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APPROBATION OF CALIX[4]ARENE AS AN ANTITHROMBOTIC AGENT *in vivo*

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Intravascular thrombosis is one of the main causes of mortality in the working-age population of the world. There are no antithrombotic drugs that act directly on the final stage of thrombosis — fibrin polymerization. However, a new compound of the calix[4]arene series, calix[4]arene C-145, which directly interacts with the fibrin polymerization site 'A-knob' thus blocking formation of polymeric fibrin and preventing thrombosis.

So, the *purpose* of this work was to study the calix[4]arene C-145 series as antithrombotic agents *in vivo* using different animals and types of administration.

Materials and methods. Laboratory animals (rats, mice and rabbits) were used for C-145 testing *in vivo*. Activated partial thromboplastin time and platelet aggregation were measured to determine the anticoagulant action after intravenous or *per os* administration.

Results. Per os way of administration was selected as the optimal one. We showed the substantial prolongation of clotting time in APTT test that was observed starting from the 2nd hour after the *per os* administration, reached the maximum on 6th hour and eliminated in 24 hours. The effect of C-145 on platelets reached maximum on 4–6 hours and eliminated in 12 hours.

Conclusions. C-145 was proven to be prospective antithrombotic drug that can be administered *per os*. Further investigations must be focused on the study of C-145 pharmacodynamics and metabolism. Such data would allow fast implementation of the tested compound into practice.

Key words: calix[4]arene, blood plasma, platelets, thrombosis, blood coagulation, noncovalent complex.

Intravascular thrombosis is one of the main causes of mortality in the working-age population of the world. It consists in the formation of fibrin-platelet thrombus, which blocks the lumen of the vessel, preventing blood supply to tissues and organs and causing severe pathologies such as myocardial infarction, thrombotic brain stroke, pulmonary embolism, etc.

Therefore, the search for ways to effectively prevent thrombus formation in the vessel is an important issue of modern medicine and biochemistry. The antithrombotic drugs are used for rapid and controlled inhibition of the process of activation of the blood coagulation system. According to the direction of action there are anticoagulants, inhibiting blood clotting at different stages, and fibrinolytics, aimed at accelerating the destruction of the fibrin clot (t-PA, streptokinase, urokinase). The most common among anticoagulants are acting directly on thrombin (dabigatran), factor Xa (rivaraxaban), factors VIIIa and V (drotrekogin), blocking the blood coagulation cascade at the stages of activation of factor X, prothrombin or conversion of fibrinogen to fibrin. A separate class of compounds that block the formation of fibrin-platelet thrombus are platelet aggregation inhibitors [1].

As of today, there are no antithrombotic drugs that act on the final stage of thrombosis, that is fibrin polymerization. As a direct inhibitor of fibrin polymerization, silver

nanoparticles or inhibitor peptides (in particular, GPRP) conjugated with albumin have been proposed. No such agents have passed preclinical trials [2].

A new compound of the calix[4]arene series [3] created at the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine, calix[4]arene C-145, which directly interacts with the fibrin polymerization site 'A-knob' due to its hydrophobic cup, was characterized in the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine [4]. Therefore, calix[4] arene C-145 directly inhibits the formation of fibrin protofibrils (IC_{50} = 2.5×10^{-6}), preventing the formation of polymeric fibrin [5]. Its efficacy both *in vitro* and *in vivo* has been shown [6].

This work was aimed to the study of the calix[4]arene C-145 series as antithrombotic agents *in vivo* using different animals and types of administration.

Materials and Methods

Materials. Chemicals: APTT (activated partial thromboplastin time) reagent and $CaCl_2$ solution were purchased from Siemens (Germany), ADP was purchased from Sigma-Aldrich (USA).

Calix[4]arene C-145 was syntesized by the reaction of tetraformylcalixarene with sodium salt of diisopropylphosphite with formation of tetrakisbisphosphonate, which after subsequent dealkylation due to treatment with trimetylbromosilane and methanol gives C-192 and its sodium salt C-145 [7]. Sample of calix[4]arene C-145 was kindly provided by Institute of Organic Chemistry of National Academy of Sciences of Ukraine. It was dissolved in 0.9% NaCl solution.

Animal keeping. Male Wistar rats (170 ± 4 g, n = 60) and Balb mice (30 ± 1 g, n = 30) were kept in the animal house of Palladin Institute of biochemistry of NAS of Ukraine. The animals were kept in standard cages (5 animals in each cage) under controlled conditions (22 °C ± 2, 12-h/12-h light/dark cycle) with unlimited access to drinking water and food. Outbreed male rabbits (3.5 ± 0.5 g, n = 9) were kept in the animal house of Bila Tserkva National Agrarian University on a standard diet. This study was carried out with the approval of animal care and use committee of the Palladin Institute of Biochemistry of NAS of Ukraine (Protocol #10/11-2019).

