EXPERIMENTAL WORKS

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CALIX[4]ARENE CHALCONE AMIDE C-1011 ELICITS DIFFERENTIAL EFFECTS ON THE VIABILITY OF 4T1 MOUSE BREAST ADENOCARCINOMA CELLS WITH DIFFERENT LEVELS OF ADAPTOR PROTEIN RUK/CIN85 EXPRESSION

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According to our earlier data, calix[4]arene chalcone amides modulate Ca ions exchange in the myometrium mitochondria and the level of inner membrane polarization that can potentially affect cell survival. To test this hypothesis, we studied the effect of calix[4]arene with 4 chalcone amide groups on mitochondria membrane polarization and viability of 4T1 mouse breast adenocarcinoma cells, a surrogate model of human triple-negative breast cancer, and on its highly malignant subline overexpressing the adaptor protein Ruk/CIN85. Mitochondria membrane potential was measured by flow cytometry, and cell viability was assessed using Trypan blue dye exclusion. It was shown that mitochondrial membranes of control (Mock) cells had a higher polarization level (67.80 ± 8.82 r.u., n = 5) compared to 4T1 cells with up-regulation of Ruk/CIN85 (RukUp cells) (25.42 ± 2.58 r.u., n = 4). Upon incubation of cells with 1 μM calix[4]arene C-1011, the CCCP-sensitive component of mitochondrial membranes polarization decreased (by almost 50%) in 4T1 Mock cells and did not change in RukUp cells compared with the control. It was demonstrated that 1 μM calix[4]arene C-1011 suppressed the viability of 4T1 Mock cells by 45%, but did not affect RukUp cells considerably. It was suggested that calix[4]arene chalcone amide C-1011 decreased mouse breast adenocarcinoma 4T1 cell viability at least by affecting mitochondrial membrane polarization.The data obtained indicate the prospects of further studies of calix[4]arene chalcone amide as a potential anticancer drug candidate.

K e y w o r d s: calix[4]arene chalcone amide, mitochondria membrane potential, breast cancer, adaptor protein Ruk/CIN85.

Calixarenes are macrocyclic molecules, the biological activity of which is determined by chemical groups on the upper or lower rim. Calixarenes are widely studied and used in bioorganic chemistry and biochemistry [1, 2]. Due to their hydrophobic properties, they are able to dissolve in the lipid phase of cells, which provides them with the role of carriers of biologically active compounds. In addition, it is possible to attach functionally active groups in different amounts to the calixarenes. These groups include, in particular, chalcones - aromatic ketones, members of flavonoids class, which are characterized by a wide spectrum of biological activity [3-7]. We have previously shown that calix[4]arene chalcone amides can affect both the level of polarization of mitochondrial membranes and the concentration of Ca^{2+} in the myometrium mitochondria matrix [8]. It was also found that short-term incubation of myometrial mitochondria with calix[4]arene chalcone amides is accompanied by an increase in the average hydrodynamic diameter of mitochondria, which indirectly indicates swelling of these organelles. The effect of calix[4]arene chalcone amides on the hydrodynamic diameter of mitochondria increased with an increase in the number of chalcone amide groups [9]. By using ca-

lix[4]arene chalcone amide C-1070 (the fluorescent equivalent of C-1011), it was proven that these compounds penetrate myometrial cells [9]. The modulatory effects of calix[4]arene chalcone amide with two chalcone amide groups on the polarization of mitochondrial membranes were shown using a primary culture of myometrial cells and a potential-sensitive probe JC-1 [9]. It should be noted that chalcones constitute a group of phenolic compounds that are of increasing interest in cancer research [4].

Our previous studies have demonstrated that overexpression of adaptor protein Ruk/CIN85 in breast cancer cells is closely associated with increased survival, motility, and invasiveness [10-12]. Therefore, the aim of this research was to study the effect of calix[4]arene with four chalcone amide groups (C-1011) on mitochondria membrane polarization and viability of 4T1 mouse breast adenocarcinoma cells with different levels of Ruk/CIN85 expression.

