diseases, sepsis, etc. Overactivation of fibrinolytic system has threatening value during extensive wound and burn injuries, surgical operations on heart with artificial blood circulation or on lungs and other parenchymatous organs [3]. Plasmin formation also involved in other physiological and pathological processes including cell metastasis, cell proliferation, angiogenesis, and embryo implantation [1, 3].

Plasmin inhibition is critical in preventing adverse consequences of plasmin overactivation. Aprotinin was widely used as an antifibrinolytic drug before its discontinuation in 2008. Tranexamic acid and ϵ -aminocaproic acid, lysine analogs that inhibit plasmin by binding to plasmin's kringle domains, are currently used in the clinic. Other developing types of plasmin inhibitors include reactive cyclohexanones, nitrile warheads, peptidomimetics and polypeptides of the Kunitz and Kazal-type, sulfated glycosaminoglycan mimetics that bind to plasmin's catalytic domain [4]. One of the perspective directions of highly specific low molecular weight plasmin inhibitors search is calixarenes. Calixarenes are nanoscale cyclic oligomers with a vase shape produced synthetically by precise cycl-condensation of substituted phenols and formaldehyde, which possess intramolecular lipophilic cavities formed by aromatic rings of the macrocyclic skeleton, and may be easily functionalized. Calixarenes have low level of toxicity and can form supramolecular complexes with wide range of biopolymers. Promising substances for calixarenes structure modification are bisphosphonates — structural analogues of natural pyrophosphate. These compounds demonstrate versatile bioactivities [5, 6]. Calixarene structure modification by methylene bisphosphonates leads to acquirement of specific bioactivity [7, 8].

In the present study we have estimated the effect of calix[4]arenes derivatives of methylene bisphosphonic acid on plasminogen/plasmin system activity.

Materials and Methods

Materials. Pooled human blood plasma for plasminogen and fibrinogen isolation was purchased from local hospital bank.

Plasminogen with an amino-terminal glutamic acid residue (Glu-plasminogen) was

prepared from fresh citrate donor plasma by affinity chromatography using the Lysine-sepharose 4B (Sigma Aldrich, USA) [9]. Glu-plasminogen purity was tested by 11,5% PAGE at pH 3.2.

Plasmin was prepared by activation of Glu-plasminogen with urokinase (HS Medac, Germany), immobilized to BrCN-activated Sepharose 4B (Sigma Aldrich, USA). 1 mg of proenzyme was incubated with 0.5 ml of urokinase-sepharose gel (1250 IU/ml) during 1 hour at 37 °C in 50 mM sodium-phosphate buffer solution pH 7.4 with 25% glycerol. Plasmin was stored in 50 mM sodium-phosphate buffer solution pH 7.4 with 50% glycerol at 20 °C. Activation efficiency was evaluated by plasmin caseinolytic and amidolytic activity and 10% PAGE with SDS in the presence of 2% β -mercaptoethanol.

Fibrinogen was purified from fresh citrate human plasma with 1000 KIU/ml aprotinine (Merckle, Germany) and 20 mM ϵ -aminocaproic acid (Sigma Aldrich, USA) by fractionation with sodium sulfate. Before fractionation plasma was twice treated by barium sulfate (60 mg/ml) by the method of Smith [10], heated to 25 °C and mixed with 1 M glycine buffer solution pH 9.0 in 1/9 volume ratio. 16% sodium sulfate was added to 5.7% saturation for plasma albumins fraction elimination and the precipitate was removed by centrifugation at 5000 g. Supernatant was slowly mixed with 16% sodium sulfate to 8.5% saturation and centrifuged at 5000 g, precipitate was dissolved in 0.2 M sodium chloride (10/1 part of initial plasma volume). Fibrinogen solution was treated by equal volume of 16% sodium sulfate after addition of 5/1 volume of 0.5 M monopotassium phosphate and centrifuged, precipitate was diluted in 0.15 M sodium chloride (10/1 part of initial plasma volume). After overnight incubation in ice fibrinogen solution was centrifuged at 5000 g for cryofibrinogen elimination and then mixed with equal volume of 16% sodium sulfate. Fibrinogen precipitate was separate by centrifugation at 5000 g, dissolved in 0.15 M sodium chloride and stored at 20 °C.

desAB-fibrin was obtained by dissolving of fibrin clot formed by thrombin (EC 3.4.21.5) — 1 NIH per 1 mg of fibrinogen (Sigma Aldrich, USA) in the presence of 50 mM ϵ -aminocaproic acid and sodium parahydroxy mercury benzoate (0.35 mg/ml) (Sigma Aldrich, USA) in 20 mM acetic acid as described elsewhere [11].

