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Anti-thrombotic Activity of Water-soluble Calix[n]arenes

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Abstract

The parent *p*-sulfonato-calix[n]arenes and six O-monosubstituted derivatives were investigated *in vitro* for anticoagulant activity. Different concentrations of calixarenes were tested, showing that the compound 49-mono-(2-carboxymethoxy)-5,11,17,23,29,35,41,47-octa-sulfonato-calix[8]arene (C8SMA) has a significantly strong prolongation on the activated partial thromboplastin time (APTT) and on the thrombin time (TT) than the other calixarenes. Secondly, investigation of whether the anticoagulant behaviour was via interaction with antithrombin or Heparin Cofactor II was determined. Thrombin inhibition mediated by antithrombin (AT) and Heparin Cofactor II (HCII) activation was investigated in comparison to the biological activators, Heparin (Hep) and Dermatan sulfate (DS). The results show that the 49-mono-(2-carboxymethoxy)-5,11,17,23,29,35,41,47-octa-sulfonato-calix[8]arene (C8SMA) and 5,11,17,23,29,35-hexa-sulfonato-calix[6]arene (C6S) produce activation of HCII at 500 μM comparable to that induced by DS at 100 μM. However, activation of AT by all of the investigated calixarenes is between 10 and 50 times lower than that observed in the presence of heparin. The mechanism of the anticoagulant effect of these calixarenes is as activators of HCII and not as activators of AT.

Abbreviations: APTT – activated partial thromboplastin time; AT – antithrombin; DS – dermatan sulfate; GAG – glycosaminoglycan; HCII – Heparin Cofactor II; TT – thrombin time

Introduction

Biomimetic chemistry has been one of the key points of supramolecular chemistry since the discovery of the cation transport properties of the crown-ethers by Pedersen in 1967 [1]. However, true biological activity of supramolecular compounds is less well defined, indeed the cholesterol complexation properties at the cellular level by the cyclodextrins have proved a major stumbling block to their application. Perhaps the first report of the bioactivity of supramolecular systems was in 1955 by Cornforth [2] on the anti-tubercular activity of polyphenols in which, at the time un-recognized, the calix[n]arenes were included.

The calix[n]arene sulfonates have recently shown bioactivity as chloride ion channel blockers [3] and have been reported to act as anti-viral agents [4] and as anti-thrombotics [5]. The initially proposed mechanism for the anti-thrombotic activity involved the endothelial cell activation but a slightly later patent proposed a mechanism via protease inhibition.

The coagulation cascade involves sequential limited proteolysis of successive clotting factors [6]. The proteolysis reveals the serine protease activity of these factors, leading finally to generation of thrombin (IIa). The thrombin transforms fibrinogen to fibrin which interacts with cells to form a clot [7]. Depending on the triggering events, one of the two pathways is involved, either the intrinsic (initiated by blood contact with negatively charged surface) or the extrinsic (initiated by tissue factor exposure when a blood vessel is damaged) [8].

The spatial expansion of the coagulation event is controlled by both cell surface inhibitors (thrombomodulin, heparansulfate proteoglycan, etc) and circulating inhibitors [9]. Among the circulating plasmatic inhibitors, Antithrombin (AT) and Heparin Cofactor II (HCII) belong to the class of serpin inhibitors [10]. This class acts as false substrates for serine protease, the serpin establishes an irreversible covalent linkage with the enzyme giving to an inactive complex. Thus the inhibitory effect is dramatically increased (thousand fold) by glycosaminoglycan sulfates (GAG sulfates), located on cell surfaces and in the extracellular matrix [9]. The amplification of the activity of AT and HC II arises by recognition of the GAG sulfates binding site, located mainly in the D helix of the protein in a region particularly rich in Lysine and Arginine. AT is activated only by heparin [11–13], while HCII is activated by many polyanionic macromolecules including dermatan sulfate, that increase the rate of thrombin inhibition [14–16].