Materials and Methods

C-1011 synthesis. Calix[4]arene chalcone amide (C-1011) was synthesized according to the previously described scheme [13]. The macrocycle of calixarene is in the *cone* conformation and contains four chalcone amide groups on the lower rim (Fig. 1).

Mitochondria membrane polarization. To record mitochondria membrane polarization of Mock and RukUp cells, a COULTER EPICS XL™ (Beckman Coulter, United States) flow cytometer with an argon laser (λ_{ex} 488 nm) was used. Experimental data were analyzed using the SYSTEM II™ Software (Beckman Coulter). Relative values of mitochondria membrane potential ($\Delta\psi$) were assayed using a voltage-sensitive fluorescent probe TMRM (Invitrogen) (λ_{ex} 488 nm, λ_{em} 590 nm) in the medium of the following composition: 20 mM HEPES (pH 7.4); 125 mM KCl; 25 mM NaCl; 2 mM K^+ phosphate buffer (pH 7.4); 5 mM sodium succinate; and 0.1 mg/ml digitonin (Merck). It should be noted that permeabilization of the plasma membrane with 0.01% digitonin excludes the contribution of its polarization to the probe signal. Cells suspension (2-2.5·10^5 cells/ml) were incubated with calix[4]arene chalcone amide C-1011 for 5 min at 37°C followed by loading with 100 nM TMRM and then immediately analyzed on the flow cytometer at a wavelength of 590 nm (channel FL_2). Each measurement is represented as the average fluorescent intensity of 10 000 events and expressed in relative units: average fluorescence intensity value of a sample minus average fluorescence intensity value of a sample upon addition of 10 μM CCCP.

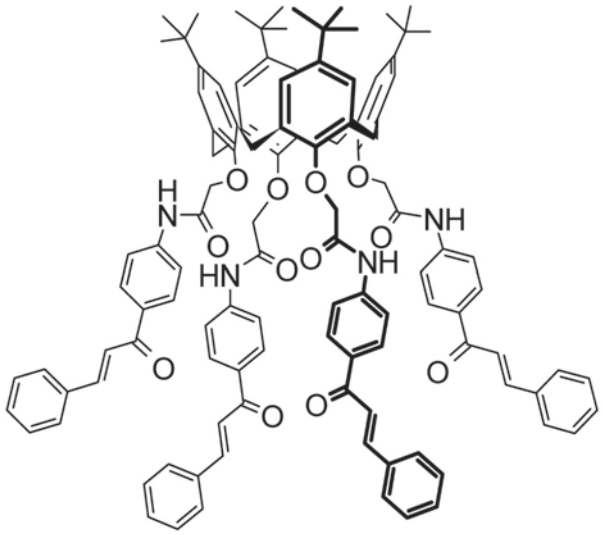

Fig. 1. Structural formula of calix[4]arene chalcone amide C-1011

Cell culture. Mouse breast adenocarcinoma 4T1 cells with stable overexpression of adaptor protein Ruk/CIN85 (RukUp subline) and control cells (Mock subline) were used in experiments [12]. Cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Hy-Clone), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), in a humidified atmosphere containing 5% CO_2 at 37°C.

Cells survival. Cells were seeded on 24-well plate (5×10^4 cells/well) in a complete medium, and were incubated in the presence of 0.1% DMFA (Control), 0,1 μM or 1 μM calix[4]arene C-1011 for 24 hours. Then, cells were detached with 0.25% trypsin-EDTA (Gibco), resuspended in 0.4% Trypan blue dye, and counted using Neubauer chamber hemocytometer. Results are presented as a percentage of control, $n = 4$.

Statistical analysis. For data analysis, one-way ANOVA followed by Fisher LSD post-hoc test was used. The difference between groups was considered significant at $P < 0.05$.