Calix[4]arenes C145-, C296-, C425-, C427- and 4-hydroxyphenyl methylenbisphosphonic acid were kindly provided by Dr. V. I. Kalchenko

and were synthesized as described in [7]. C-145 is a sodium salt of calix[4]arene 5,11,17,23-tetrakis-methylene bisphosphonic acid, C-296 — 5,17-bis(dihydroxyphosphonylmethyl)-11,23-dibrom-25,26,27,28-tetrapropoxycalix[4]arene, C-425 — 5,17-bis(1-hydroxymethyl-1,1-bis(dihydroxyphosphoryl)-25,26-propoxycalix[4]arene, C-427 — 5-(1-hydroxymethyl-1,1-bis(dihydroxyphosphoryl)-17-(1-keto-1-dihydroxyphosphorylmethyl)-25,27-dipropoxycalix[4]arene (Fig. 1). C-145 and 4-hydroxymethylenbisphosphonic acid stock solutions (1 mM) were prepared in working buffers. Stock solutions of calix[4]arenes C-296, C-425 and C427 with concentration 1 mM were prepared in distilled water and then were used for working solutions preparing in working buffers.

All inorganic chemicals, amino acids and acetic acid were purchased from Synbias, Ukraine. PAGE chemicals were purchased from Sigma Aldrich, USA.

Methods. Amidolytic activity assay. Plasmin activity was evaluated by amidolytic activity assay. Assessment of amidolytic activity was performed by optical density changes as result of chromogenic substrate S2251 (H-D-Val-

L-Leu-L-Lys-p-nitroaniline, Chromogenix, Sweden) cleavage by plasmin or plasminogen, activated by streptokinase (Kabikinase, Pharmacia, Sweden). The reaction mixture contained 0.03 μM plasmin (or 0.03 μM plasminogen) and 0.3 mM S2251 in 50 mM tris buffer solution, pH 7.4 with 150 mM NaCl. The assay was performed in 96-wells plate at 37 °C. The amidolytic activity was determined by measurement of the absorbance at 405 nm using 96-well plate reader (Multiskan Titertek, Finstruments, Finland).

Fibrinolytic activity assay. For the estimation of fibrinolytic activity of plasmin or plasminogen, activated by tissue type plasminogen activator (Actylise, Boeringher Ingelheim, Germany), streptokinase (Kabikinase, Pharmacia, Sweden), the turbidimetric method was applied as described by Bouvier [12]. The final concentration of desAB-fibrin was 0.6 μM , plasmin — 0.02 μM . The rate of fibrin clot lysis by plasmin was calculated as $V=1/t_{50\%}$. Half-time of clot lysis ($t_{50\%}$) was calculated as period of time from the beginning of clot formation to the point when the turbidity of clot was 1/2 from the maximal.

