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## CO-RELEASING MOLECULE (CORM-2) IN THE REGULATION OF $Ca^{2+}$ -DEPENDENT $K^{+}$ -PERMEABILITY OF ERYTHROCYTE

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In recent decades, the scientists have discovered the existence of a new class of biologically active substances – gaseous intermediaries, which perform a signaling function in the cells and with a high specificity are involved in intercellular and intracellular communication. A special place is occupied by carbon monoxide.

We conducted an experimental study of the effect of the donor of carbon monoxide CORM-2 on the change in the volume of red blood cells after cultivation in solutions with different osmotic forces. It is known that the change in the volume of red blood cells is controlled by  $Ca^{2+}$ -activated  $K^{+}$  channels ( $K^{+}(Ca^{2+})$ ) or Gardos channels.

To study the effect of CORM-2 on  $K^{+}(Ca^{2+})$  erythrocyte channels, donor blood was used. The red blood cells were pre-washed in phosphate buffered saline with glucose. To prove the effect of CORM-2 on  $K^{+}(Ca^{2+})$  channels in a parallel sample, these channels were blocked with clotrimazole, a known blocker. To clarify the activity of  $K^{+}(Ca^{2+})$  channels after incubation, red blood cells were placed in the media with different osmotic strengths: 220, 320, 420, and 520 mosm. After that, the degree of light transmission was measured. The cultivation of red blood cells with different concentrations showed a dose-dependent effect of CORM-2 on the  $K^{+}(Ca^{2+})$  channels of red blood cells.

As a result of the studies, it was found that CORM-2 is able to block  $K^{+}(Ca^{2+})$  channels. This confirms that the light transmission of the erythrocyte suspension (increase in red blood cell volume) after treatment with CORM-2 was the same as after treatment with clotrimazole. It should be noted that the effects of CORM-2 are dose-dependent. The maximum blocking effect of CORM-2 on  $K^{+}(Ca^{2+})$  channels was observed

at a concentration of 200 and 10  $\mu$ M. At a concentration of 100  $\mu$ M in a hypotonic solution of 220 mosm, the opposite effect was observed – water leakage from red blood cells (a decrease in the volume of red blood cells indicates this). This phenomenon can be explained by the effect of red blood cells on aquaporins.

**Keywords:** Gardos channel, CO-releasing molecule, carbon monoxide, erythrocytes.

**Research relation to the programs, plans, and department themes.** The work is a fragment of research work “The effect of certain vasoactive substances on the central and peripheral lymphoid organs of white mice”, the state registration number is 0117U001764.

**Introduction.** Red blood cells (RBCs) are the main component of blood and perform three main functions: transport (transport of  $O_2$  and  $CO_2$ , amino acids, polypeptides, proteins, carbohydrates, enzymes, cholesterol, prostaglandins, leukotrienes, etc.), protective (participation in vascular-platelet hemostasis, coagulation blood), regulatory (regulation of blood pH, ionic composition of plasma, water metabolism). RBCs are also able to influence the processes of microcirculation in organs and tissues due to changes in the structural and functional properties of the membrane, which determine its deformation, geometry, viscosity and fluidity [1]. The implementation of these functions is possible due to the full functioning of the membrane.

In recent decades, the existence of a new class of biologically active substances – gaseous mediators (gas transmitters), which perform a signaling function in the cells and participate in cell-to-cell and intracellular communication with high specificity, has been

discovered [2-4]. A special place is occupied by carbon monoxide (CO).

CO poisoning causes mitochondrial dysfunction, chronic intoxication leads to the appearance of diseases of the cardiovascular system [5]. However, it was found that CO is synthesized in the body of higher animals [6, 7]. The studies showed that CO was formed after the decomposition of heme to biliverdin under the action of the hemoxygenase enzyme [8].

Thus, CO in low concentrations is promising for therapeutic purposes. Subsequent studies have shown that CO plays an important role in the regulation of the cardiovascular system: relaxes the smooth muscles of blood vessels [5], stimulates angiogenesis [9], and regulates apoptosis [5]. However, the problem is that therapeutic use of CO is very difficult. It's very difficult to dose. The only way out is to use carbon monoxide donors [10].