The generation of the inactive Thrombin-Antithrombin complex (TAT) leads to the heparin release thus considered acting as an indirect anticoagulant.

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However, thrombin binds to fibrin and is not inhibited by the antithrombin-heparin complex [17]. Direct thrombin inhibitor (DTI) has been used to interact directly with the thrombin-fibrin complex and inhibit its activity [18]. DTI exists as univalent inhibitors that interact with catalytic site of thrombin such as melagatran and bivalent inhibitors such as hirudin and bivalirudin that interact with both; the catalytic site and the binding site of fibrin to the thrombin [19]. Warfarin (antagonist of vitamine K) [20] and heparin [21, 22] are the most important therapeutic agents for the treatment of thrombosis [23]. Anticoagulant activities have been widely studied properties for sulfated macromolecules such as polyphenols [24], chitosan sulfate [25, 26] and semi-synthetic poly-saccharides [27, 28]. The complexation ability of *para*-sulfonato-calix[n]arenes towards the (key) positively charged amino acids Arginine and Lysine [29, 30] present in the heparin recognition peptide sequences has been clearly demonstrated. The structure of the supramolecular complexes between calyx[4]arene sulfonate and Lysine [31] has been reported. A series of novel *para*-sulfonato-calix[n]arenes bearing a pendant group such as carboxylic acid or amine at the lower rim of calixarene has been synthesized [32]. The presence of these functional groups is of interest as a possible means to increase the interaction between *para*-sulfonato-calix[n]arenes and antithrombin.

The anti-thrombotic activity of the *para*-sulfonato-calix[n]arene derivatives is believed to proceed by a heparinoid like inhibitory effect on protease activity in the coagulation cascade and two most likely target proteins for this interaction would be AT or HC II.

Materials and methods

Reagents

The parent *para*-sulfonato-calix[n]arenes and their derivatives were synthesized according to the literature method and were purified by RP HPLC prior to use [32]. The time of plasma coagulation is accurately measured using an optical and a mechanical measurement Coagulometer 2 channel COA-Titer 2 purchased from CGA STRUMENTI SCIENTIFICI S.P.A. (Firenze, Italy) and ensured very high measurement sensivity up to the coagulation point. Optical density at 405 nm was determined using a UV-Spectrometry Beckman UV-DU640. The analysis of anti-Thrombin activity was determined using Stachrom HCII and Stachrom ATIII (Diagnostica Stago S.A.,Taverny, France).

Plasma samples

For the different experiments tested, the citrated platelet plasma (0.105 M trisodium citrate; 1:9 v:v) is supplied by the blood bank from freshly drawn human blood from healthy donors without history of bleeding or thrombosis (15 min of time centrifugation at room temperature). For each plasma obtained, the blood provided by five donors is pooled. Blood was centrifuged at 2500 g during 25 min and the plasma is directly used for studying the coagulation time. Five experiments needed and performed for determining the effect for each concentration of calixarene on the different coagulation tests.

Coagulation time activated by Ca^{2+} (Activated partial thromboplastin time-APTT)

Increasing concentrations of *para*-sulfonato-calix[n] arene derivatives (10–130 μM) were added to 0.1 ml of fresh human pool plasma and 0.1 ml of negatively charged material (Kaolin, CGA Strumenti Scientifici S.P.A., Italy) and incubated during 3 min at 37 °C. At this time, the factor XII is autoactivated and converts prekallikrein (PK) to kallikrein which activates factor XII to XIIa. The blood cascade coagulation resulting in thrombin generation and conversion of fibrinogen to fibrin was started by adding 0.025 M of $CaCl_2$. The coagulation time is measured two times for each sample to confirm the reproducibility.

