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ANTIHERPETIC ACTION OF CERIUM SALTS *IN VITRO*

Compounds based on cerium are highly promising objects in biotechnology regarding their high biological activities such as antiviral, antibacterial, antifungal, neuro- and radioprotective action, and antioxidant activity. On their basis it is possible to develop compositions capable of activating the systems of cellular and humoral immune defense and use them for the prevention and therapy of viral diseases, which makes it achievable to use them for the development of potential antiherpetic agents. Despite the success of their application in biotechnological fields, the mechanism of their action on biological objects requires detailed research.

The work aimed to verify in vitro anti-HSV-1/2 activity of trivalent and tetravalent cerium salts (1 mM–0.01 nM) according to the preventive and therapeutic regimen. Methods: virological, cytological, statistical. The therapeutic regime was noneffective. In the preventive regime, salt $(NH_4)_2Ce(NO_3)_6$ in vitro forms antiviral resistance in the range of investigated concentrations, while the salt $CeCl_3{\cdot}7H_2O$ forms a non-linear, sinusoidal-like concentration-dependent anti-HSV-1/2 response of cells. Cerium salts (III and IV) can cause the formation of a state of antiviral resistance in the model system MA-104 – HSV-1/2 during their previous 24 h of contact with test cells. Cerium salt (IV) provides 50 % inhibition of the cytopathic action of HSV-1/2 at a concentration of 1 μM. It is assumed that the shown antiviral activity of cerium salts may be due to their effect on the interferon system and the formation of antiviral resistance in cells.

Keywords: herpes simplex virus (HSV-1/2), Ce^{3+} and Ce^{4+} salts, antiviral activity in vitro, preventive and treatment regimen.

Introduction. Cerium is the most common rare earth element in the lanthanide series and is found in the earth's crust. It can exist in both trivalent and tetravalent states [1]. Today, compounds based on cerium are highly promising objects in biotechnology regarding their high biological activities such as antiviral, antibacterial, antifungal, neuro- and radioprotective action, and antioxidant activity, and these compounds can also increase the lifespan of micro- and macroorganisms [2-4]. On their basis it is possible to develop nanocomposites capable of activating the systems of cellular and humoral immune defense, operate as drug/gene delivery systems, and antidiabetic drugs, and use them for the prevention and therapy of viral diseases, which makes it achievable to use them for the development of potential antiherpetic agents [1, 5].

However, there are only limited reviews describing synthetic methodologies and biomedical applications of cerium salts and they do not offer complete information on their biological applications [1].

Cerium can show stability in the tetravalent state, while other lanthanides are stable only in the trivalent state [6]. The activity of tetravalent cerium is higher than trivalent [2, 7]. Cerium has several unique properties associated with the stable state of Ce^{4+}: redox activity, small ionic radius, and high charge density [2].

It should be noted that water-soluble salts of cerium Ce^{3+} in biological fluids are easily hydrolyzed to form a hydroxy compound. At pH 7-8, Ce^{3+} ions are rapidly oxidized by dissolved oxygen with the formation of Ce^{4+} ions, which are even more prone to hydrolysis, so that they immediately form insoluble Ce^{4+} hydroxide and the latter dehydrates to cerium. In other words, almost all cerium that enters the organism in the form of water-soluble salts is converted into insoluble cerium dioxide and cerium dioxide forms individual or aggregated nanoparticles in the presence of biopolymers (proteins, polysaccharides, and others) that perform stabilizing functions. That is, the biological activity of cerium compounds is largely due to CeO_2 nanoparticles [8].

Cerium oxide or nano cerium can switch the oxidation state between + 3 and +4 depending on the environment. It can exist as CeO_2 and Ce_2O_3 and exhibits catalytic activity due to the redox behavior of cerium [1].

Also, nano cerium and cerium ions have oxidoreductase-like and phosphatase-like properties and catalase and peroxidase activity [2, 9]. The beneficial biological effects of nano cerium and cerium ions are largely identical and are observed at low concentrations. Cerium ions can cause direct oxidation (especially in acidic environments), which also depends on ligands. At high concentrations, cerium ions inactivate enzymes and inhibit biologically active molecules. At appropriate (low) concentrations, cerium ions may increase the activity of some enzymes. Many lanthanides have a similar effect on living things, although due to the properties of the tetravalent cerium ion in oxide/hydroxide compounds, it is most suitable for biomedical use [2].