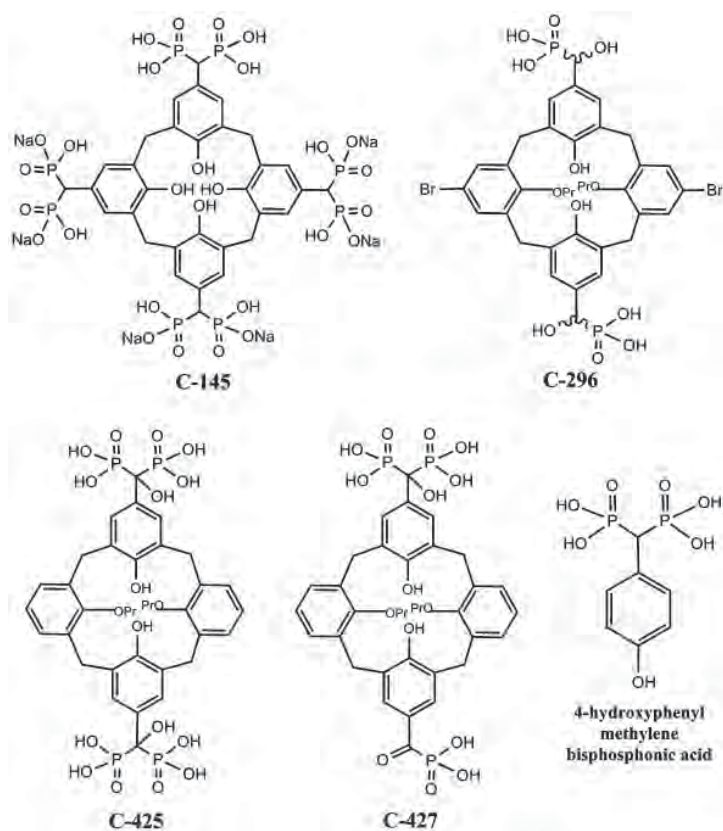


Fig. 1. Structure of calix[4]arenes C-145, C-296, C-427, C-425 and monomeric molecule 4-hydroxyphenyl methylene bisphosphonic acid

Caseinolytic activity assay. Casein (Sigma, USA) dissolved in 70 mM PBS pH 7,4 to the final concentration 2% was hydrolyzed by 30 μ M plasmin during 30 min at 37 °C. The reaction was terminated by adding of 10% trichloroacetic acid in the 3:2 volume ratio. Precipitated protein was removed by centrifugation at 12,000 rpm and 4 °C for 10 min. The absorbance of the supernatant at 280 nm was measured using a LambdaBio+ spectrophotometer (Perkin Elmer, USA). One unit (U) of enzyme activity was defined as the amount of enzyme required to increase the absorbance at 280 nm by 0.001 AU per minute under the aforementioned assay conditions [13]. Specific caseinolytic activity was calculated as caseinolytic activity per 1 mg of plasmin.

Inhibition constant calculation. For inhibition type determination and inhibition constant calculation Dixon method was applied [14].

Statistical data analysis. Kinetic curves are typical for series of experiment ($n \geq 3$). Data was analyzed using GraphPad Prism 7 software. Enzymes activity is expressed as mean \pm SEM. ANOVA Dunnett test was used for P-value assessment where it is applicable. $P < 0.05$ was considered as a level of significance.

Results and Discussion

Calix[4]arenes with various number of phosphonic acid residues inhibit plasmin fibrinolytic activity with different intensity. C-145 — sodium salt of calix[4]arene methylen bisphosphonic acid — demonstrates most effective inhibition of plasmin. At minimal used concentration C-145 (1 μ M) decreases clot lysis rate by 46%, whereas maximal (10 μ M) totally inactivate plasmin and fibrin is not hydrolyzed. C-425 and C-427 decrease plasmin activity by respectively 1.5% and 17% at 1 μ M and by 70% and 77% at 10 μ M. C-296 does not affect fibrin clot lysis in concentration range 1 — 5 μ M, but at 10 μ M decreases lysis rate by 30% (Fig. 2). The data demonstrates that the less phosphonic groups contains calix[4]arene, the less fibrinolytic inhibition activity it has. However, 4-hydroxyphenyl-methylen bisphosphonic acid, which represents 1/4 part of C-145 does not inhibit plasmin fibrinolytic activity at any used concentration. Obviously, full calix[4]arene molecule is necessary for plasmin inhibiting action, probably its spatial “basket” structure provides optimal orientation of negatively charged phosphonic groups.

C-145 has most efficient inhibiting action on fibrinolysis therefore in further we investigated this calix[4]arene interaction with plasminogen/plasmin system proteins.