Among CO donors, tricarbonyldichlororuthenium (II) dimer (CORM-2) is isolated [11, 12]. CORM-2 has been used in biological systems for releasing CO in a controlled way without markedly altering carboxyhemoglobin (CO-Hb) levels [12]. CORM-2 also showed anti-inflammatory effect [13, 14]; it suppresses the lipopolysaccharide induced airway [15], enhances plasmatic coagulation and attenuates fibrinolysis *in vitro* plasma [16].

RBCs are an important intermediate that CO directly affects. They are an important model for studying membrane transport. Oxygenation of cells and body tissues largely depends not only on the ability of hemoglobin to bind and release oxygen, but also on the rheological properties of blood. They are largely determined by the ability of red blood cells to deform and aggregate, since their functions are carried out through the free surface of the membranes. An important role in the RBC is played by the cell membrane, which passes gases, ions and water. Erythrocyte membrane has specialized channels. There are  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ -channels of RBCs among them. They play an important role in the programmed cell death and their deformability [17, 18].

**The purpose of the study** was to determine how CORM-2 interacts with the erythrocyte membrane and affects the course of  $\text{K}^+(\text{Ca}^{2+})$  – permeability of RBCs.

#### **Materials and methods**

##### **Collection of human blood**

Human venous blood was obtained from 30 donors (male, aged 28 to 40 years). Blood was collected into Vacutainers coated with sodium heparin (25 IU/ml), (Greiner, Kremmunster, Austria). Samples were centrifuged (320 g for 15 min, 21°C), the platelet-rich plasma and the white blood cells coat were removed. The erythrocyte sediment was washed twice with three parts of an isoosmotic NaCl solution (150 mM

containing 5 mM Na-phosphate buffer (pH 7.4) under the same centrifugation conditions. Lastly, the erythrocytes were washed with medium (containing 150 mM NaCl, 1 mM KCl, 1 mM  $\text{MgCl}_2$ , 10 mM glucose), under the same centrifugation conditions. After that, packed RBCs were transferred to ice and stored for no more than 12 hours [18].

All experiments were conducted in accordance with the Council of Europe Convention "On the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine Application of Biological and Medicine Achievements (ETS No. 164)" dated 04.04.1997, and the Helsinki Declaration of the World Medical Association (2008).

##### **Light transmission measurements**

The washed RBCs were divided into 2 parts. They (0.350 ml aliquots, Hb: 0.2 g/dl) were placed in a medium (volume 3.150 ml) of various osmolarity:

*Protocol 1:* erythrocytes in isotonic incubation solution. Erythrocytes were suspended in isoosmotic (320 mOsm) salt solution, containing (in mM): NaCl 150, KCl 1.0,  $\text{MgCl}_2$  1.0, glucose 10.

*Protocol 2:* shrinking of red blood cells. Hypertonic (420 and 520 mOsm) salt solution (SS), containing (in mM): NaCl 150, KCl 1.0,  $\text{MgCl}_2$  1.0,  $\text{C}_{12}\text{H}_{22}\text{O}_{11}$  100 (420 mOsm) or 200 (520 mOsm), glucose 10.

*Protocol 3:* swelling of red blood cells. Hypotonic (220 mOsm) SS, containing (in mM) NaCl 100, KCl 1.0,  $\text{MgCl}_2$  1.0, glucose 10.

The erythrocyte incubation time in solutions of various osmolarity was 5 minutes, in a thermostat at a temperature of 37 °C. Similar conditions were maintained during incubation with a blocker  $\text{K}^+(\text{Ca}^{2+})$  channels (Clotrimazole, 3 $\mu\text{M}$ ; Sigma Aldrich, Steinheim, Germany), as well as a CO donor (CORM-2).

Aliquots of the suspension with RBCs were filled into quartz cuvettes and placed in the wells of a spectrophotometer. Light scattering of samples to light, with wave length 800 nm, relative to the value passing through a suspension of quiescent RBCs incubated in different solutions, was measured at 21°C in a spectrophotometer that offers the possibility of online registration (Shimadzu UV-2600, Japan).