Materials and methods. *Cerium salts.* The following compounds were investigated: $CeCl_3{\cdot}7H_2O$ (Sigma, USA) and $(NH_4)_2Ce(NO_3)_6$ (Sigma, USA), in which cerium has a valence of +3 and +4 respectively. Solutions of $CeCl_3{\cdot}7H_2O$ and $(NH_4)_2Ce(NO_3)_6$ were used with an initial concentration of 0.1 M and 1 M in accordance. Cerium salts were dissolved in sterile water to obtain a concentration of 10 mM.

Cell Culture. The cell culture used in the research is African green monkey kidney cell culture MA-104 obtained in collaboration with *GlaxoSmithKline Biologicals S.A.* (Belgium). Cells were grown in monolayer culture in glass and plastic culture vials with a bottom area of 25 and 75 cm^2 and 96-well plates in DMEM medium with 10 % fetal bovine serum (Sigma, USA) and incubated at 37 °C, 5 % CO_2. Cells were established at $2{\cdot}10^6$ cells/mL and subcultures when confluence reached 100 % every 2 to 3 days.

Virus. Used to determine the antiviral activity of cerium salts was herpes simplex virus (HSV-1/2) isolate "GMM" from the virus collection of D.K. Zabolotny Institute of Microbiology and Virology of the NASU. Adsorption of HSV-1/2 on the cell occurs within 60 minutes. The appearance of the cytopathic effect of the virus was observed with an optical microscope 18–20 hours after the infection of cell cultures. The virus is characterized by high cytotoxicity, which is manifested by a characteristic cytopathic effect on cell culture after 24–72 h of cultivation. The CPE of the herpes virus develops in the form of enlarged, refractive, round cells. CPE begins focally, but spreads rapidly, affecting other parts of the monolayer [10].

The dose of the virus used in the research is equal to $TCID_{50}$. $TCID_{50}$ is the dose of the virus that destroys 50 % of the cell monolayer. The $TCID_{50}$ was calculated by determining the titer of the virus by tenfold titration in a 96-well plate on the appropriate cell line. The logarithm of the virus titer was calculated by a modified method of Reed–Muench [11]. The antiviral efficacy of the investigated compounds was

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evaluated by determining the median effective dose ED_{50} – the investigated compound that protects 50 % of the cell monolayer. This indicator was determined relative to the cell control which is intact cells of the appropriate cell culture on which the study was performed. To determine the antiviral activity of cerium salts preventive and therapeutic regimens were used. According to the preventive regimen, cerium salt samples were added to the MA-104 cell culture 24 h before the infection of HSV-1/2. The antiviral effect of the researched compounds was determined in the tenfold concentration range from 100 μM to 0.01 nM. According to the treatment regimen, salt samples were added to MA-104 cells 60 min after infection with HSV-1/2. In this regimen, the antiviral effect of cerium salts was determined in the tenfold concentration range an order of magnitude higher than in the preventive regimen from 1.0 mM to 0.1 nM. Used HSV-1/2 with the multiplicity of infection 1×10^3 TCD_{50} in 100 μl of medium DMEM (Sigma, USA). Crystal violet staining was used to determine the total number of adherent viable cells [12].

Crystal Violet Assay Procedure. The assay is based on determining cell adhesion by staining attached cells with crystal violet, which binds to proteins and DNA. Dead cells lose the ability to attach, which leads to a decrease in the amount of dye in cell culture. The optical density of the bound dye is measured using a spectrophotometer [13]. Summary of the methodology: The culture medium was removed from 96-well cells in which cells were cultured. To each well was added 50 μl of 0.5 % solution of Crystal violet (Sigma-Aldrich, USA) in 30 % ethanol and incubated plates at room temperature for 10 min. After 10 min, the unbound dye was removed by rinsing the wells four times with tap water. The plates were inverted on filter paper to remove excess water and then air-dried for 1-2 hours [14]. Then, the absorbance values at 540 nm were recorded using a plate reader. And all samples were analyzed in four replicates.

Statistical Analysis. Experimental data were processed by conventional methods of variation statistics. The obtained experimental data in the comparison groups are presented in the form of median and statistical error. All comparisons were considered with a significant level of $P<0.05$. The results presented graphically were obtained using Microsoft Office Excel 2010 (Microsoft Corporation, USA).

Results and discussion. The addition of Ce^{3+} and Ce^{4+} salts to cells that were previously infected with HSV-1/2 (treatment regimen) did not protect against the development of the cytodestructive change. Only adding Ce^{4+} salt to the cells in the maximum investigated concentration – 1 mM – showed more than 50 % survival of infected cells ($P<0.01$). However, if an order of magnitude lower concentration of Ce^{4+} salt was added to the medium, the number of viable cells in the experimental wells did not differ from the control infected ones (Fig. 1, A).