For the explanation of C-145 action on fibrinolytic activity we have investigated its effect on polymeric fibrin hydrolysis by streptokinase- and tPA-activated plasminogen. The calix[4]arene inhibits fibrin clot lysis by streptokinase- and tPA-activated plasminogen, as well as plasmin, in dose-dependent manner (Fig. 3, A–C). At minimal used concentration (1 μ M) C-145 decrease the rate of clot lysis by streptokinase- and tPA-activated plasminogen on 40 and 45% respectively (Fig. 3, D). At the presence of maximal concentration of C-145 (10 μ M) the clot is not hydrolyzed. The same pattern of fibrin clot hydrolysis inhibition for the activated by tPA and streptokinase and plasmin indicates that C-145 interacts with the active enzyme and decline its interaction with physiological substrate — fibrin, resulting in dramatically decreased fibrinolytic activity.

Plasmin in blood circulation catalyzes hydrolysis not only of fibrin, but also of other proteins — fibrinogen, fibronectine, thrombospondine, von Willebrand factor, activates collagenases, induces the generation of bradykinine [1]. Peptide chromogenic substrate assay is used for assessment of non-fibrinolytic hydrolytic activity of plasmin. We investigated amidolytic activity of plasmin against S2251 in the presence of C-145. The

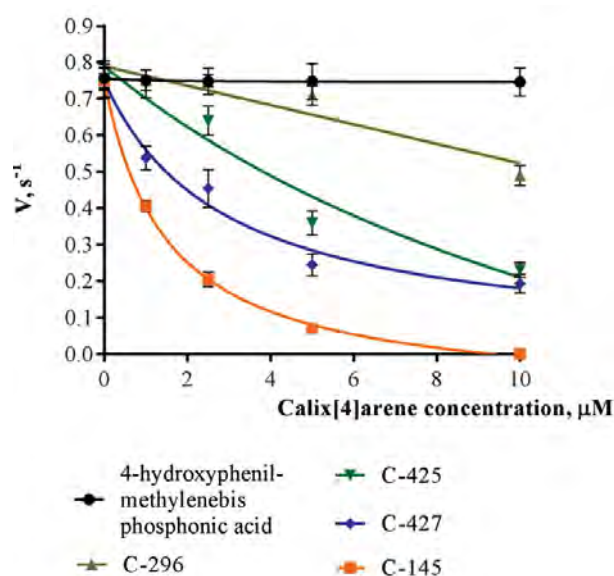


Fig. 2. Rate of fibrin clot hydrolysis by plasmin in the presence of calix[4]arenes: reaction rate was calculated as reciprocal value of clot half-lysis time