Administration of calix[4]arene C-145. Solution of C-145 was administered per os to mice (in a volume of 0.1 ml) and to rats (in a volume of 0.5 ml). Solution of C-145 was also injected into the rat's lateral tail vein and into rabbit's marginal vein of the ear. The dosage was standard in all experiments (12 mg/kg).

Blood sampling. Samples of rat or mice blood were collected by heart puncture. Pentobarbital (Nembutal) anesthesia (dosage was 50 mg/kg of body weight) was used intraperitoneally according to ethical standards and principles. A 3.8 % sodium citrate solution was added to the blood samples immediately after collection. Animals were decapitated immediately after blood collection still being under anesthesia. Rabbit blood samples were collected using Wenflon catheter (Becton Dickinson, USA), G22 (0.8 mm). 3.8 % Sodium Citrate added immediately after collection to whole blood in 1:9 ratio was used as an anticoagulant.

Blood plasma preparation. Platelet rich plasma (PRP) was prepared by centrifuging blood at 160 g for 20 min at 25 °C. Platelets were spun-down at 300 g for 15 min at 25 °C for obtaining of platelet poor plasma (PPP). PRP was analyzed immediately after collection. PPP was fresh frozen at −20 °C and stored no longer than 2 months.

Activated partial thromboplastin time. Activated partial thromboplastin time (APTT) was performed according to the following procedure. 0.1 ml of blood plasma was mixed with an equal volume of APTT-reagent and incubated for 3 min at 37 °C. Then the coagulation was initiated by adding 0.1 ml of 0.025 M solution of $CaCl_2$. Clotting time was monitored by the Coagulometer Solar CGL-2410 (Solar, Minsk, Belarus).

Platelet aggregation. Platelet aggregation measurements were based on changes in the turbidity of human PRP. Aggregation was registered for 10 min using the Aggregometer Solar AP2110 (Solar, Minsk, Belarus). Platelet count was estimated using the same device. We measured the initial rate and final level of aggregation at 37 °C. In a typical experiment, 0.25 ml of PRP was incubated with $CaCl_2$ (0.010 mM) activated by platelet agonist ADP (0.012 mM) at 37 °C.

Statistical data analysis. Statistical data analysis was performed using Microsoft Excel. All assays were performed in series of three replicates and the data were fitted with standard errors using "Statistica 7". Results are presented as means ± standard deviation. The difference between the groups was analyzed by one way ANOVA. Data were considered significant when $p < 0.05$.

Results and Discussion

Intravenous administration. Intravenous administration is a useful method for drugs that must be administered fast and are used urgently [8]. It is also useful for initial testing as far as this way does not depend on metabolization and delivers drug to the bloodstream directly [9].

In our study we applied C-145 intravenously to rabbits and rats and determined the anticoagulant effect of the compound using APTT test. Prolongation of clotting time in APTT test was an evidence of the presence of C-145 into the bloodstream.

For both rats and rabbits the anticoagulant effect was observed 2–12 h after injection and completely eliminated after 24 h (Fig. 1, 2). However it was more pronounced in the case of rats (Fig. 2).

Analyzing the rat model, we also detected the dramatic decrease of platelet aggregation rate after the C-145 administration (Fig. 3). Platelet aggregation was insulted in 2 h after C-145 injection, aggregation rate was constantly low in 2–12 h and remained decreased after 24 h.

Results of APTT and platelet aggregation testing in rats allowed us to conclude that the used dosage was higher than it was needed for this kind of animals. Also, the anti-platelet effect of C-145 was undesirable. We presume that the per os administration of C-145 would allow to avoid this effect.

Per os administration. Administration per os is the best way for the use of deugs of regular administration. As non-invasive method, it could be recommended the preferential method of drug delivery [10].

Testing of per os administration of C-145 was performed on mice and rats models. We showed the substantial prolongation of clotting time in APTT test in the case of both species (Fig. 4, Fig. 5).

Anticoagulant effect was observed starting from the 2nd hour after the administration, reached the maximum on 6th hour and eliminated in 24 hours.

Even more important finding was the moderate effect of C-145 on platelets. In both animal models the effect of C-145 on platelets reached maximum on 4-6 hours and eliminated in 12 hours (Fig. 6, 7).