To determine the effect of CO on the activity of  $\text{K}^+(\text{Ca}^{2+})$  channels, a freshly prepared solution was added to the resulting solution CORM-2 (tricarbonyldichlororuthenium (II) dimer, 6, 10, 50, 100 и 200  $\mu\text{M}$ ; Sigma Aldrich, Saint Lois, USA), was dissolved in DMSO (in the final solution, the concentration of DMSO did not exceed 0.1%). After 5 minutes, measurements were made on a spectrophotometer.

##### **Statistical analysis**

All data of absorbance measurements are presented as means  $\pm$  standard error of the mean (SEM).

In each single curve shown under a given condition, the average is from 12 experiments on RBC prepared from a given donor. Maximal rate of change of light scattering (slope) following the acute osmotic challenge was determined separately from each recording. Since average slopes under a given condition did not differ significantly between those individual donors at our disposal, it was possible to combine maximal-slope results obtained with RBC of different donors. Statistical analysis of slope data was performed by one-way analysis of variance (ANOVA) for repeated measures, and P-values corrected for multiple comparisons by the Bonferroni-Holm procedure  $< 0.05$  were taken as statistically significant.

**Results and discussion**

The full functioning of red blood cells is provided by a change in their volume (and, accordingly, ion transport through the membrane).  $Ca^{2+}$ -activated  $K^+$  channels take part in this process. To prove the effect on  $K^+$  ( $Ca^{2+}$ ) channels of CORM-2, a known blocker of these channels, clotrimazole, was introduced in parallel.

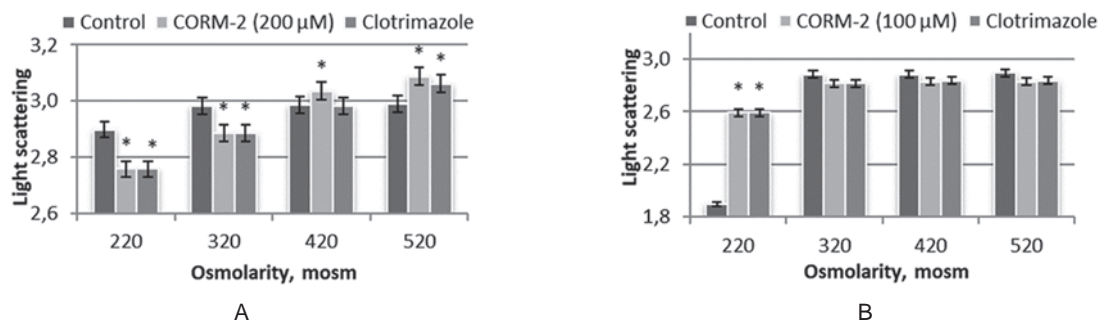
In a hypotonic solution (220 mosm), CORM-2 (at a concentration of 200  $\mu$ M) (like clotrimazole) blocked  $K^+$  ( $Ca^{2+}$ ) channels. This is evidenced by an increase in the volume of red blood cells (compared with control) by  $4.8 \pm 0.2\%$ . The fact that cells do not restore

their volume in a hypotonic solution is evidence that  $K^+$  ( $Ca^{2+}$ ) channels are blocked (Figure 1-A).

In an isotonic solution (320 mosm), the addition of CORM-2 (200  $\mu$ M) to the erythrocyte suspension also showed a decrease in the light scattering by  $3.2 \pm \pm 0.2 \%$ . This indicates an increase in their volume. In a hypertonic solution (420 mosm), the addition of CORM-2 caused a decrease in the volume of red blood cells, as evidenced by an increase in the light scattering by  $1.6 \pm 0.08 \%$ . In a solution of 520 mosm CORM-2 acted on red blood cells – reduced their volume by  $3.17 \pm 0.16 \%$ . Clotrimazole influenced them in a similar way.