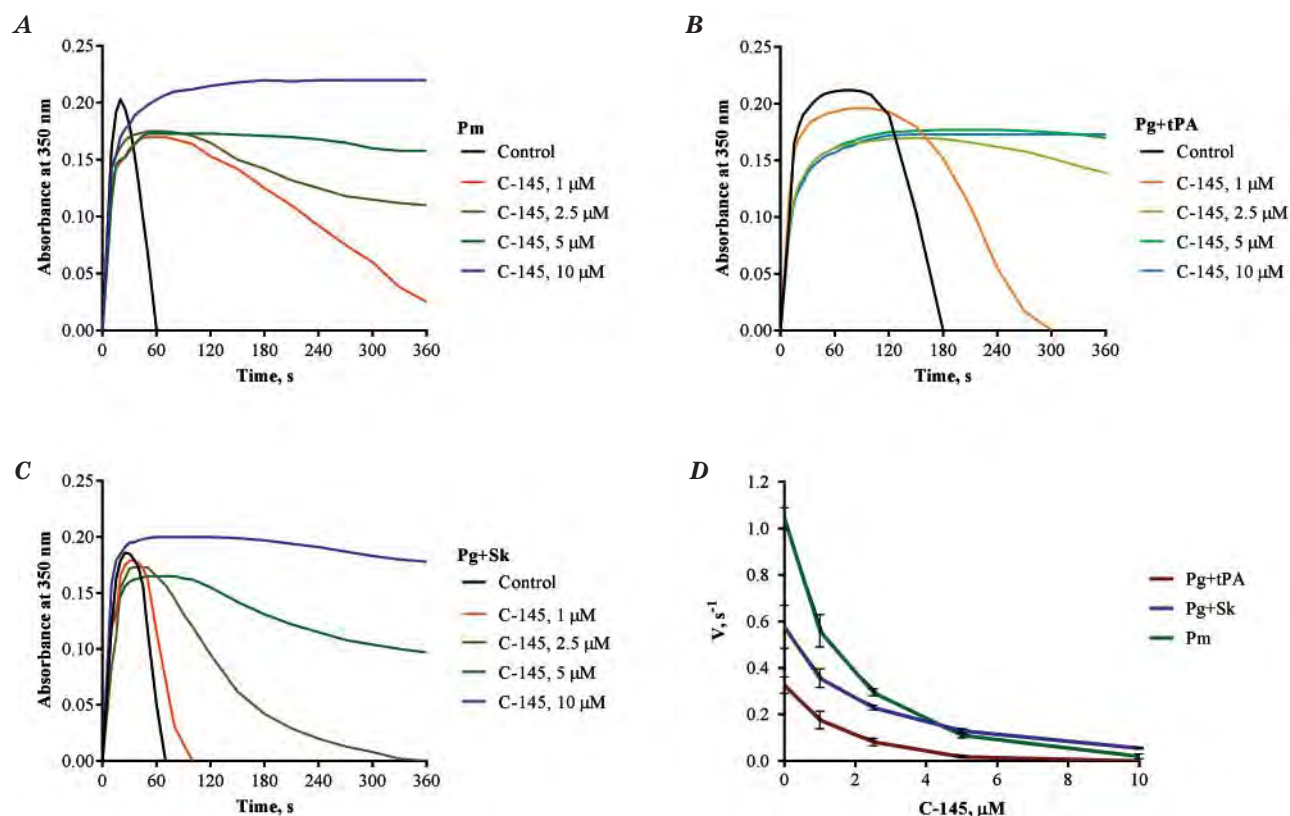


Fig. 3. desAB fibrin hydrolysis by plasmin and tPA- and streptokinase-activated plasminogen in the presence of calixarene C-145 (1–10 μM):

A–C — typical kinetic curves; D — fibrin hydrolysis reaction rate, represented as reverse value of half-lysis time ($1/t_{50\%}$)

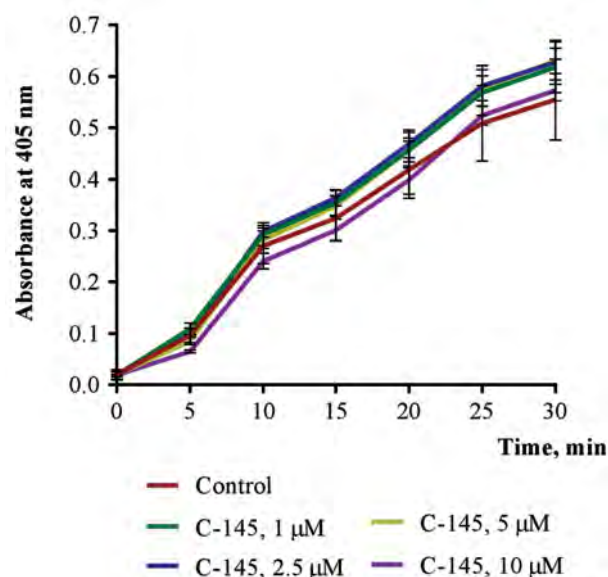


Fig. 4. Amidolytic activity of plasmin in the presence of calix[4]arene C-145 (concentration range 0–10 μM):

ANOVA Dunnett test confirms no significant effect of C-145 on chromogenic substrate hydrolysis by the enzyme measured as optical density increase as result of H-D-Val-L-Leu-L-Lys-*p*-nitroaniline formation

calix[4]arene has no effect on chromogenic substrate hydrolysis at any concentration used in the study (Fig. 4).

It should be mentioned that C-296, C-425 and C-427 also do not effect amidolytic activity of plasmin.

Casein is non-specific high molecular weight protein substrate for plasmin and is used for plasmin activity determination. It has linear structure and contains high amount of tyrosine residues. Investigation of casein hydrolysis by plasmin in the presence of C-145 (1–10 μM) have demonstrated that the calix[4]arene does not change plasmin caseinolytic activity.