Thrombin time bioassay (Thrombin clotting time)

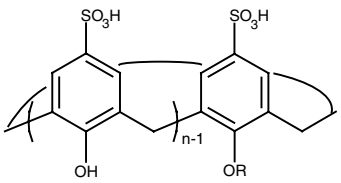
Increasing concentrations of *para*-sulfonato-calix[n]arene derivatives (100–1.5 mM) were added to 0.2 ml of fresh human pool plasma and incubated during 2 min at 37 °C. Coagulation is started by added 0.2 ml of a solution of bovine Thrombin s in HEPES buffer (3–4 units NIH/ml, Kit Thromboquik, bioMérieux, France).

Anti-Thrombin activity measurement (chromogenic assay)

Thrombin activity is quantified through the proteolysis of a specific chromogenic substrate (CBS 61.50, Diagnostica Stago S.A., France) leading to the release of a para-nitroaniline group absorbing at 405 nm. About 10 μl of human HCII (1.5 μM) or bovine AT (2 μM) is incubated at 37 °C with 200 μl of Dermatan sulfate (DS) at 0.1 mg/ml, Heparin at 0.1 mg/ml or *para*-sulfonato-calix[n]arene derivatives at 100 and 500 μM during 60 s is added at 200 μl of phosphate buffer salt pH 7,4. About 200 μl of thrombin (5.5 NIH units in 2 ml) is added and incubated during 120 s followed by addition of 200 μl of a solution of chromogenic substrate (2.5 μmoles in 2 ml). The enzymatic reaction is stopped by adding 200 μl of 100% acetic acid 90 s later. Absorbance is quantified at 405 nm against a control without anticoagulant factor.

Results

The structures and abbreviated names of the *para*-sulfonato-calix[n]arenes used in this study are given in Figure 1.



1a-c (n=4), **2a-c** (n=6), **3a-c** (n=8)
R= -H (a); -CH_2COOH (b); -$CH_2CH_2NH_2$ (c)
1a = C4S **1b** = C4SMA **1c** = C4SMN
2a = C6S **2b** = C6SMA **2c** = C6SMN
3a = C8S **3b** = C8SMA **3c** = C8SMN

Figure 1. Molecular structure of *para*-sulfonato-calix[n]arene derivatives.

Activated partial thromboplastin time

The presence of different concentrations of all *para*-sulfonato-calix[n]arenes and their derivatives, except for *para*-sulfonato-calix[4]arene and its derivatives, produces an effect on the prolongation of APTT in presence of calcium (required to initiate the activity of proteases in the blood cascade coagulation). The resultant coagulation time activated by Ca^{2+} is shown in Figure 2. The value of coagulation time in the absence of the anticoagulant agent is 70 ± 5 s. The reaction time of APTT is observed until 400 s. The highest activity of calixarene is observed for C8SMA showing a ratio $APTT_{calix}/APTT_{without\ calix}$ of 7 at 50 μM. For all the concentrations tested, the *para*-sulfonato-calix[8]arene derivatives, C8SMA and C8S, showed larger effects than other calixarenes. *Para*-sulfonato-calix[8]arene mono amine, C8SMN, has a lower effect than C8SMA and C8S, comparable to that of C6SMN. No significant difference in relation to the prolongation of clotting was observed for C4S, C4SMA and C4SMN between 10 μM and 1 mM. Moreover, the prolongation of APTT by increasing concentration of *para*-sulfonato-phenol is the same of prolongation of APTT in absence of anticoagulant (APTT = 60 s). The results show a clear dependence of the prolongation as a function of the macrocycle size.

Thrombin Time

In the presence of added thrombin, in plasma incubated at 37 °C, the coagulation time in the absence of anticoagulant is extremely rapid with a time of clot formation being 12 ± 0.2 s. In the presence of various calix[n]arenes derivatives, the prolongation of Thrombin Time is observed except with the calix[4]arene derivatives (Figure 3). The compounds C8S and C8SMA present the same anticoagulant effect and full inhibition of coagulation is observed at 200 μM and the inhibition of coagulation occurs at a concentration of 600 μM for C8SMN. C6S, C6SMA and C6SMN present full anticoagulation activity at concentrations of 0.9, 1.1 and 1.3 mM, respectively. Again, the anticoagulant properties increase with increasing macrocycle size.