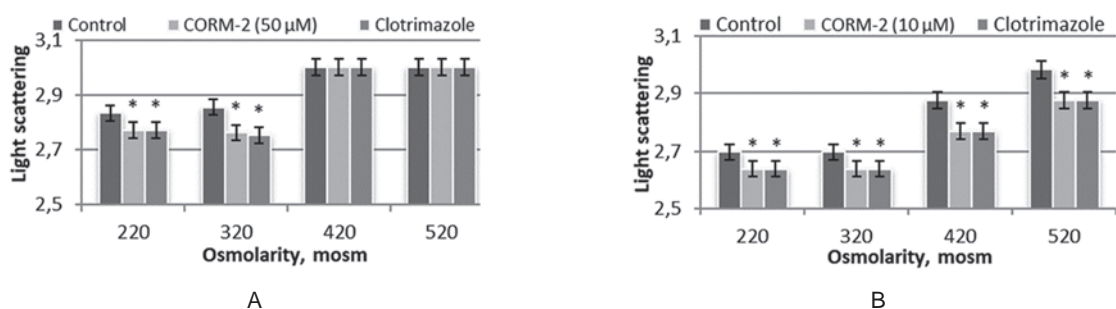
The cultivation of RBCs in a hypotonic solution after treatment with CORM-2 at a concentration of 100  $\mu$ M and clotrimazole led to an even greater leakage of water from the cells (the volume of red blood cells decreased by  $27 \pm 1.35 \%$  compared to intact). However, the RBC volume after adding CORM-2 to other suspensions did not significantly change (Figure 1-B).

In a hypertonic solution, the addition of CORM-2 (at a concentration of 50  $\mu$ M) or clotrimazole also led to an increase in cell volume (an increase of  $2.1 \pm 0.1 \%$ , respectively). We observed a decrease in volume by  $3.2 \pm 0.2 \%$  in isotonic solution (Figure 2-A). There were no differences observed in hypertonic solutions.



**Figure 1.** The dependence of the light scattering index of the erythrocyte suspension on the osmolarity of the incubation medium after incubation with CORM-2 (A - 200  $\mu$ M, B - 100  $\mu$ M) and clotrimazole (3  $\mu$ M)

**Note:** \* – indicators of light scattering of erythrocytes significantly ( $p \leq 0.01$ ) differ from the intact sample.

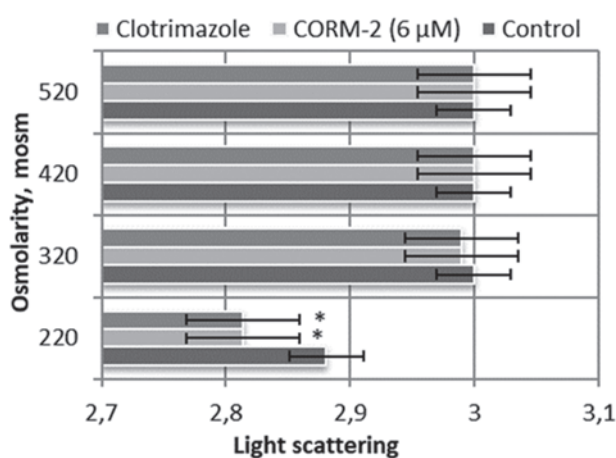


**Figure 2.** The dependence of the light scattering index of the erythrocyte suspension on the osmolarity of the incubation medium after incubation with CORM-2 (A - 50  $\mu$ M, B - 10  $\mu$ M) and clotrimazole (3  $\mu$ M)

**Note:** \* – indicators of light scattering of erythrocytes significantly ( $p \leq 0.01$ ) differ from the intact sample.

The addition of CORM-2 at a concentration of 10  $\mu\text{M}$  (as well as the addition of clotrimazole) led to an increase in the volume of red blood cells in different incubation media of different molarity. In the medium of 220 mosm – the volume increased by  $2.2 \pm 0.1 \%$ , in the medium of 320 mosm – by  $2.2 \pm 0.2 \%$ , in the medium of 420 mosm – by  $3.7 \pm 0.2 \%$ , 520 mosm – by  $3.6 \pm 0.1 \%$  (Fig. 2-B). Thus, CORM-2 at this concentration blocked calcium-dependent potassium channels in red blood cells in media with different osmotic strengths.

In contrast, CORM-2 at a concentration of 6  $\mu\text{M}$  affected the  $\text{K}^+(\text{Ca}^{2+})$  erythrocyte channels only in a hypoosmotic solution of 220 mosm. The erythrocyte volume increased by  $2.3 \pm 0.1 \%$  (Figure 3).