Due to the absence of inhibiting effect of C-145 on amidolytic and caseinolytic activity of plasmin, the calix[4]arene is probably specific inhibitor of plasmin hydrolytic activity against its physiological substrate fibrin.

To determine the calix[4]arene inhibition constant and inhibition type we have evaluated rate of desAB fibrin lysis by plasmin at different concentration of the inhibitor (0, 1, 2.5, 5 μM) and at two

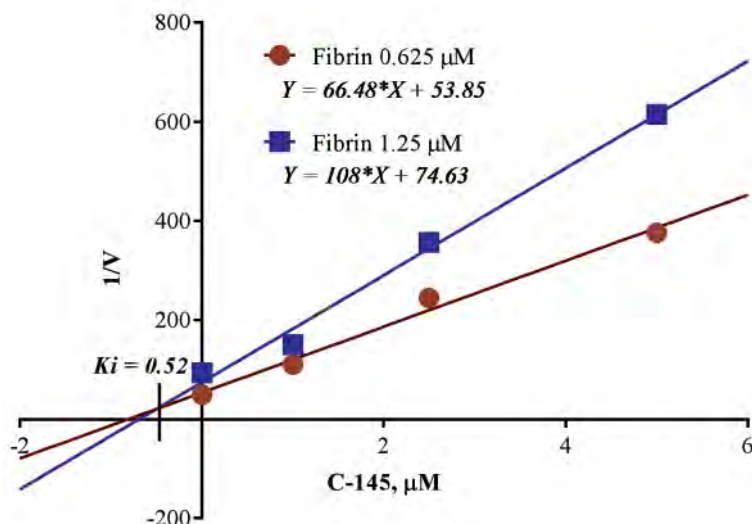


Fig. 5. Determination of inhibition constant for C-145 delaying of desAB fibrin plasmin-mediated clot lysis using Dixon plot:

the inhibitor was used in concentration range 0 – 5 μM, substrate — 0.625 and 1.25 μM. K_i is calculated as reverse X coordinate value of lines interception point

concentration of substrate (0.625 and 1.25 μM of fibrin). Obtained data was used for Dixon plots fitting and K_i calculation (Fig. 5). The plot indicates competitive nature of plasmin inhibition by C-145 with inhibition constant $K_i = 0.52$ μM.

Probably calixarene C-145 forms supramolecular complex with plasmin molecule near the active site and obstructs high molecular weight substrates into the enzyme catalytic pocket, resulting in fibrinolytic, but not amidolytic, activity inhibition.

It is well known, that streptokinase binds to plasminogen kringles 4 and 5 and catalytic domain [15]. Streptokinase binding results in conformational and substrate specificity change of the proenzyme and streptokinase-plasminogen complex do not hydrolyzes fibrin despite of having the proteolytic activity towards plasminogen activation loop [16], as well as towards chromogenic substrate S2251. In catalytic domain of the proenzyme streptokinase occupies positively charged amino acids cluster near active site. We assumed the similar pattern of interaction of streptokinase and C-145 due to the strong negative charge of the calix[4]arene, provided by eight phosphonic groups. To test this hypothesis, we have evaluated the rate of plasminogen activation by streptokinase, using chromogenic substrate S2251. Because of disability of C-145 to inhibit amidolytic activity of plasmin despite of fibrinolysis

inhibition, such way is informative for the plasminogen activators activity. As was demonstrated, the calix[4]arene inhibits the proenzyme activation by streptokinase in dose-dependent manner (Fig. 6) probably preventing the interaction between these two proteins. In the presence of 50 μM of C-145 plasmin formation is fully inhibited.

