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# Effect of tilorone and its analogues on the change of mitochondrial potential of rat hepatocytes

*The influence of tilorone dihydrochloryde and its analogues – diphenyl derivatives on the changes of transmembrane potential of mitochondrial membranes of the isolated rat hepatocytes has been estimated. Authors have shown a significant increase in mitochondrial potential thirty minutes after the introduction of the test compounds to the cells using the fluorescent probe JC-1. These results indicate the rapid activation with tilorone and its analog – dihydrochloryde 4,4'-bis-[2-(diethylamino)ethoxy]diphenyl – of the RLR signaling pathway. The final stage of this pathway is the cell production of IFN type I. The authors concluded that there is an increasing of the organelles resistance to the extra/intracellular damaging agents under the influence of the test compounds.*
*Key words: tilorone, diphenyl derivatives, mitochondrial potential, fluorescent probes, rat hepatocytes.*

## INTRODUCTION

Mitochondria are important cellular organelles which undergo the dynamic cycles of synthesis and division. Beside the energy supply of the cell mitochondria are involved in mammals' cellular reactions of innate antiviral immunity. These reactions include a cascade response which final stage is the production of type I interferons [16]. Mitochondrial antiviral signaling (MAVS) in particular adaptor protein of the mitochondria outer membrane plays an important role in this process. MAVS-mediated is the activation of transcription factors – interferon-regulatory factor-3 (IRF-3) and nuclear factor κB (NF-κB). The regulation of mitochondria-mediated antiviral response is associated with MAVS structural characteristics [19]. In addition, it was shown a close correlation between the antiviral response of the cells and mitochondrial membrane potential ($\Delta\psi m$): the reduction of $\Delta\psi m$ correlates with the decrease of the antiviral response [10]. The authors have shown that $\Delta\psi m$ dissipation does not affect on the activation of interferon-regulatory factor-3 (IRF-3) after the activation of the MAVS. This indicates the close relationship of $\Delta\psi m$ and MAVS in RLR signaling pathway. I.e. the physiological functions of mitochondria play a key role in innate antiviral immunity.

One way to enhance innate antiviral immunity for the prevention and treatment of viral infections is the use of interferon (IFN) inducers. Among them is the well-known low-molecular synthetic inducer Amixine IC (tilorone dihydrochloryde) which has, due to its structure, both antiviral and immunomodulating properties [7]. Being double cyclic compounds with aminoalkoxyl groups, diphenyl derivatives are structurally similar to tilorone dihydrochloryde. Antiviral and interferon-inducing activities were shown for these compounds as for tilorone [3]. The result of the effect of these substances in the cell is the IFN production. That is why the aim of our study was to investigate their influence on the physiological functions of mitochondria, in particular, the dynamics of $\Delta\psi m$ changes in rat hepatocytes.

## METHODS

In our experiments we used dihydrochloryde 2,7-bis-[2-(diethylamino)ethoxy]fluoren-9-one (tilorone dihydrochloryde, drug Amixine IC) [2] and its analogues – dihydrochloryde 4,4'-bis-[2-(diethylamino)ethoxy]diphenyl (compound 1) and dihydrochloryde 2-metoksycarbonil-4-4'-bis-[2-(diethylamino) ethoxy]-diphenyl (compound 2) [9] (kindly provided for research by Dr. S.A. Lyakhov, O.V. Bogatsky Physico-Chemical Institute of National Academy of Sciences of Ukraine), Table 1. To prepare basic solution, the specified compounds are dissolved in distilled water to a concentration of 1,0 mg/ml.

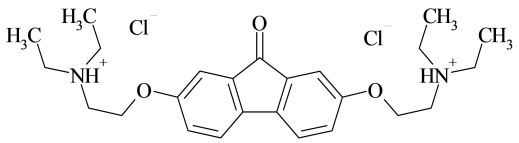
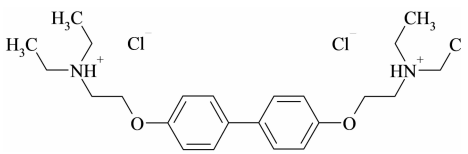
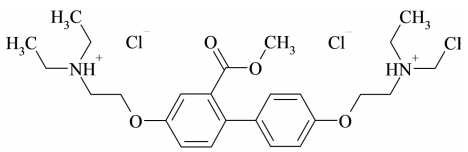
Isolated liver hepatocytes of 3 month old white male Wistar population rats were used in the work. The cells were isolated by non-enzyme method using the perfusion medium, containing 2 mM EDTA [8]. The cell viability was assessed on the staining with trypan blue. Viability of freshly isolated hepatocytes made about 90%.

