EXPERIMENTAL ARTICLES

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CALIX[4]ARENE C-145 EFFECTS ON CELLULAR HAEMOSTASIS

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The aim of the research was to study a potential antithrombotic sodium salt of calix[4]arene-methylene-bis-phosphonic acid (C-145) — on activation and aggregation of platelets *in vivo*, as well as on proliferation and apoptosis of endothelial cells in the cell culture.

Effects of calix[4]arene C-145 estimated *in vitro* after addition to the platelet rich plasma, and *in vivo* after intravenous injection into rabbit bloodstream in equivalent amounts (46 μM). Aggregation of platelets was induced by adenosine diphosphate and detected using aggregometer Solar AP2110. Platelet shape and cytoplasmic granularity were monitored on COULTER EPICS XL Flow Cytometer. The level of tissue-type plasminogen activator — tPA — was estimated using enzyme-linked immunosorbent assay ELISA. Effects of calix[4]arene C-145 on culture of endotelial cells cells was studied using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide — MTT-test. The population of proliferative pool of cells (G_2/M+S) was determined using flow cytometry.

Aggregometry and flow cytometry showed that calix[4]arene C-145 did not activate platelets nor affect their aggregation *in vitro*. However intravenous injection of calix[4]arene C-145 into the bloodstream of healthy rabbits leads to strong inhibition of platelet aggregation and changes of shape and granularity of most of the platelets after 2 hours of administration. Any additional appearance of endothelial cells activation marker tPA *in vivo* and any inhibition of calix[4]arene C-145 on proliferation of endothelial cells in cell culture did not observe.

So calix[4]arene C-145 had strong anti-platelet effect *in vivo* that was not a result of their direct action on platelets or endothelial cells *in vitro*. This allowed to assume the possibility of calix[4]arene C-145 use as an effective antithrombotic agent.

Key words: calix[4]arene, haemostasis, antithrombotic drugs, fibrin polymerization.

Calix[4]arene-methylene-bis-phosphonic acids are synthetic macrocyclic compounds obtained by cyclocondensation of para-substituted phenols and formaldehyde. Aromatic rings of calix[4]arenes form a lipophilic "cup", an interface tailored to handle macromolecules through hydrogen bonds, hydrophobicity or electrostatic interactions. Calix[4]arene C-192 and it's sodium salt C-145 are inhibitors of blood coagulation. These calix[4]arenes selectively and with high affinity inhibit the first stage of fibrin polymerization — formation of protofibrils ($IC_{50} = 0{,}5 \cdot 10^{-6}$ and $IC_{50} = 2{,}5 \cdot 10^{-6}$ respectively). It was reported that this inhibition occurred as a consequence of direct binding of calix[4]arene with "A"-knob of fibrin molecule [1].

In vivo studies demonstrated that C-145, being injected intravenously into rabbit's bloodstream, acts as effective anticoagulant agent and its effects correspond to those shown *in vitro*. We did not find any significant effects of C-145 on total level of prothrombin, fibrinogen, activity of protein C and other main protein compounds of coagulation and fibrinolysis [2]. The effects of C-145 on cellular haemostasis that include platelets and endothelium remained unstudied.

Platelets and endothelium are the most sensitive compounds of haemostasis that being activated immediately after stimulation of coagulation cascade and take part in the formation of thrombus [3]. Study of platelet or endothelium responce on the action of any potential antithrombotic drug is one of the main conditions of the estimation of its effectiveness [4, 5].

That is why the aim of present work was to study a potential antithrombotic sodium salt of calix[4]arene-methylene-bis-phosphonic acid (C-145) — on activation and aggregation of platelets in vivo, as well as on proliferation and apoptosis of endothelial cells in the cell culture.