**Figure 3.** The dependence of the light scattering index of the erythrocyte suspension on the osmolarity of the incubation medium after incubation with CORM-2 (6  $\mu\text{M}$ ) and clotrimazole (3  $\mu\text{M}$ )

**Note:** \* – indicators of light scattering of erythrocytes significantly ( $p < 0.01$ ) differ from the intact sample.

RBCs are universal and affordable model for studying processes in the membrane. It contains only one type of channel, namely,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels of medium conductivity, or Gardos channels [19].

When they are open,  $\text{K}^+$  ions exit. Because of this, hyperpolarization of the erythrocyte membrane occurs. This  $\text{K}^+(\text{Ca}^{2+})$  channel plays an important role in the process of erythrocyte death.

As a result of the studies, it was found out that CORM-2 was able to block  $\text{K}^+(\text{Ca}^{2+})$  channels. This confirms that the light transmission of the erythrocyte suspension after treatment with CORM-2 was the same as after treatment with clotrimazole. It should be noted that the effects of CORM-2 are dose-dependent. The maximum blocking effect of CORM-2 on  $\text{K}^+(\text{Ca}^{2+})$  channels was observed at a concentration of 200 and 10  $\mu\text{M}$ . At a concentration of 100  $\mu\text{M}$  in a hypotonic solution of 220 mosm, the opposite effect was observed – water leakage from RBCs (a decrease in the volume of RBCs indicates this). We explain this phenomenon by the fact that CO acts on the aquaporins of RBCs. This requires further research.

### Conclusion

1. Carbon monoxide, which is released from CORM-2, affects the activity of  $\text{K}^+(\text{Ca}^{2+})$  – medium-conductivity channels (or Gardos-channels) in the erythrocyte membrane after incubation in solutions with different osmotic forces.
2. CORM-2 blocks  $\text{K}^+(\text{Ca}^{2+})$  red blood cell channels. This confirms that the light transmission of the erythrocyte suspension after treatment with CORM-2 was the same as after treatment with clotrimazole.
3. The effects of CORM-2 are dose-dependent. The maximum blocking effect of CORM-2 on  $\text{K}^+(\text{Ca}^{2+})$  channels was observed at a concentration of 200 and 10  $\mu\text{M}$ . At a concentration of 100  $\mu\text{M}$  in a hypotonic solution of 220 mosm, the opposite effect was observed – water leakage from red blood cells, a decrease in the volume of red blood cells. This phenomenon can be explained by the effect of red blood cells on aquaporins.

### The prospect for further scientific research.

The results indicate that it is necessary to study the effect of CORM-2 on erythrocyte aquaporins. This study will explain how CORM-2 at a concentration of 100  $\mu\text{M}$  in a hypotonic solution of 220 mosm led to a decrease in the volume of red blood cells.

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**РОЛЬ ДОНОРА МОНООКСИДУ КАРБОНУ (CORM-2)  
В РЕГУЛЯЦІЇ СА<sup>2+</sup>-ЗАЛЕЖНОЇ К<sup>+</sup> - ПРОНИКНОСТІ ЕРИТРОЦИТІВ  
Бесчасний С. П., Гасюк О. М.**

**Резюме.** В останні десятиліття було виявлено існування нового класу біологічно активних сполук - газоподібних посередників, які виконують у клітинах сигнальну функцію та з надзвичайно високою специфічністю приймають участь у міжклітинній та внутрішньоклітинній комунікації. Особливе місце серед них займає монооксид карбону.