Mitochondrial membrane potential ($\Delta\psi m$) of single cells was assessed by the accumulation JC-1 fluorescent probe aggregates within cells by quantitative microfluorimetry [5]. JC-1 (5,5',6,6'-tetrachlor-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) was synthesized in the Laboratory of Nanodisperse Materials of the Institute for Scintillation Materials of the National Academy of Sciences of Ukraine (Kharkov) by standard methods [6]. JC-1 has two band fluorescence with maxima at 510 nm (monomeric form) and 585 nm (J-aggregates). Initial solution (1 mM) of the studied probe in DMSO was diluted directly prior to the experiment up to the concentration required. The cells ($5 \cdot 10^5$ cells/ml) were incubated with the probe JC-1 ($10^{-6}$ M) in Eagle's medium with 10% fetal calf serum (BioloT, Russia) at 22°C during 60 min (incubation media produced by Institute of Poliomyelitis and Virus Encephalitis of Russian Academy of Medical Sciences). The experimental compounds were added in a concentration of 0,01 mg/ml of the cell suspension and incubated for 30 and 120 min.

Luminescent objects were examined and imaged by means of luminescent microscope Olympus IX71 and digital camera Olympus C-5060 (Japan). The luminescence spectra of single hepatocytes stained by the probe were recorded with a spectrometer USB4000 (Ocean Optics, USA).

The results were statistically processed by means of software Statistika v. 5.0 (StatSoft, USA) and Origin 6.1 (Origin Lab Corporation, USA) using the Student's t-criterion. The results significantly differed in statistics at $P < 0,05$.

**The structural formulas of the experimental compounds**

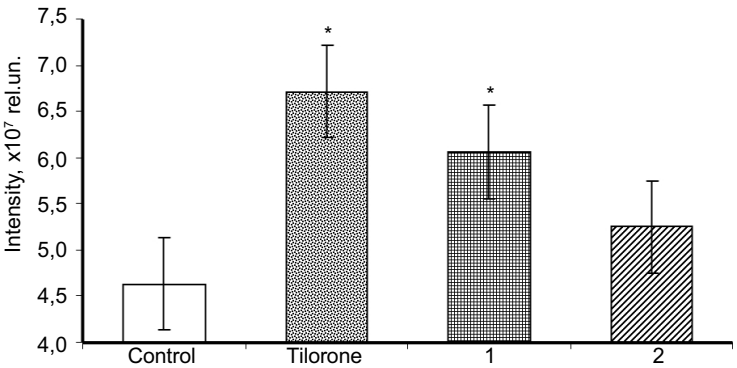
| Name of the compound | The structural formula |
|---|---|
| dihydrochloryde 2,7-bis-[2-(diethylamino)ethoxy]fluoren-9-one (tilorone dihydrochloryde, drug Amixine IC) |  |
| dihydrochloryde 4,4'-bis-[2-(diethylamino)ethoxy]diphenyl (compound 1) |  |
| dihydrochloryde 2-metoksycarbonil-4-4'-bis-[2-(diethylamino)ethoxy]diphenyl (compound 2) |  |

## RESULTS AND DISCUSSION

Fluorescent microscopy allows us to study mitochondrial function at the level of single cells. This is important in the study of subtle mechanisms which determine individual characteristics of cellular reaction to regulatory effects [14]. The accumulation of the fluorescent probe JC-1 in the mitochondria leads to the formation of J-aggregates depending on the value of $\Delta\psi m$. This process is accompanied by a shift of the fluorescence emission from green color which inherent to monomers to red color which inherent to J-aggregates [15]. JC-1 allows us to study the local changes in the value of $\Delta\psi m$ and to visualize mitochondria with high and low membrane potential, unlike other dyes [17]. In studies using JC-1 probe for the characterization of mitochondrial processes are often used both the fluorescence intensity of the monomers and of the J-aggregates, as well as their ratio. However, some researchers believe that the fluorescence intensity of the J-aggregates is more accurate and adequate criterion for assessing $\Delta\psi m$ [11, 12]. Earlier we showed in isolated rat hepatocytes the applicability of this approach to assess mitochondrial function in intact cells and at various regulatory agents' influences [1, 5].