Materials and Methods

Materials. ADP (Merck, Germany); anti-tPA (tissue-type plasminogen activator), clone GMA-043 mouse monoclonal IgG; goat anti-mouse IgG (H+L) alkaline phosphatase conjugate, PNPP (*para*-Nitrophenylphosphate), DMEM (Dulbecco's Modified Eagle's medium), MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dimethyl sulfoxide were purchased from Sigma (USA. Cell line PAE (Porcine Aortic Endothelial Cells)) was a kind gift of Prof. I. T. Goot (University of London).

Methods. Sample of Calix[4]arene C-145 was dissolved in 0.9% NaCl solution to the final concentration 15 mg/ml and was injected in the marginal ear vein of rabbit in the dose of 7.5 mg/kg using Wenflon catheter (Becton Dickinson, USA), G22 (0.8 mm). This study was approved by the institution's Ethics Committee.

Blood samples were collected using Wenflon catheter (Becton Dickinson, USA), G22 (0.8 mm) before the injection and after 2, 4 and 24 h after injection. 3.8% Sodium Citrate added immediately after collection to whole blood in 1:9 ratio was used as an anticoagulant.

Platelet rich blood plasma (PRP) was prepared from human citrated blood by centrifugation at 1 000 rpm during 30 min. Platelet poor blood plasma as obtained by spinning-down PRP at 1 500 rpm during 30 min [6].

Platelet shape and cytoplasmic granularity were monitored on COULTER EPICS XL Flow Cytometer [7]. Samples of PRP (1 ml) before and after the administration of C-145 were analyzed. Scattered and transmitted light were monitored to detect any changes of platelet granularity and shape respectively.

Platelet aggregation measurements were based on changes in the turbidity of platelet-rich plasma. Aggregation was registered for 10 min using Aggregometer Solar AP2110 (Belorussia). We estimated the initial rate and final level of aggregation at 37 °C. In typical experiment 250 µl of PRP was activated by ADP (12.5 µM) [8].

The level of tPA was estimated using ELISA according to [9]. Mixture was contain 2 µl of blood plasma in 0.2 ml of TBS. Monoclonal mouse anti-tPA (clone GMA-043) IgG antibody and Goat anti-mouse IgG conjugated to alkaline phosphatase were used for the detection. PNP formed after PNPP cleavage by alkaline phosphatase was determined at 405 nm using multiplate reader Multiscan EX.

Cell line of PAE (Porcine Aortic Endothelial Cells) was incubated in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and 40 mg/ml gentamicin at standard conditions (at 37 °C in 5% CO_2) during two days. C-145 in the final concentration 300 µM in TBS was added to the culture medium. Equivalent volume of TBS was added to control samples.

Cell viability was measured by MTT-colorymetric test. The biochemical essence of this method is based on the fact that mitochondrial dehydrogenases of living cells are capable to cleave MTT (3-(4, 5-diethylthiazoly-2-yl)-2,5-diphenyltetrazolium bromide) rings with formation of insoluble purple crystals (formazan). Cells were plated at a density of PAE cells per well in 96-well plates. The cells were incubated with 20 µl complete medium containing 1 mg/ml MTT at 37 °C for 4 h followed by solubilization with 100 µl dimethyl sulfoxide. The absorbance at 540 nm was measured with a microplate reader. The cell proliferation was expressed as percentage of the viable cell number of the control (non-treated cells) and C-145-treated cells. Cell proliferation rate was calculated as $[(1-OD_{\text{experimental group}})/OD_{\text{control group}}] \times 100\%$ [10].

Cell cycle determination was measured by flow cytometry [11]. For this purpose the samples were stained with propidium iodide (PI), which selectively joins with intercalating places in DNA. Cytometry was carried out on the FACS Calibur (Becton Dickinson, United

States). Special mathematical program Mod Fit LT 2.0 (BDIS, United States) for Macintosh computers was used for acquisition and data analysis. Narrowband filter 585/42 nm was used in order to measure the fluorescence of PI.