Thrombin inhibition by HCII and AT

The inhibition of thrombin in presence of physiological protein inhibitor like HCII and AT has been investigated in presence of Dermatan Sulfate (Figure 4) and Heparin (Figure 5), respectively. The anticoagulant effect of calixarene is measured by the percentage of thrombin inhibition against the biological polysaccharides. In Figure 4, C8SMA and C6S present respectively 95 and 83% of thrombin inhibition at 500 μM. In comparison full thrombin inhibition occurs at concentration of 100 μM for DS, the activity is two times lower for C8SMA and C6S and three times lower for C8S and C8SMN. In Figure 5, it is shown that the calixarenes tested in the presence of AT induced a lower thrombin inhibition than Heparin. At a concentration of 100 μM, only C6S produces a significant inhibition of thrombin

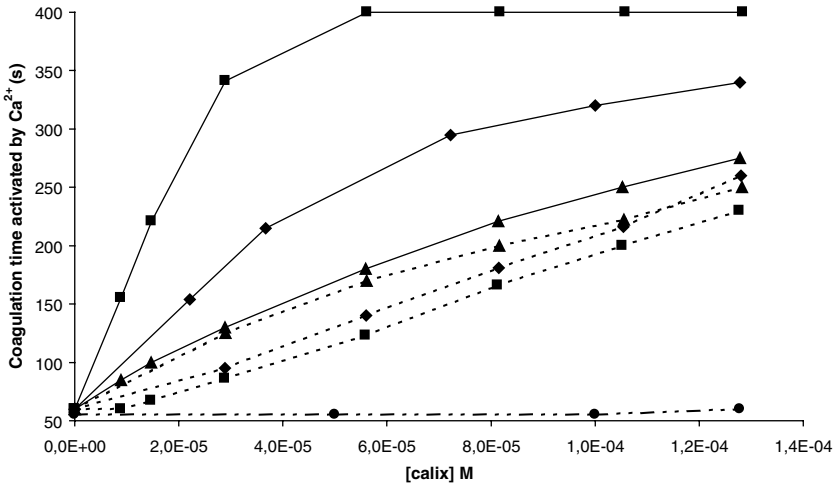


Figure 2. Inhibition of blood cascade coagulation in presence of calcium and the calixarenes derivatives: C8S (–♦–), C8SMA (–■–), C8SMN (–▲–), C6S (–♦–), C6SMA (–■–), C6SMN (–▲–), C4S; C4SMA and C4SMN (–●–). The standard deviation for each point is 2 and 5% for respectively low and high concentrations of calixarene, the error bars are omitted in the graph for clarity.