Results and Discussion

4T1 cells are a highly invasive mouse breast adenocarcinoma cell line that represents an animal model of triple-negative human breast cancer [14]. It has been previously demonstrated that overex-

pression of adaptor protein Ruk/CIN85 in breast adenocarcinoma cells is accompanied by enhanced malignancy in comparison to parental cells [10, 12]. Available data suggest that Ruk/CIN85 may play a potential role in the control of metastasis *in vivo* [12]. Given the above, Ruk/CIN85-overexpressing 4T1 cells (RukUp) and mock-transfected control cells (Mock) were used in the present study.

Mitochondria are not only a source of energy in cancer cells, but also dynamic regulators of their survival and apoptosis, proliferation, motility and invasiveness, as well as the development of stemness characteristics. Like the cells that contain them, mitochondria are able to adapt to the tumor environment and are likely to evolve into "oncogenic mitochondria" capable of transmitting malignant properties to recipient cells. The broader search for cancer metabolic modulators has already identified therapies that target mitochondria in cancer cells, but the field is still in its infancy [15-18].

In our experiments, we focused on studying the effect of calix[4]arene chalcone amide C-1011 on the polarization of mitochondrial membranes in Mock and RukUp cells, which differ in the degree of malignancy.

We have previously shown that short-term incubation of digitonin-perforated myometrial cells with 10 μM C-137 (calix[4]arene chalcone amide with two chalcone amide groups) was accompanied by hyperpolarization of mitochondrial membranes [8]. It is known that hyperpolarization of the mitochondrial membrane is the first stage on the way to depolarization leading to cell death [19]. We also studied the effect of calix[4]arene chalcone amide C-137 on the membrane potential of myometrial mitochondria using confocal microscopy [9]. Myometrial cells loaded with JC-1 dye were incubated with C-137 calix[4]arene chalcone amides (10 μM) for 20 min. Using this approach, we were able to register the phase of mitochondrial membrane hyperpolarization followed by depolarization. These data are consistent with those published by Gutierrez et al. [20] that chalcones are substances that facilitate the initiation of apoptosis along the mitochondrial pathway.

Recording of mitochondrial membrane potential was performed on digitonin-perforated Mock and RukUp cells. Permeabilization of cell plasma membranes with digitonin, firstly, eliminates the contribution of its polarization to the probe signal, and secondly, ensures the entry of medium components into cells. As can be seen from Fig. 2, cell autofluorescence is registered (Fig. 2, *a*, *b*, blue graph). Cells were then loaded with 100 nM TMRM for 5 min. Incubation of cells with the probe leads to an increase in fluorescence intensity, as evidenced by a signal shift to the right. (Fig. 2, *a*, *b*, red graph). Consequently, there was an accumulation of a positively charged probe in the mitochondria. Further introduction of CCCP into the incubation medium led to a decrease in the fluorescence intensity, which indicates the release of the probe from mitochondria due to the depolarization of mitochondrial membrane (Fig. 2, *a*, *b*, green graph).

Calculations of the CCCP-sensitive component of the fluorescence intensity of TMRM-loaded cells showed that the mitochondrial membranes of Mock cells have a higher level of polarization (67.80 ± 8.82 r.u., $n = 5$) compared to RukUp cells (25.42 ± 2.58 r.u., $n = 4$).

In subsequent experiments, we studied the effect of C-1011 calix[4]arene on the mitochondrial membrane potential of Mock and RukUp cells. As can be seen from Fig. 2, *c*, incubation of Mock cells in the presence of 1 μM calix[4]arene C-1011 was accompanied by a decrease in the fluorescence intensity of the potential-sensitive TMRM probe loaded into these cells. Calculations of the CCCP-sensitive polarization component of mitochondrial membranes of Mock cells indicate a decrease (almost by 50%) in this parameter compared with the control upon incubation of organelles with 1 μM calix[4]arene C-1011 (Fig. 2, *e*).