Results and Discussion

Effects of C-145 on platelets. Action of C-145 on platelet branch of haemostatic system was estimated by the study of ADP-induced platelet aggregation in PRP. Number of platelets before the C-145 administration and after 2, 4 and 24.0 h after its injection into the rabbit's bloodstream was also calculated. PRP of rabbit taken before the injection of C-145 was used as the control probe. It was shown that in 2 hours after the administration of 46 μM of C-145 platelets lost their ability for aggregation. After 24 h the rate of platelet aggregation was not higher then 9 ± 5% against 35 ± 8% in PRP of control group (Fig. 1, *A*).

Number of platelets after the administration of C-145 was dramatically decreased from 180±30 thousands per 1 μl. The restoration of platelets count was observed in 24 h after injection of C-145 but nor number of platelets, nor their ability to aggregate did not restored completely till 48 h (Fig. 1, *B*).

Flow cytometry study showed that in 2 h after C-145 injection the pool of intact platelets in PRP decreased more then in 2 times (Fig. 1, *C*). Number of intact platelets in PRP did not reach control value (80 ± 4%) in 24 and 48 h.

In vitro studies demonstrated that C-145, being added to the PRP of healthy rabbit at the final concentration of 46 μM, did not change shape or granularity during incubation up to 3 h (Fig. 2, *B*) and also did not decrease the platelet aggregation (Fig. 2, *E*). The effects of C-145 injected intravenously in the equivalent amount are shown on the Fig. 2 (panels *C* and *F*).

Presented data allowed us to assume that C-145 injected into the rabbit's bloodstream