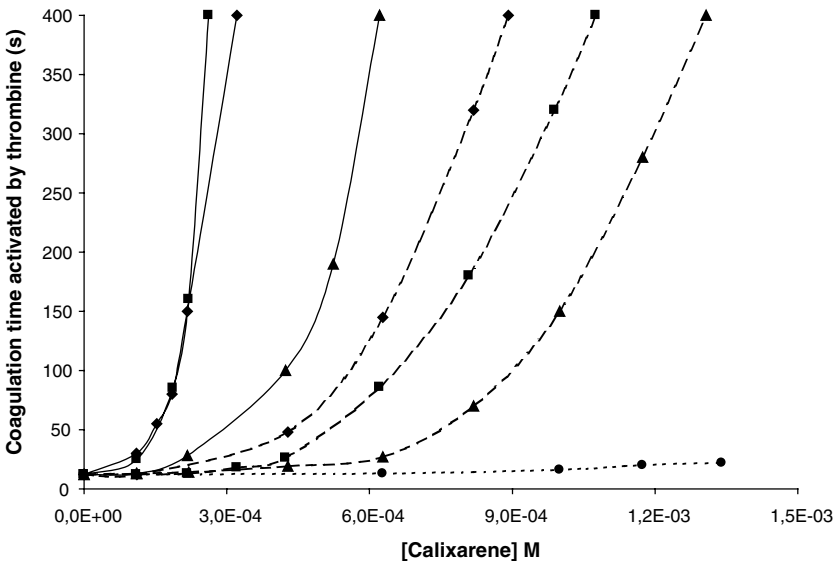


Figure 3. Inhibition of blood cascade coagulation in presence of Thrombin and the calixarenes derivatives: C8S (–♦–), C8SMA (–■–), C8SMN (–▲–), C6S (–♦–), C6SMA (–■–), C6SMN (–▲–), C4S; C4SMA and C4SMN (–•–). The standard deviation for each point is 2 and 5% for respectively low and high concentrations of calixarene, the error bars are omitted in the graph for clarity.

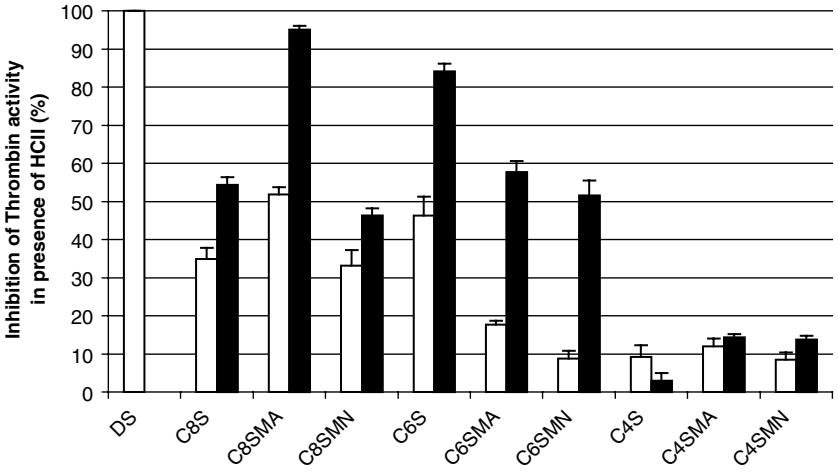


Figure 4. HCII activation by calixarene derivatives at 100 μM (□) and 500 μM (■) compared to DS (100 μM).

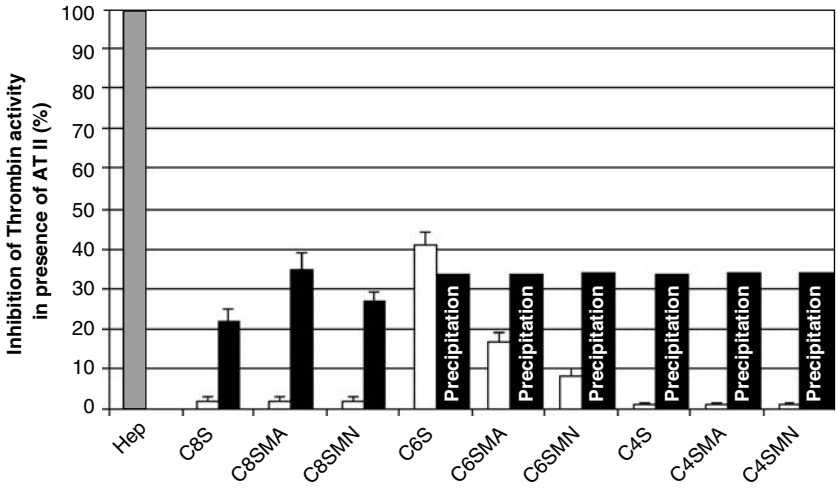


Figure 5. AT activation by calixarene derivatives at 100 μM (white) and 500 μM (black) compared to Heparin (gray color, 10 μM); observation of precipitation at 500 μM.

activity. At a concentration of C6S of 500 μM, the formation of a precipitate occurs in the presence of thrombin and AT. For the other calixarenes, no activation of AT is observed for a concentration of 100 μM. C8SMA presents an approximately comparable activity at a concentration of 500 μM to that induced by C6S at 100 μM.