Also, it should be noted that anticoagulant effect of C-145 was too high. Actually 1.5-times prolongation of clotting time would be enough [11]. So, we expect that C-145 dosage can be decreased substantially in the future studies that also is an important issue in the light of future implementation of this antithrombotic drug.

Antithrombotic action of C-145 through its action of fibrin was reported earlier [12]. This inhibition of fibrin polymerization occurs as a result of direct non-covalent complex formation of C-145 and GPRP motive of fibrin Aα-chain (polymerization site 'A'-knob) [4, 5].

Important outcome of the study is the direct demonstration of C-145 anticlotting activity in blood plasma of animals. While its efficacy after intravenous injection was predictable, high effect during oral administration was a remarkable finding. Prolongation of clotting time of blood plasma of rats after C-145 oral

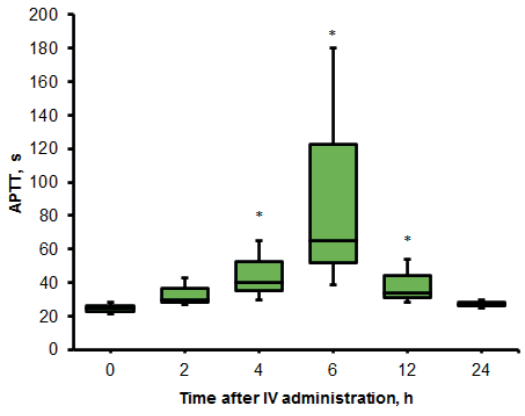

Fig. 1. **Activated partial thromboplastin time of blood plasma clotting after intravenous (IV) administration of C-145 to rabbits (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \le 0.05$

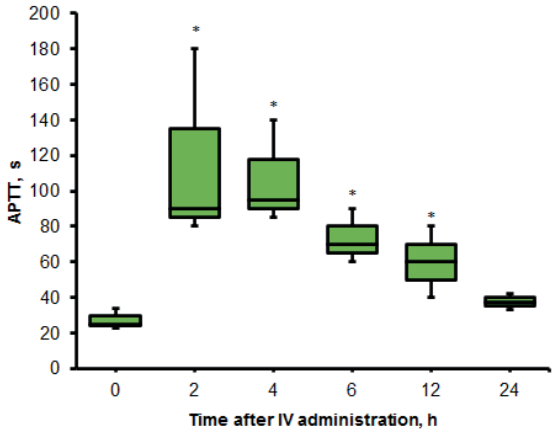

Fig. 2. **Activated partial thromboplastin time of blood plasma clotting after intravenous (IV) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \le 0.05$

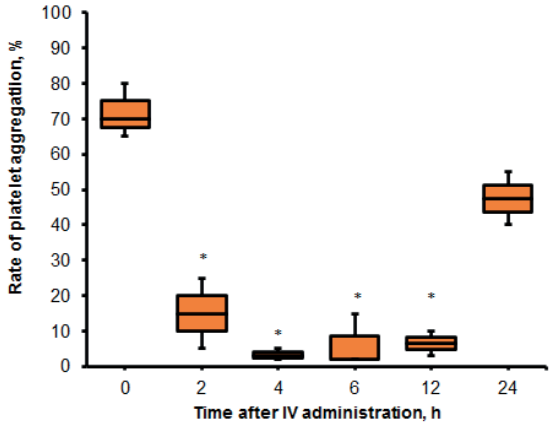

Fig. 3. **The rate of platelet aggregation after intravenous (IV) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

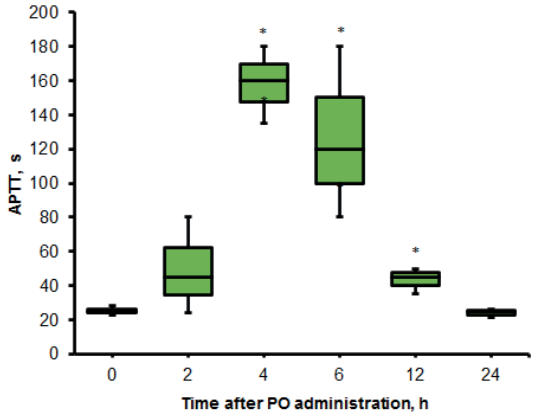

Fig. 4. **Activated partial thromboplastin time of blood plasma clotting after per os (PO) administration of C-145 to mice (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