The results demonstrate that calix(4)arene C-145 containing four methylene bisphosphonic acid residues suppresses plasmin fibrinolytic activity, but does not affect the hydrolysis of

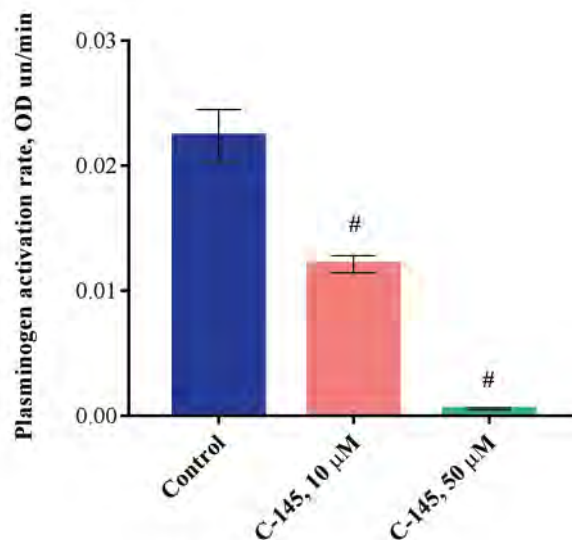


Fig. 6. Rate of Glu-plasminogen activation by streptokinase in the presence of C-145:
— $P < 0,0001$ compared to control

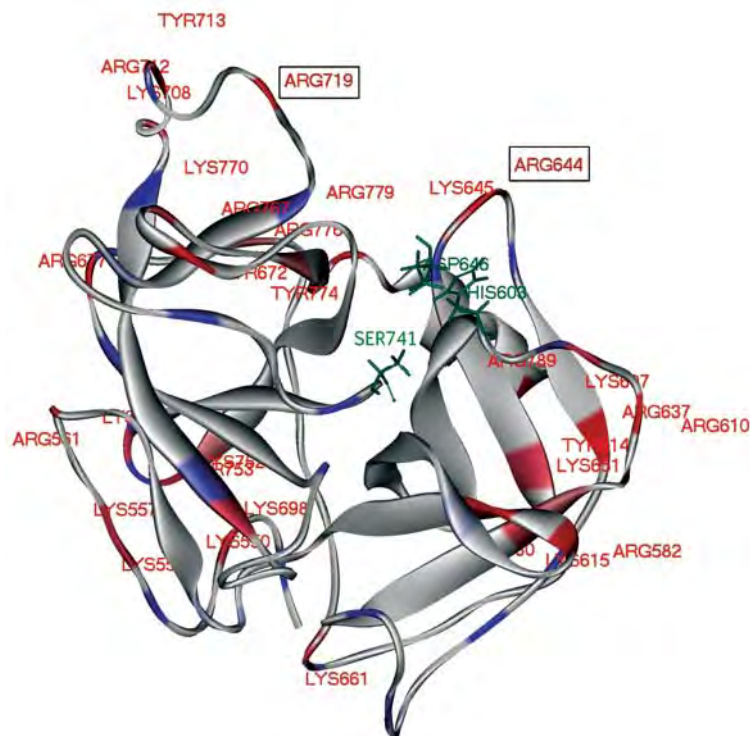


Fig. 7. Catalytic domain of human plasminogen:

(figure was generated using Discovery Studio 2016 software and 1DDJ.pdb).

Positively charged amino acids highlighted by red. Amino acids in black boxes are involved in plasminogen-streptokinase interaction.

Catalytic triade (green): His 603, Asp 646, Ser 741

chromogenic peptides and denaturated protein substrates. It indicates that C-145 blocks high molecular weight substrates entering into the enzyme active site. The calix(4)arene structure implies its interaction with positively charged groups. Catalytic domain of plasmin contains 12 arginine and 9 lysine residues clustered near the active site [17] (Fig. 7).

Noncovalent interaction between the calix[4]arene and side chains of these amino acids results in obstruction of plasmin catalytic domain interaction with substrate, which is necessary for the substrate orientation in catalytic pocket. Low molecular weight substrates do not interact with the clusters and reach the catalytic pocket directly, that is why calix[4]arenes demonstrate no inhibition activity towards chromogenic substrate cleavage by plasmin.

Two positively charged amino acids near active site of plasmin — Arg 644 and Arg 719 — are involved in plasminogen-streptokinase complex formation [17]. Blocking of them attenuates streptokinase binding to the proenzyme and plasminogen activation. Prevention of plasminogen activation by

streptokinase in the presence of C-145 confirms that phosphonic groups of the calix[4]arene bind to positively charged residues in plasminogen catalytic domain including Arg 644 and Arg 719, obstructing intermolecular interactions.