Incubation of RukUp cells with 1 μM calix[4]arene C-1011 did not affect the fluorescence intensity of the potential-sensitive TMRM probe loaded into these cells (Fig. 2, *d*). This conclusion was confirmed by calculations of the CCCP-sensitive component of mitochondrial membrane polarization in RukUp cells. Incubation of RukUp cells with 1 μM calix[4]arene C-1011 was not accompanied by statistically significant changes in membrane potential compared to the control (Fig. 2, *e*).

Considering that the level of inner membrane polarization can potentially affect cell survival, we also examined the viability of Mock and RukUp cells when treated with 0.1 and 1 μM calix[4]arene C-1011. As shown in Fig. 3, 0.1 μM calix[4]arene C-1011 suppressed the viability of the Mock and RukUp sublines by 19%. Increasing the concentration of calix[4]arene C-1011 to 1 μM led to the suppression of Mock cells by 45%, but had no sig-

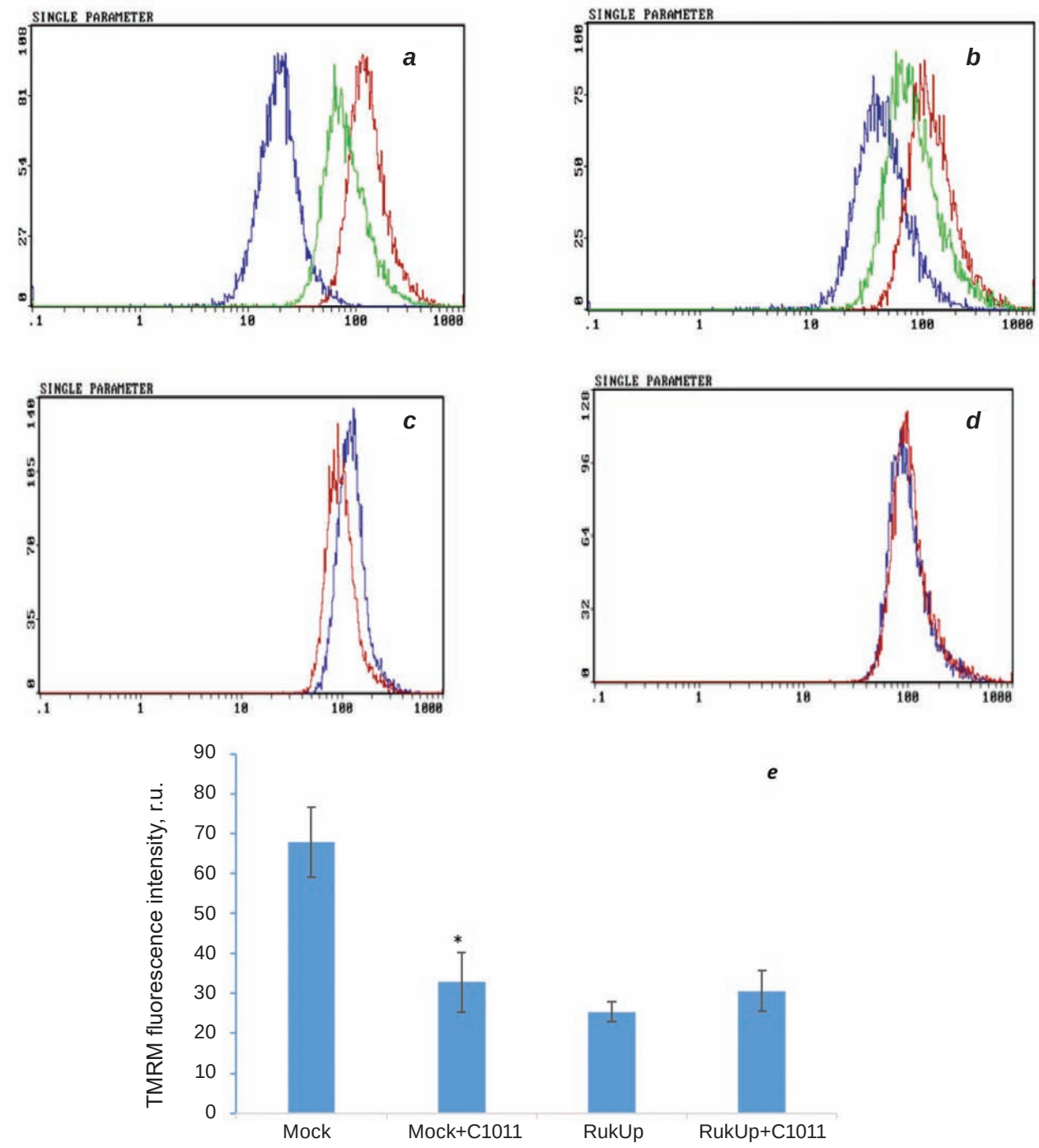

*Fig. 2. Calix[4]arene C-1011 induced a decrease in the mitochondrial membrane potential in Mock control cells and did not change it in Mock cells overexpressing Ruk/CIN85. Fluorescence intensity of Mock cells (**a**) and RukUp cells (**b**): blue graph - autofluorescence; red graph – after incubation with 100 nM TMRM; green graph – after incubation with 10 μM CCCP loaded with a fluorescent probe cells. Effect of 1 μM calix[4]arene C-1011 on the fluorescence intensity of a potential sensitive probe TMRM loaded into Mock cells (**c**) and RukUp cells (**d**). Blue graph – control, red graph – + 1 μM calix[4]arene C-1011. Typical results are