That is, it can be argued that in the treatment regimen of cerium salts III and IV valence *in vitro* certain limited antiherpetic activity is shown only for Ce^{4+} salt in the maximum studied concentration of 1 mM. The percentage of living cells relative to the control intact cells was 70.4 % [54.4–90.7 %] ($P<0.01$). The absence of antiviral effect manifestations of the Ce^{3+} salt under the studied conditions is likely to be associated with its chemical properties, which are different from the Ce^{4+} salt.

Study results of the antiviral resistance state formation in MA-104 cells after 24 h of contact with various concentrations of Ce^{3+} and Ce^{4+} salts (preventive regimen) are shown in Fig. 1, B.

The presence of Ce^{4+} salt in the cell culture medium for 24 h before infection with HSV-1/2 ensured the formation of an antiviral resistance state in the entire range of studied concentrations with a maximum of ~ 50 % protection of cells from the viral cytopathic action at a concentration of 1 μM (high concentrations did not provide better protection, which is probably due to their certain toxic effects). More than 30 % protection was found under the condition of the Ce^{4+} salt present in the cell culture medium at concentrations of 0.1–0.01 μM and ~25 % protection was provided by the presence of Ce^{4+} salt at 0.1–1.0 nM concentrations ($P<0.01$). Even a 10 pM concentration of Ce^{4+} salt provided significantly higher ($P<0.05$) than in infected control cells resistance to the cytopathic effect development under conditions of HSV-1/2 infection.

The nature of the effect of 24 h of Ce^{3+} salt contact on the state of resistance of HSV-1/2 infected cells is ambiguous. A nonlinear, oscillating response was observed when the concentration of the Ce^{3+} salt changed by one order of magnitude. This phenomenon could be attributed to methodological errors, but since in the experiment the change in the reaction is traced four times, in addition, it is reproduced in repeated experiments, the question arises as to the mechanism of such a reaction of cells. Most likely, the fact we discovered is due to the ability of $CeCl_3\cdot7H_2O$ salt at different concentrations to interact differently with the components of the nutrient medium, changing their characteristics, which after 24 hours of contact with cells can affect their metabolism and reactivity.

That is, if the effect of Ce^{4+} salt on cells is concentration-dependent and linear, then Ce^{3+} salt affects the state of cells indirectly: we observe a series of sharp changes (jumps) in the appearance and loss of activity associated with a 10-fold change in its concentration. Interestingly, the maximum manifestation of antiviral resistance in cells is shown when nanomolar concentrations of Ce^{3+} salt (0.1 nM and 10 nM, $P<0.01$) are introduced into the culture medium.

The absence of the effect of antiviral protection in the presence of Ce^{3+} salt in the culture medium at intermediate concentrations of 1 nM and 0.01 nM ($P>0.05$) indicates a high sensitivity of the object to exposure to Ce^{3+} ions or products of their reaction with environmental components. The obtained results are very interesting from the point of view of the analysis of the mechanisms of antiviral state formation in cells in the presence of microelements in their microenvironment and rare earth elements in particular. In addition, the detected fluctuation concentration dependence of the formation of antiviral resistance leads to a certain parallel regarding the similarity of the detected phenomenon to the self-oscillation reaction created by B. Belousov and studied in detail by A. Jabotinsky, where the main component that provided the self-oscillating process was the Ce^{3+} ion [15]. The facts obtained and the parallels drawn out require a deep and thorough study.

Thus, under *in vitro* conditions, the use of III and IV valence cerium salts according to the treatment regimen does not provide antiherpetic effects. While the presence of Ce^{4+} salts in the culture medium causes the formation of an antiviral resistance state: the effect linearly depends on the salt concentration. The $CeCl_3\cdot7H_2O$ salt effect was found to fluctuate (disappear and appear) depending on the change in the concentration of ions in the culture medium. Table 1 shows a certain range of effective concentrations of $CeCl_3\cdot7H_2O$ and $(NH_4)_2Ce(NO_3)_6$ salts.