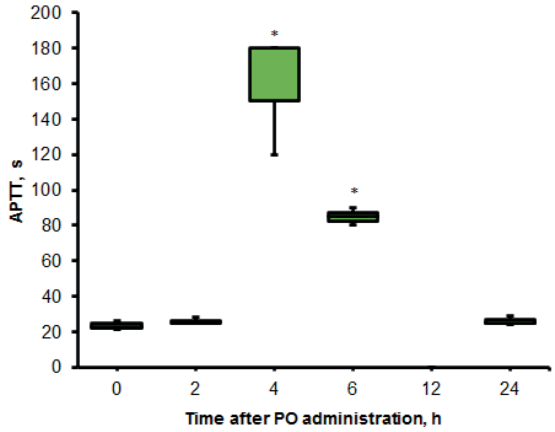

Fig. 5. **Activated partial thromboplastin time of blood plasma clotting after per os (PO) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

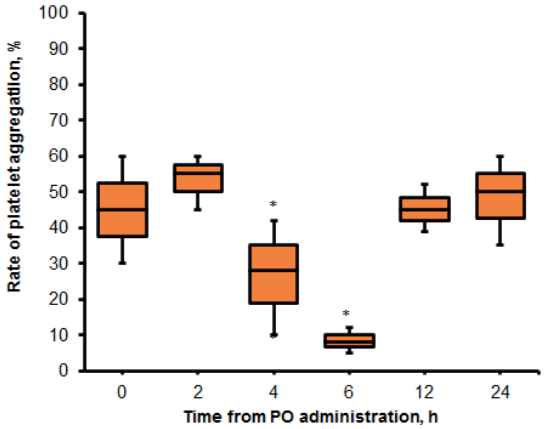

Fig. 6. **The rate of platelet aggregation after oral administration of C-145 to mice (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

administration indicates the appearance of active compound into the bloodstream in unchanged active form. Observing the stable anti-clotting effect up to 12 hours after the administration is evidence of the continuing presence of C-145. The normalization of clotting time after 24 hours indicates its clearance from the bloodstream.

In conclusion, C-145 was proven to be a prospective antithrombotic drug that can be administered per oral. Its anticoagulant effect slightly differed for different animal species. We presume that the selected concentration (12 mg/kg) can be decreased substantially. Also, antiplatelet effect that was observed after C-145 administration can be minimized by the dosage selection. Further investigations must be concentrated on the study of C-145 pharmacodynamics and metabolization. Such data would allow fast implementation of the development compound into practice.

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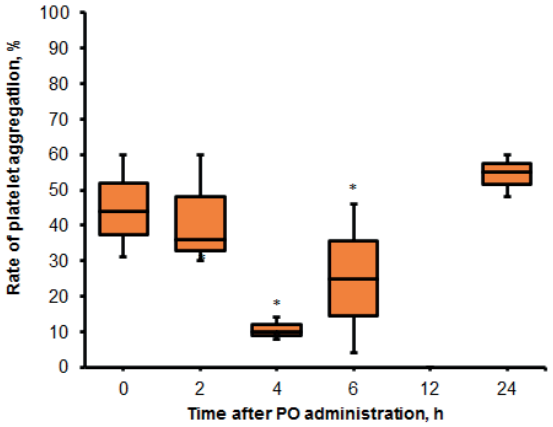

Fig. 7. **The rate of platelet aggregation after per oral (PO) administration of C-145 to rats (12 mg/kg)**
0 — clotting time before the C-145 administration. 2–24 — time of clotting of blood plasma after the C-145 administration. $n = 5$. * $P \leq 0.05$