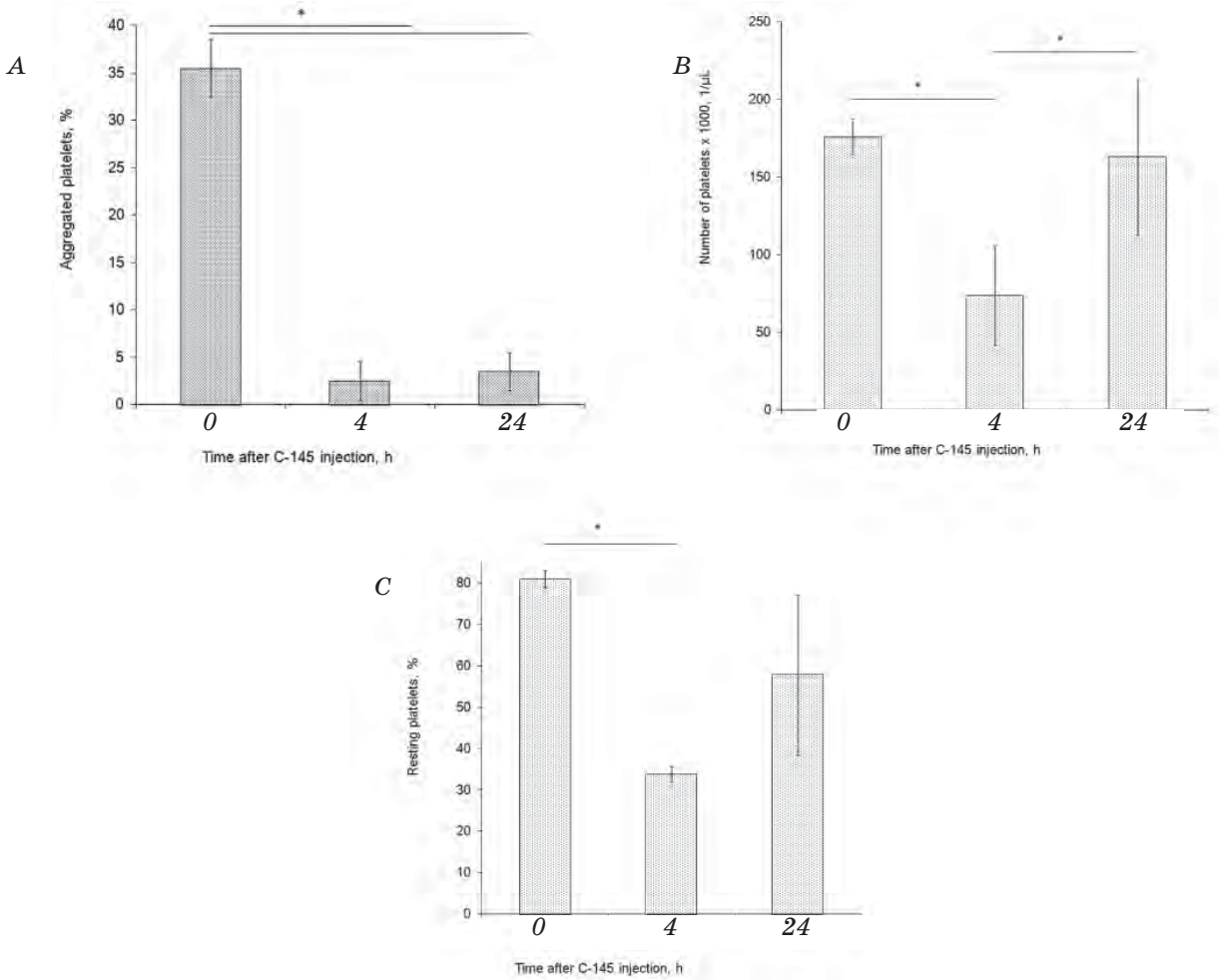

Fig. 1. **Platelet aggregation rate (*A*), number of platelets (*B*) and value of intact platelets (*C*) in platelet rich plasma of rabbits after intravenous administration of 46 μM of C-145:**
0 — parameters before the injection (control); *4* — after 4 h; *24* — after 24 h after injection. Data represent the means ± SE, **P* < 0.05 as compared to the control