Discussion

In a recent study, we have demonstrated that the *para*-sulfonato-calix[n]arenes tested do not present haemolytic effects against red blood cells at concentration up to 50 mM [33]. In view of this result, the purified *para*-sulfonato-calix[n]arenes may be used in blood for measuring the anticoagulant activity, without risk of interference for haemolysis of the red blood cells.

In order to understand the mechanism of thrombin inhibition, all the *para*-sulfonato-calix[n]arenes have been tested in presence or in absence of plasma. The APTT and TT results show that C8S allows the prolongation time of APTT and TT and is a better anticoagulant than the other parent *para*-sulfonato-calix[n]arenes (Figures 2 and 3). The introduction of a pendant group containing a carboxylate function increases the anticoagulant activity in the case of C8SMA with regard to the parent C8S. In this case, the presence of a carboxylate function may modify the affinity of calixarene for antithrombin proteins. However, the presence of a pendant group in the case of C6SMA and C6SMN decreases the anticoagulant properties in the case of TT and does not change the properties in the case of APTT measurements. The size of calixarene plays a role on the inactivation of blood cascade coagulation; C8SX > C6SX $\gg$ C4SX. Inhibition of coagulation is observed for C8SMA and C6S at concentrations approximatively half of these observed for sulfated quercetin derivatives [24].

In the second part, the direct inhibition measurement of thrombin by HCII in the presence of different polyanionic systems (DS or calixarene) shows that C8SMA and C6S activate HCII. In the case of *para*-sulfonato-calix[8]arenes, C8SMA shows 50% activity compared to DS at 100 μM and at 500 μM has comparable activity to DS. Both C8S and C8SMN show reduced activity compared with C8SMA. However with the *para*-sulfonato-calix[6]arenes, the underivatised C6S shows the greatest activity, and addition of a pendant arm, C6SMA and C6SMN reduces HCII activation. The difference in macrocycle size may allow different interactions between C8SMA and C6S to occur at the recognition site on HCII. In the case of AT, only C6S appears to interact with the protein, at 100 μM, approximatively 40% AT activation occurs for this compound. All other derivatives show neglible or zero AT activation. For a concentration at 500 μM for the *para*-sulfonato-calix[4]arene, *para*-sulfonato-calix[6] arene and their derivatives, the formation of a precipitate prevents determination of the thrombin activity. The concentrations above 200 μM, the turbidity of the solution prevents to obtention of reproducible results. The heparinoid activity of C6S with regard to the AT chromogenic substrate is, however, a factor of 20 less than that of the heparin used in experiments. Moreover, the study carried on the thrombin activity in presence of chromogenic substrate and calixarenes derivatives and in the absence of HCII or AT show that the calixarenes do not inhibit directly the thrombin. Thus the mechanism of the antithrombotic activity of the *para*-sulfonato-calix[n]arenes proceeds by interaction with either AT or HCII, two of the major circulating inhibitors of protease activity in the coagulation cascade. This mechanism appears to be mediated primarily *via* interaction with HC II.

The complexation between these calixarenes and Bovine Serum Albumin (major protein in plasma) has been investigated by Electrospray mass spectrometry. The results show that the weakest complexation is for *para*-sulfonato-calix[8]arene and its derivatives. For possible use as an anticoagulant, the calixarene of choice will be C8SMA, which has a greater effect on HCII activation than the other *para*-sulfonato-calix[n] arenes.

Conclusion

In conclusion, we have established that mono-substitution of *para*-sulfonato-calix[n]arenes can lead to an increase in their anticoagulant properties with the highest activity observed for C8SMA. The mechanism of the anticoagulant properties proceeds *via* interaction with the serine proteases inhibitors HCII and AT. Chromogenic test indicate that interaction between the *para*-sulfonato-calix[n]arenes and HCII is the major factor in the activity of these molecules.

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