Calix[4]arenes methylene bisphosphonic acids demonstrate different level of inhibiting action on plasmin fibrinolytic activity. Calix[4]arene C-145 is a most effective plasmin inhibitor due to its structure with four phosphonic acid residues. C-145 delays fibrin clot hydrolysis by plasmin and plasminogen, activated by tissue type activator and streptokinase.

Since C-145 is an effective specific fibrinolysis inhibitor, it is perspective as potential novel pharmaceutical agent for clinical intervention against fibrinolytic system overactivation disorders.

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ВПЛИВ КАЛІКС[4]АРЕНІВ МЕТИЛЕНБІСФОСФОНОВИХ КИСЛОТ НА АКТИВНІСТЬ ПЛАЗМІНУ

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Метою роботи було дослідити інгібувальні властивості калікс[4]аренів, функціоналізованих різною кількістю залишків фосфонові кислоти, стосовно плазміну. Використовували такі методи: турбідиметрію, аналіз ензимної активності за хромогенним субстратом, оцінювання казеїнолітичної активності за вивільненням тирозину з казеїну, метод Діксона для визначення типу та константи інгібування. Було виявлено, що калікс[4]арени С 296, С-425, С-427 та С-145 інгібують лізис фібринового згустку плазмином залежно від дози, і швидкість інгібування пропорційна кількості фосфонових груп. С-145 є найефективнішим інгібітором плазміну (конкурентний механізм, $K_i = 0,52$ мкМ). Проте жоден з досліджених калікс[4]аренів не впливає на амідолітичну та казеїнолітичну активність плазміну, однак інгібує активацію плазміногену стрептокіназою. Ми припускаємо, що механізм селективності калікс[4]аренів до фібринолізу і його здатність перешкоджати взаємодії плазміноген-стрептокінази забезпечується комплексоутворенням між негативно зарядженими фосфоновими групами калікс[4]арену та позитивно зарядженими амінокислотами в екзосайтах активного центру плазміну, що розпізнають субстрат. Таким чином, із досліджених калікс[4]аренів С-145 є найбільш ефективним інгібітором фібринолітичної активності плазміну і перспективним для подальшого вивчення як антифібринолітичний засіб.

Ключові слова: фібриноліз, плазмін, плазміноген, інгібітор плазміну, активація плазміногену, стрептокіназа.

ВЛИЯНИЕ КАЛІКС[4]АРЕНОВ МЕТИЛЕНБІСФОСФОНОВИХ КИСЛОТ НА АКТИВНОСТЬ ПЛАЗМИНА

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Целью работы было исследование ингибирующих свойств калікс[4]аренов, функционализированных разным количеством остатков фосфоновой кислоты, относительно плазмина. Использовали следующие методы: турбидиметрия, анализ энзимной активности по хромогенным субстратам, оценка казеинолитической активности по высвобождению тирозина из казеина, метод Диксона для определения типа и константы ингибирования. Было обнаружено, что калікс[4]арены С 296, С-425, С-427 и С-145 ингибируют лизис фибринового сгустка плазмином в зависимости от дозы, и скорость ингибирования пропорциональна количеству фосфоновых групп. С-145 является наиболее эффективным ингибитором плазмина (конкурентный механизм, $K_i = 0,52$ мкМ). Однако ни один из исследованных калікс[4]аренов не влияет на амидолитическую и казеинолитическую активность плазмина, но ингибирует активацию плазминогена стрептокиназой. Мы предполагаем, что механизм селективности калікс[4]аренов к фибринолизу и его способность препятствовать взаимодействию плазминоген-стрептокиназы обеспечивается комплексообразованием между отрицательно заряженными фосфоновыми группами калікс[4]арена и положительно заряженными аминокислотами в экзосайтах активного центра плазмина, которые распознают субстрат. Таким образом, из исследованных калікс[4]аренов С-145 является наиболее эффективным ингибитором фибринолитической активности плазмина и перспективен для дальнейшего изучения как антифибринолитическое средство.

Ключевые слова: фибринолиз, плазмин, плазминоген, ингибитор плазмина, активация плазминогена, стрептокиназа.