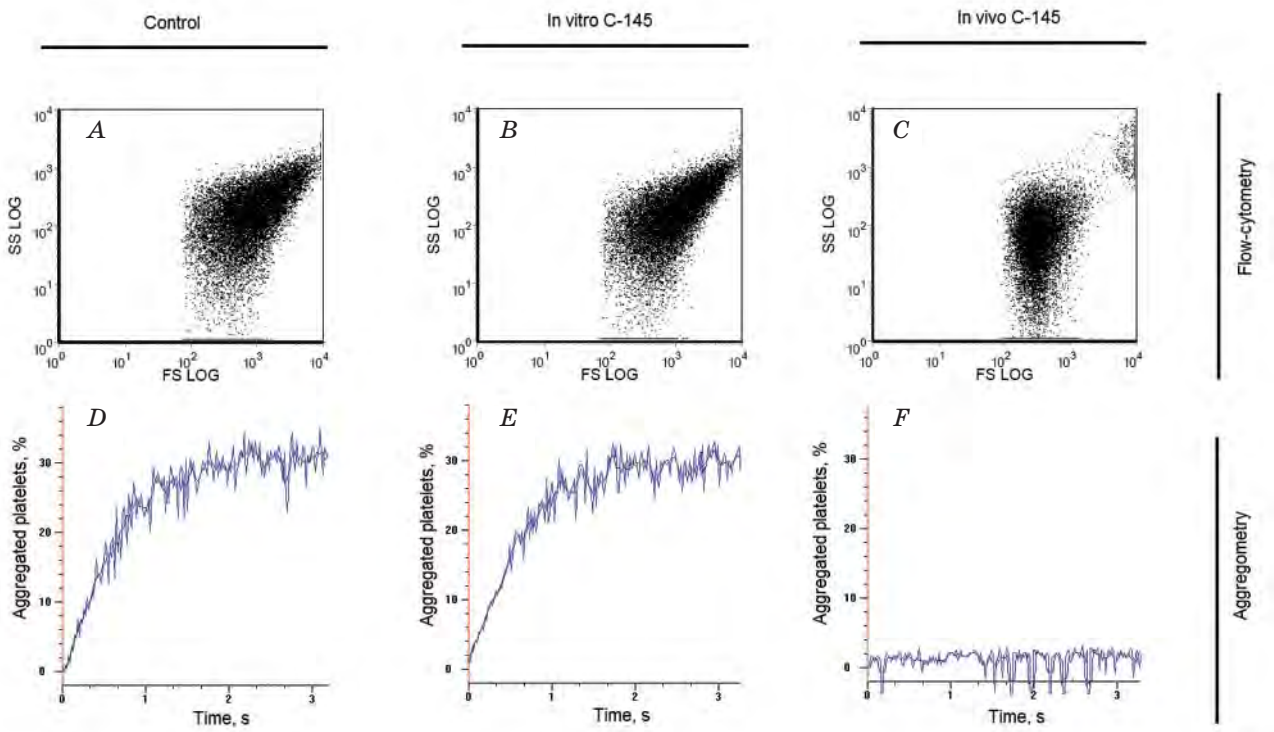

Fig. 2. **Flow cytometry and aggregation study of platelets during C-145 administration *in vitro* and *in vivo*:** Flow cytometry of resting platelets of rabbit in platelet rich blood plasma (*A*), after addition of 46 μM of C-145 to platelet rich blood plasma (PRP) *in vitro* (*B*) and after intravenous administration of equivalent C-145 *in vivo* (*C*). The distribution of platelets according to the correlation between shape and granulation. LgSS — parameter of platelets granulation: LgFS — parameter of platelets shape. Traces are typical for 3 independent experiments. Aggregation of rabbit platelets in PRP (*D*), after addition of 46 μM of C-145 to platelet rich blood plasma (PRP) *in vitro* (*E*) and after intravenous administration of equivalent C-145 *in vivo* (*F*). Traces are typical for 3 independent experiments

at the final concentration of 46 μM has strong anti-platelet action. Using flow cytometry and aggregometry we did not observed any similar effects of equivalent concentrations of C-145 *in vitro*.

Effects of C-145 on endothelial cells. As far as C-145 demonstrated prominent effects on platelets, it was very important to investigate its effects on another compound of cellular haemostasis — endothelium [12].

For the evaluation of C-145 effects on endothelial cells *in vivo* we determined the level of tPA in blood plasma of rabbits after intravenous administration of 46 μM of C-145. Tissue-type plasminogen activator is secreted into the bloodstream and is common marker of endothelial cells activation [13].

As it is shown on Fig. 3, confident difference between level of tPA before and after the C-145 administration was not observed. Thus we can assume that endothelial cells remained intact and were not activated after C-145 administration.

Direct study of C-145 action on endothelial cells was carried-out using PAE cell culture that was synchronized in G_0/G_1 phase of cell cycle. For this cells were planted 500 000 ml^{-1} in 100 ml and cultivated during 2 days. Model of used cells culture leaded to the formation of monolayer and transition most of the endothelial cells in G_0/G_1 phase of cell cycle. Cells were cultivated in the presence of 300 μM of C-145 during 48 h. The cell viability after treatment with C-145 was evaluated by the MTT assay. It was shown that the level of apoptotic cells in the presence of C-145 decreased to 19 ± 1% against 24 ± 2.1 in control. The population of proliferative pool of cells (G_2/M+S) in the presence of C-145 was increased in 4 times (Table) compared to control probes.

Thus, any activating action of C-145 on endothelium *in vivo* was observed. However cell culture studies demonstrated antiapoptotic and proliferation-stimulating effects of C-145 on PAE that could be a new promising feature of C-145 that has to be studied more precisely.