given, n = 4-5. Effect of calix[4]arene C-1011 (1 μM) on the CCCP-sensitive component of TMRM fluorescence intensity of Mock cells (M ± m, n = 5, P < 0.05 in comparison to the untreated Mock cells) and of RukUp cells (M ± m, n = 4); (**e**)*

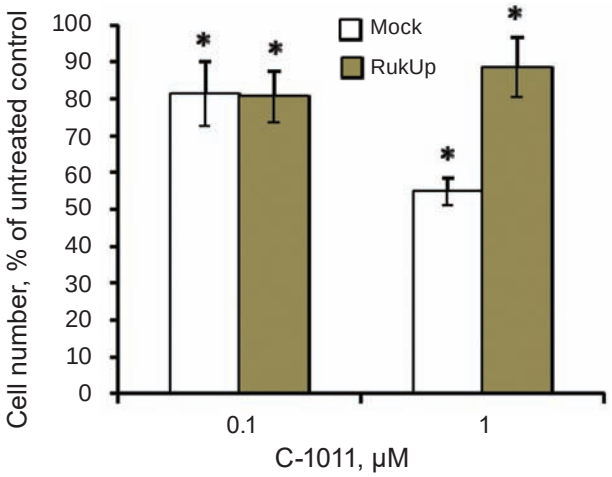

*Fig. 3. Calix[4]arene C-1011 decreased the survival of Mock control cells and did not change it of Mock cells overexpressing Ruk/CIN85. Survival of Mock and RukUp cells in the presence of C-1011 at different concentrations, n = 4, *P < 0.05 in comparison to untreated cells*

nificant effect on RukUp cells compared to a concentration of 0.1 μM.

We suggested that calix[4]arene chalcone amide C-1011 decreased mouse breast adenocarcinoma 4T1 control cells survival at least by affecting mitochondrial membrane polarization. Thus, it has been shown that calix[4]arene chalcone amide C-1011 is able to suppress the viability of 4T1 breast adenocarcinoma cells depending on their genetic context associated with the degree of malignancy. Mitochondria have been regarded as essential cell elements that fuel the metabolic needs of most cancer cell types [21]. Currently, it is well established that highly aggressive cancer cells rely on glycolysis instead of mitochondrial oxidative phosphorylation of glucose, and one marker of this metabotype known as the Warburg effect is a decrease in mitochondrial transmembrane potential [22-23]. To recognize the precise molecular mechanisms underlying mitochondrial dysfunction in Ruk/CIN85 overexpressing cells and consequent resistance to C-1011 action, further studies are needed.