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REFERENCES

1. *Chernyshenko V.O.* Mechanisms of intravascular and extravascular blood clotting: fundamental studies for clinical practice. *Visn. Nac. Acad. Nauk Ukr.* 2019, 4, 50–56. https://doi.org/10.15407/visn2019.04.050
2. *Chernyshenko V. O, Lugovska N. E.* Molecular mechanisms of intravascular inhibition and stimulation of extravascular thrombosis. *Biotechnologia Acta.* 2021, 14(6), pp. 5–22. https://doi.org/10.15407/biotech14.06.005
3. *Asfari M. Z., Bohmer V., Harrowfield J., Vicens J. (eds).* Calixarenes 2001. *Kluwer academic Publishers.* 2001, 3–684.
4. *Komisarenko S. V., Kosterin S. O., Lugovskoy E. V., Kalchenko V. I.* Calixarene methylene bisphosphonic acids as promising effectors of biochemical processes. *Ukr. Biochem. J.* 2013, 85(6), 106–128. http://dx.doi.org/10.15407/ubj85.06.106
5. *Lugovskoy E. V., Gritsenko P. G., Koshel T. A., Koliesnik I O., Cherenok S. O., Kalchenko O. I., Kalchenko V. I., Komisarenko S. V.* Calix[4]arene methylenebisphosphonic acids as inhibitors of fibrin polymerization. *FEBS J.* 2011, 278(8), 1244–51. https://doi.org/10.1111/j.1742-4658.2011.08045.x
6. *Chernyshenko V. O., Korolova D. S., Dosenko V. E., Pashevin D. O., Kalchenko V. I., Pirogova L. V., Chernyshenko T. M., Lugovska O. E., Kravchenko N. A., Makogonenko Y. M., Lugovskoy E. V., Komisarenko S. V.* Calix[4]arene C-145 Effects on Plasma Haemostasis. *Pharmaceutica Analytica Acta.* 2015, 6(8), 1–5. https://doi.org/10.4172/2153-2435.1000406
7. *Rodik R. V., Boyko V. I., Kalchenko V. I.* Calixarenes in bio-medical researches. *Current Medicinal Chemistry.* 2009, 16(13), 1630–1655. https://doi.org/10.2174/092986709788186219
8. *Bansal S, Sangha KS, Khatri P.* Drug treatment of acute ischemic stroke. *Am J Cardiovasc Drugs.* 2013, 13(1), 57–69. https://doi.org/10.1007/s40256-013-0007-6.
9. *Alsenaidy M.A., Kazi M., Ahmad M.Z.* Advances in Oral Drug Delivery. *Sec. Translational Pharmacology.* 2021, 12, 618411. https://doi.org/10.3389/fphar.2021.618411
10. *Homayun B., Lin X., Choi H.J.* Challenges and Recent Progress in Oral Drug Delivery Systems for Biopharmaceuticals. *Pharmaceutics.* 2019, 11(3), 29. https://doi.org/10.3390/pharmaceutics11030129.
11. *Hartmann S., Biliouris K., Lesko L.J., Nowak-Göttl U., Trame M.N.* Quantitative Systems Pharmacology Model-Based Predictions of Clinical Endpoints to Optimize Warfarin and Rivaroxaban Anti-Thrombosis Therapy. *Front Pharmacol.* 2020, 14(11), 1041. https://doi.org/10.3389/fphar.2020.01041.
12. *Chernyshenko V., Pirogova L., Didkivskyi V., Cherenok S., Dosenko V., Pashevin D., Kalchenko V., Makogonenko E., Lugovskoy E.* Effects of calix[4]arene C-145 on overall haemostatic potential of blood plasma in vitro and in vivo. *Journal of International Research in Medical and Pharmaceutical Sciences.* 2016. 10(3), 146–151.

АПРОБАЦІЯ КАЛІКС[4]АРЕНУ ЯК АНТИТРОМБОТИЧНОГО ЗАСОБУ *in vivo*

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Внутрішньосудинні тромбози є однією з основних причин смертності працездатного населення світу. Антитромботичних препаратів, що впливають безпосередньо на кінцеву стадію тромбозу — полімеризацію фібрину, наразі не створено. Однако раніше було описано нову сполуку калікс[4] аренового ряду — калікс[4]арен С-145, яка безпосередньо взаємодіє з центром полімеризації фібрину «А».

Отже, *метою* цієї роботи було вивчення калікс[4]арену С-145 як антитромботичного агенту *in vivo* з використанням різних видів тварин і способів введення.

Матеріали та методи. Для дослідження калікс[4]арену С-145 *in vivo* використовували лабораторних тварин (щурів, мишей і кроликів). Для визначення антикоагулянтної дії після внутрішньовенного або перорального введення вимірювали активований частковий тромбопластиновий час і агрегацію тромбоцитів.

Результати. Оптимальним був визначений пероральний спосіб застосування. Показано значне подовження часу згортання в тесті АЧТЧ, яке спостерігалося, починаючи з 2-ї години після перорального прийому, досягало максимуму на 6-й годині та припинялася через 24 години. Дія калікс[4]арену С-145 на тромбоцити досягала максимуму через 4–6 годин і припинялася через 12 годин.

Висновки. Було доведено, що калікс[4]арен С-145 є перспективним антитромботичним препаратом, який можна вводити перорально. Подальші дослідження мають бути зосереджені на вивченні фармакодинаміки та метаболізму калікс[4]арену С-145. Такі дані потрібні для якомога швидкого впровадження створено комплексу на практиці.

Ключові слова: калікс[4]арен; плазма крові; тромбоцити; тромбоз; згортання крові; нековалентний комплекс.