It is known that some medications injected intravenously could provoke decrease of platelet count that is known as drug-induced thrombocytopenia [14]. In most cases they

Density of apoptotic cells and distribution of endothelial cells in the presence of C-145 (300 μM)

	Apoptotic cells, %	Cell cycle stages		
		G_0/G_1	G_2/M	S
Control	24 ± 2.1	85.71 ± 2.3	4.85 ± 0.5	9.71 ± 0.7
C-145	19 ± 1	34.13 ± 1.4	33.19 ± 1.8	32.68 ± 1.3

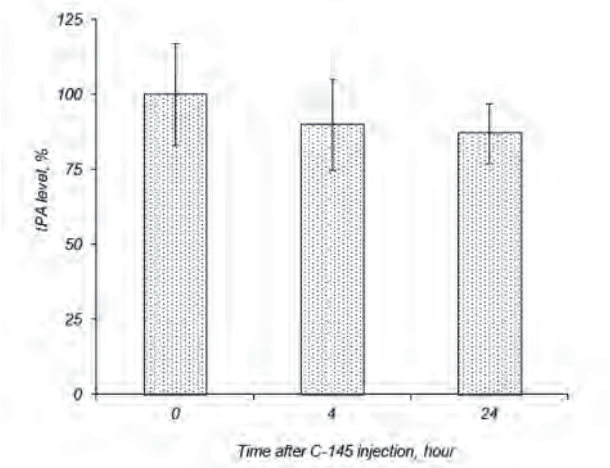

Fig. 3. **The level of tPA in rabbit plasma following intravenous administration of 46 μM of C-145**

induce the production of antibodies specific to surface molecules of platelets which are active only in the presence of drug [15]. Thrombocytopenia is common for the use of heparin and low-molecular weight heparin (HIT, heparin-induced thrombocytopenia [16]) and antagonists of GPIIbIIIa-receptors [17]. Last ones could induce the appearance of ligand-induced binding sites on the surface of platelet receptor GPIIbIIIa that are the targets to auto-antibodies [18].

Another reason of thrombocytopenia could be the disorder of platelet production by megakaryocytes [19] or fast degradation of platelets [20]. Recently demonstrated anti-platelet effect of C-145 *in vivo* could not be explained by the affection of platelet production as far as it was observed only after 2 hours of C-145 administration. In the same time the period of life of platelet in the bloodstream could last to 100 hours [21].

Fast decrease of platelets count by C-145 could provide additional anticoagulant effect but on the other hand it made obligatory the instant control of platelet viability during treatment with C-145 and, possibly, concomitant therapy targeted to platelet restoration.

Some anticoagulant medication can act on endothelial cells mainly by increasing or inhibiting their proliferation [22], but sometimes drug-induced activation of endothelium leading to bleeding is also observed [23].

Intravenous administration of C-145 did not affect endothelial cells that was confirmed by determining the level of tPA which is the marker of endothelial activation. However the study of C-145 effects on endothelial cells culture allowed to conclude anti-apoptotic and proliferation-stimulating effects of C-145 that could be promising during revascularization of tissues after ischemia.

So we have analyzed the effects of intravenously administrated calix[4]arene C-145 (46 μM) on cellular haemostasis *in vivo*. Our findings (composed with data on C-145 effects on protein haemostasis) allow us to conclude the possibility of its use in anticoagulant therapy. Anti-platelet action of C-145 can significantly increase its anticoagulant action. Instant monitoring of platelet functionality could minimize risk of possible by-effects of C-145 administration.

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ДІЯ КАЛІКС[4]АРЕНУ С-145 НА КЛІТИННУ ЛАНКУ СИСТЕМИ ГЕМОСТАЗУ

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Метою роботи було вивчення дії потенційного антитромботичного агента — калікс[4]арен-метилен-біс-фосфонової кислоти — калікс[4]арену С-145 на активацію та агрегацію тромбоцитів *in vivo*, а також на проліферацію та апоптоз ендотеліоцитів у тканинній культурі.