In summary, our results indicate the following:

• Mock cell mitochondrial membranes have a higher level of polarization (67.80 ± 8.82 r.u., n = 5) compared to those of RukUp cells (25.42 ± 2.58 r.u., n = 4).

• the CCCP-sensitive component of mitochondrial membranes polarization of Mock cells decreased (by almost 50%) compared with the control upon incubation of organelles with 1 μM calix[4]arene C-1011.

• incubation of RukUp cells with 1 μM calix[4]arene C-1011 did not affect mitochondrial membranes polarization compared with the control.

• 1 μM calix[4]arene C-1011 suppressed the viability of Mock cells by 45%, but did not affect RukUp cells considerably.

• since mitochondria play an important role in cell survival and death, the obtained results indicate the prospects of further studies of calix[4]arene chalcone amides as a potential anticancer drug candidate at the early stages of carcinogenesis.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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КАЛІКС[4]АРЕНХАЛКОНАМІД С-1011 МАЄ ДИФЕРЕНЦІЙНИЙ ВПЛИВ НА ЖИТТЄЗДАТНІСТЬ КЛІТИН АДЕНОКАРЦИНОМИ МОЛОЧНОЇ ЗАЛОЗИ МИШІ 4Т1 З РІЗНИМИ РІВНЯМИ ЕКСПРЕСІЇ АДАПТЕРНОГО ПРОТЕЇНУ RUK/CIN85

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Згідно з нашими попередніми даними, калікс[4]аренхалконаміди модулюють обмін іонів Са в мітохондріях міометрія та рівень поляризації внутрішньої мембрани, що потенційно може впливати на життєздатність клітин. Щоб перевірити цю гіпотезу, ми дослідили вплив калікс[4]арену з 4 халконамідними групами на поляризацію мембрани мітохондрій і життєздатність клітин аденокарциноми молочної залози миші 4Т1, сурогатної моделі тричі негативного раку молочної залози люди-

ни, а також на його високозлоякісну сублінію з надекспресією адаптерного протеїну Ruk/CIN85. Мембранний потенціал мітохондрій вимірювали методом проточної цитометрії, а життєздатність клітин оцінювали за допомогою прямого підрахунку з трипановим синім. Показано, що мітохондрійні мембрани контрольних (Mock) клітин мали вищий рівень поляризації (67,80 ± 8,82 в.о., n = 5) порівняно з клітинами 4T1 із надекспресією Ruk/CIN85 (клітини RukUp) (25,42 ± 2,58 в.о., n = 4). Після інкубації клітин із 1 мкМ калікс[4]ареном С-1011 СССР-чутливий компонент поляризації мітохондрійних мембран зменшився (майже на 50%) у клітинах 4T1 Mock і не змінився у клітинах RukUp порівняно з контролем. Продемонстровано, що 1 мкМ калікс[4]арен С-1011 пригнічував виживаність клітин 4T1 Mock на 45%, але не впливав суттєво на клітини RukUp. Висловлюється припущення, що калікс[4]аренхалконамід С-1011 знижує життєздатність клітин аденокарциноми молочної залози миші 4T1, принаймні, за рахунок впливу на поляризацію мітохондрійних мембран. Отримані дані свідчать про перспективи подальших досліджень калікс[4]аренхалконаміду як потенційного протипухлинного препарату.

Ключові слова: калікс[4]аренхалконамід, мембранний потенціал мітохондрій, рак молочної залози, адаптерний протеїн Ruk/CIN85.

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