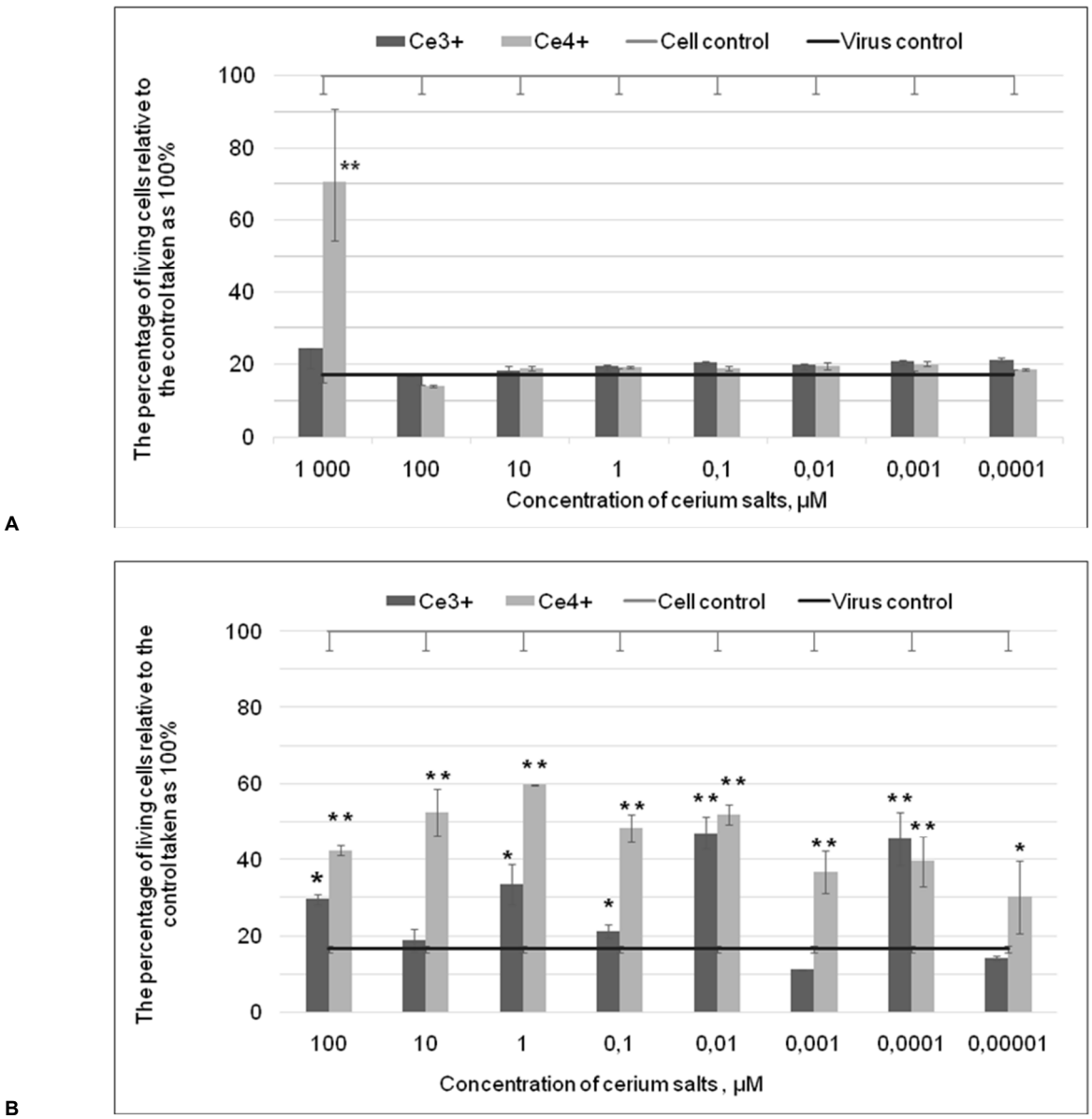

Fig. 1. Antiherpetic action of Ce^{3+} and Ce^{4+} salts. Notes:
A – treatment regimen, B – preventive regimen; * – $P < 0.05$; ** – $P < 0.01$, n=4,
results presented as the median and the interval between the first and third quartiles [Q1–Q3]

Table* 1. Indicators of effective antiherpetic concentrations of cerium salts according to the preventive regimen *in vitro

Effective concentration, nM	EC_{10}	EC_{25}	EC_{50}	EC_{75}	EC_{90}
$CeCl_3 \cdot 7H_2O$	0.05	0.08	–	–	–
$(NH_4)_2Ce(NO_3)_6$	0.01	0.10	1 000	–	–

Note: "–" anti-virus protection is insufficient, and effective concentration has not been determined.

Since the experiment was conducted with a high multiplicity of infections, it can be assumed that with a lower multiplicity of infections, the effective concentrations will be lower.