Ефекти калікс[4]арену С-145 оцінювали *in vitro* після додавання у збагачену тромбоцитами плазму крові, а також *in vivo* після внутрішньовенного введення у кровоток кролика в еквівалентних кількостях (46 μM). Агрегацію тромбоцитів індукували ADP і встановлювали за допомогою агрегометра Solar AP2110. Форму та гранулярність цитоплазми тромбоцитів визначали протоковим цитометром COULTER EPICS XL. Рівень активатора плазміногену тканинного типу — tPA — вимірювали методом імуноензимного аналізу ELISA. Ефекти калікс[4]арену С-145 на культуру ендотеліальних клітин визначали з використанням 3-(4,5-диметилтіазол-2-іл)-2,5-диметилтетразолію броміду — MTT-тесту. Популяцію проліферативного пулу (G_2/M+S) клітин ендотелію визначали за допомогою цитометрії.

Методами агрегатометрії та цитометрії встановлено, що калікс[4]арен С-145 не активує тромбоцити та не впливає на їх агрегацію *in vitro*. Водночас вже через дві години після введення калікс[4]арену С-145 здоровим лабораторним кролям тромбоцити втрачали здатність до агрегації, а пул інтактних тромбоцитів зменшувався більш ніж удвічі. Викиду в плазму крові маркера активації ендотеліоцитів tPA *in vivo*, а також інгібування проліферації ендотеліоцитів у культурі клітин не спостерігали.

Таким чином, калікс[4]арен С-145 виявляє значний антитромбоцитарний ефект *in vivo*, не діючи безпосередньо на тромбоцити та ендотеліоцити *in vitro*. Такі властивості калікс[4]арену уможливлюють його використання як ефективного антитромботичного агента.

Ключові слова: калікс[4]арени, гемостаз, антитромботичні препарати, полімеризація фібрину.

ДЕЙСТВИЕ КАЛИКС[4]АРЕНА С-145 НА КЛЕТОЧНОЕ ЗВЕНО СИСТЕМЫ ГЕМОСТАЗА

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Целью работы было изучение действия потенциального антитромботического агента — натриевой соли каликс[4]арен-метилен-бисфосфоновой кислоты — каликс[4]арена С-145 на активацию и агрегацию тромбоцитов *in vivo*, а также на пролиферацию и апоптоз эндотелиоцитов в тканевой культуре.

Эффекты каликс[4]арена С-145 оценивали *in vitro* после добавления в плазму крови, богатую тромбоцитами, а также *in vivo* после внутривенного введения в кровоток кролика в эквивалентных количествах (46 μM). Агрегацию тромбоцитов индуцировали ADP и определяли с помощью агрегометра Solar AP2110. Форму и гранулярность цитоплазмы тромбоцитов устанавливали проточным цитометром COULTER EPICS XL. Уровень активатора плазминогена тканевого типа — tPA — измеряли методом иммуноэнзимного анализа ELISA. Эффекты каликс[4]арена С-145 на культуру эндотелиоцитов изучали с помощью 3-(4,5-диметилтиазол-2-ил)-2,5-диметилтетразолия бромида — MTT-теста. Популяцию пролиферативного пула (G_2/M+S) клеток эндотелия определяли с применением цитометрии.

Методами агрегатометрии и цитометрии установлено, что каликс[4]арен С-145 не активирует тромбоциты и не влияет на их агрегацию *in vitro*. Однако уже через два часа после введения каликс[4]арена С-145 здоровым лабораторным кролям тромбоциты теряли способность агрегировать, а пул интактных тромбоцитов уменьшался в два раза. В то же время не наблюдали выброса в плазму крови маркера активации эндотелиоцитов tPA *in vivo*, а также ингибирования пролиферации эндотелиоцитов в культуре клеток.

Таким образом, каликс[4]арен С-145 обладает значительным антитромбоцитарным эффектом *in vivo*, не действуя непосредственно на тромбоциты и эндотелиоциты *in vitro*. Такие свойства каликсарена позволяют рассматривать возможность его применения в качестве эффективного антитромботического агента.

Ключевые слова: каликс[4]арены, гемостаз, антитромботические препараты, полимеризация фибрина.