У статті наведено результати експериментального дослідження дії донора монооксиду карбону CORM-2 на зміну об'єму еритроцитів після культивування у розчинах з різною осмотичною силою. Відомо, що зміна об'єму еритроцитів контролюється кальцій-активуючими калієвими К<sup>+</sup>(Ca<sup>2+</sup>) або Гардош-каналами. Для вивчення дії CORM-2 на К<sup>+</sup>(Ca<sup>2+</sup>) канали еритроцитів використовували свіжу донорську кров. Еритроцити попередньо відмивали у фосфатному буферному розчині з глюкозою. Для того, щоб довести вплив CORM-2 на К<sup>+</sup>(Ca<sup>2+</sup>) канали, у паралельній пробі ці канали блокували клотримазолом. Для з'ясування активності К<sup>+</sup>(Ca<sup>2+</sup>) каналів, суспензію еритроцитів поміщали у середовища з різною осмотичною силою: 220, 320, 420 та 520 мосм. Після цього проводили вимірювання ступеню світлорозсіювання отриманої проби. Культивування еритроцитів з різними концентраціями CORM-2 показала дозозалежну дію останнього на К<sup>+</sup>(Ca<sup>2+</sup>) канали еритроцитів. В результаті проведених досліджень було з'ясовано, що CORM-2 здатен блокувати К<sup>+</sup>(Ca<sup>2+</sup>) канали. Це підтверджується тим, що світлорозсіювання суспензії еритроцитів після обробки CORM-2 було аналогічне показникові отриманим після обробки клотримазолом. Необхідно відмітити, що максимальний блокуючий вплив CORM-2 на К<sup>+</sup>(Ca<sup>2+</sup>) канали спостерігався у

концентрації 200 та 10  $\mu\text{M}$ . Проте, в концентрації 100  $\mu\text{M}$  у гіпотонічному розчині 220 мосм спостерігався зворотній ефект – вихід води з еритроцитів та зменшення їхнього об'єму. Зазначене явище можна пояснити впливом на аквапоринові канали еритроцитів, що потребує подальшого дослідження.

**Ключові слова:** Гардош-канали, молекули-донори CO, монооксид карбону, еритроцити.

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### **РОЛЬ ДОНОРА МОНООКСИДА УГЛЕРОДА (CORM-2) В РЕГУЛЯЦИИ $\text{Ca}^{2+}$ -ЗАВИСИМОЙ $\text{K}^+$ -ПРОНИЦАЕМОСТИ ЭРИТРОЦИТОВ**

**Бесчасный С. П., Гасюк Е. Н.**

**Резюме.** В последние десятилетия было обнаружено существование нового класса биологически активных соединений – газообразных посредников, которые выполняют в клетках сигнальную функцию и с высокой специфичностью принимают участие в межклеточной и внутриклеточной коммуникации. Особое место среди них занимает монооксид углерода.

В статье приведены результаты экспериментального исследования действия донора монооксида углерода CORM-2 на изменение объема эритроцитов после культивирования в растворах с различной осмотической силой. Известно, что изменение объема эритроцитов контролируется кальций-активирующими калиевыми  $\text{K}^+(\text{Ca}^{2+})$  или Гардош-каналами.

Для изучения действия CORM-2 на  $\text{K}^+(\text{Ca}^{2+})$  каналы эритроцитов использовали свежую донорскую кровь. Эритроциты предварительно отмывали в фосфатном буферном растворе с глюкозой. Для доказательства влияния CORM-2 на  $\text{K}^+(\text{Ca}^{2+})$  каналы, в параллельной пробе эти каналы блокировали клотримазолом. Для выяснения активности  $\text{K}^+(\text{Ca}^{2+})$  каналов, суспензию эритроцитов помещали в среды с разной осмотической силой 220, 320, 420 и 520 мосм. После этого проводили измерения степени светорассеяния полученной пробы. Культивирование эритроцитов с различными концентрациями CORM-2 показала дозозависимое действие последнего на  $\text{K}^+(\text{Ca}^{2+})$  каналы эритроцитов. В результате проведенных исследований было выяснено, что CORM-2 способен блокировать  $\text{K}^+(\text{Ca}^{2+})$  каналы. Это подтверждается тем, что светорассеяние суспензии эритроцитов после обработки CORM-2 было аналогично показателю, полученным после обработки клотримазолом. Необходимо отметить, что максимальное блокирующее влияние CORM-2 на  $\text{K}^+(\text{Ca}^{2+})$  каналы наблюдалось при концентрации 200 и 10  $\mu\text{M}$ . Однако, в концентрации 100  $\mu\text{M}$  в гипотоническом растворе (220 мосм) наблюдался обратный эффект - выход воды из эритроцитов и уменьшение их объема. Указанное явление можно объяснить влиянием на аквапориновые каналы эритроцитов, что требует дальнейшего исследования.

**Ключевые слова:** Гардош-каналы, молекулы-доноры CO, монооксид углерода, эритроциты.

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