The results of investigation of the tilorone and its analogs – diphenyl derivatives influence on the fluorescence intensity of J-aggregates in mitochondria within 30 min after the exposure with the rat hepatocytes are shown in Figure 1. Significant increase in the fluorescence intensity of J-aggregates is influenced by tilorone and compound 1 which indicates an increase in $\Delta\psi m$. The observed effect varies in the severity degree which indicates the sensitivity of this system in the differential assessment of the effects of regulatory compounds. Thus, it was shown that the most significant changes in the value of $\Delta\psi m$ occurred under the influence of tilorone (145 ± 13% compared to the control). The fluorescence intensity of J-aggregates also increased under the influence of compound 1 and amounted after 30 min incubation 131 ± 10% compared to the control. The effect of compound 2 was not expressed (113 ± 13% compared with the control cells). Subsequent incubation of the cells (up to 120 min) have not allowed to reveal a significant differences in the influence of the experimental substances on the value of $\Delta\psi m$ compared to the control. These data indicate that tilorone and its analogue – compound 1 – demonstrate the stimulating effect on the mitochondrial activity in isolated rat hepatocytes.

It is known that high membrane potential in intact mitochondria prevents the protons transport from crossing the inner membrane which is associated with the work of the respiratory chain; providing thus a low rate of mitochondria respiration. The increase in




Effect of tilorone and its analogues on the fluorescence of the J-aggregates in rat hepatocytes.
Note: * – P < 0,05 relative to the control.

the rate of respiration is due to the leakage of ions through the membrane when organelles are damaged because of hypoxia or lipid peroxidation and is accompanied by a decrease in membrane potential [4]. The discovered fact of the increase of the value of Δψm after the exposure with tilorone and compound 1 indicates that these compounds increase the membrane potential of mitochondria and may increase their resistance to the damaging agents of extra/intracellular microenvironment.

The change in mitochondrial membrane potential Δψm after the 30 min exposure of the cells with the test compounds may be considered in the context of well-known fact of the direct relation of the innate antiviral response severity on the value of mitochondrial potential Δψm which starts RLR signaling pathway (MAVS-mediated activation of IRF-3, NF-κB) which ends with production of type I IFN [10, 16, 19]. The interferon-inducing activity is known for tilorone [13, 18] and this activity is shown for its analogue – compound 1 [3, 9]. Based on the obtained results we can say that the experimental compounds at the thirtieth minute of exposure with the cells activate the classical pathway of interferon response that is required for recognition the RNA viruses in different cells.

The observed differences in the degree of the mitochondrial processes activation of the tested analogues of tilorone – compounds 1 and 2 – can be explained by the peculiarities of the cellular reception of these compounds and by the peculiarities of the initial stages of the cell activation which requires a more detailed study.

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## ВПЛИВ ТИЛОРОНУ ТА ЙОГО АНАЛОГІВ НА ЗМІНУ МІТОХОНДРІАЛЬНОГО ПОТЕНЦІАЛУ ГЕПАТОЦИТІВ ЩУРІВ

Проведено дослідження впливу тилорону дигідрохлориду та його аналогів – похідних дифенілу на зміну мембранного потенціалу мітохондрій гепатоцитів щурів. З використанням зонда JC-1 показано, що на 30-ту хвилину після внесення досліджуваних сполук до клітин спостерігається значне збільшення мітохондріального потенціалу. Отримані результати свідчать про швидку активацію за допомогою тилорону та його аналога – 4,4'-біс-[2-(діетиламіно) етокси]дифеніл дигідрохлориду – RLR сигнального шляху, завершальним етапом якого є продукція клітиною ІФН І типу. Зроблено висновок про збільшення резистентності органел до поза/внутрішньоклітинних пошкоджувальних агентів під впливом досліджуваних сполук.

Ключові слова: тилорон, похідні дифенілу, мітохондріальний потенціал, флуоресцентні зонди, гепатоцити щурів.

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## ВЛИЯНИЕ ТИЛОРОНА И ЕГО АНАЛОГОВ НА ИЗМЕНЕНИЕ МИТОХОНДРИАЛЬНОГО ПОТЕНЦИАЛА ГЕПАТОЦИТОВ КРЫС

Проведено исследование влияния тилорона дигидрохлорида и его аналогов – производных дифенила на изменение мембранного потенциала митохондрий гепатоцитов крыс. При помощи зонда JC-1 показано, что на 30-ю минуту после внесения исследуемых соединений к клеткам наблюдается значительное увеличение митохондриального потенциала. Полученные результаты свидетельствуют о быстрой активации при помощи тилорона и его аналога – 4,4'-бис-[2-(диэтиламино)этокси]дифенил дигидрохлорида – RLR сигнального пути, завершающим этапом которого является продукция клеткой ИФН I типа. Авторы пришли к выводу об увеличении резистентности органелл к вне/внутриклеточным повреждающим агентам под действием исследуемых соединений.

Ключевые слова: тилорон, производные дифенила, митохондриальный потенциал, флуоресцентные зонды, гепатоциты крыс.

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