We have shown that cerium salts are capable of providing the formation of a state of antiviral resistance against HSV-1/2, provided that they are present for 24 h in the culture medium. The treatment regimen in the same salt concentrations as the preventive regime (100 μM–0.01 nM) does no antiviral effectiveness. Salt $(NH_4)_2Ce(NO_3)_6$ *in vitro* provides the formation of an effective state of antiviral resistance, while the salt $CeCl_3 \cdot 7H_2O$ forms a non-linear, sinusoidal-like concentration-dependent anti-HSV-1/2 response of cells.

It was studied *in vitro* the antiviral effect of $CeCl_3 \cdot 7H_2O$ and $(NH_4)_2Ce(NO_3)_6$ salts at various concentrations in MA-104 cell culture at a high multiplicity of HSV-1/2 infection. It has been found that cerium salts are capable of providing antiherpetic activity.

These results correlate with the previously obtained data [16] on the antiviral activity of cerium salts against vesicular stomatitis virus *in vitro*. The detected effects correlated with the shown activation of the interferon system under the

condition of their 24h contact with L929 cell culture, which can actively produce interferon. A preventive regimen of the studied salt application can affect the activation of the interferon system in MA-104 cells and, in some way, form a state of antiviral resistance, as shown in our work. It should be noted that herpes infection can interfere with the interferon system [17, 18], which may cause the ineffectiveness of salts in the treatment regimen.

Conclusions. Antiherpetic activity of trivalent and tetravalent cerium salts ($CeCl_3 \cdot 7H_2O$ and $(NH_4)_2Ce(NO_3)_6$) was studied *in vitro* in the range of ten-fold concentrations from 1 mM to 0.01 nM.

1. It was shown that in the therapeutic regimen, the use of $CeCl_3 \cdot 7H_2O$ and $(NH_4)_2Ce(NO_3)_6$ salts in the range of studied concentrations does not protect MA-104 cells from the development of the cytopathic effect of HSV-1/2.

2. According to the preventive regimen *in vitro* (samples were added to the MA-104 cell culture 24h before the infection of HSV-1/2) $(NH_4)_2Ce(NO_3)_6$ provides the formation of an effective state of antiviral resistance, while the salt $CeCl_3 \cdot 7H_2O$ forms a non-linear, sinusoidal-like concentration-dependent anti-HSV-1/2 response of cells.

3. It has been suggested that the shown antiviral activity of cerium salts according to the preventive regimen may be due to their effect on the interferon system and the formation of antiviral resistance in cells.

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АНТИГЕРПЕТИЧНА ДІЯ СОЛЕЙ ЦЕРІЮ *IN VITRO*

Сполуки на основі церію є одними з високоперспективних об'єктів у біотехнології, що пов'язано з їхньою високою біологічною активністю: антивірусною, антибактеріальною, антифунгальною, нейро- та радіопротекторною дією, антиоксидантною активністю. На їх основі можна розробляти композиції, що здатні активувати системи клітинного та гуморального імунного захисту, і застосовувати для профілактики і терапії вірусних захворювань, що дає можливість їх використання для розроблення потенційних антигерпетичних засобів. Незважаючи на успіхи використання в біотехнологічних сферах, механізм їхньої дії на біологічні об'єкти потребує детального дослідження. Мета роботи полягала в перевірці в умовах in vitro антигерпетичної активності солей Ce^{3+} and Ce^{4+} у діапазоні концентрацій 1 мМ–0.01 нМ за профілактичною і лікувальною схемами застосування. У дослідженні були використані вірусологічні, цитологічні та статистичні методи. Результати роботи: застосування солей за лікувальною схемою є неефективним. Внесення до клітин водного розчину солі $(NH_4)_2Ce(NO_3)_6$ за профілактичною схемою супроводжувалось формуванням стану антивірусної резистентності в усьому діапазоні досліджуваних концентрацій, тоді як застосування солі $CeCl_3 \cdot 7H_2O$ – нелінійним, концентраційно-пов'язаним синусоїдоподібним формуванням у клітин резистентності до цитопатичної дії HSV-1/2.

Висновки: у модельній системі MA-104 – HSV-1/2 солі Ce^{3+} та Ce^{4+} здатні викликати формування стану антивірусної резистентності за їхнього попереднього 24-годинного контакту із клітинами. Сіль Ce^{4+} забезпечує 50 % пригнічення цитопатичної дії HSV-1/2 у концентрації 1 μM. Припускаємо, що показана антивірусна активність солей церію може бути зумовлена їхнім впливом на систему інтерферону та формуванням у клітинах стану антивірусної резистентності.

Ключові слова: вірус простого герпесу (HSV-1/2), солі Ce^{3+} та Ce^{4+}, антивірусна активність in vitro, профілактична та лікувальна схеми.