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Basic Research

Diphenyl derivatives: cytotoxicity, antiviral and IFN-inducing activities in vitro

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Abstract

Background: the search for new antiviral drugs is the pressing task ahead of scientists all over the world. Ideally, the new drugs should be less cytotoxic and act directly on the virus or induce the production of body's own interferon. Diphenyl derivatives are analogues of tilorone (known in the Ukraine, such as «Amixine IC») with less cytotoxicity and higher interferon-inducing activity. In this study, the toxicity, antiviral and interferon-inducing activities of diphenyl derivatives have been compared for the different cell cultures. **Methods:** Cytotoxicity, IFN-inducing and antiviral activity of diphenyl derivatives were studied using cell lines L929, PST and Vero. **Results:** Diphenyl derivatives were found to be less toxic when compared with tilorone. Also, these compounds induced interferon production on L929 and PST cell lines and gave 50% antiviral protection on the Vero cell line which cannot produce interferon. **Conclusions:** The compounds tested are capable of protecting cells against viral cytopathic effect, not only through interferon production, but also through some other mechanisms of antiviral activity. Further study of these mechanisms appears to be promising. IJBM 2011; 1(3): 153-157.
Key words: *antiviral drugs, interferon induction, diphenyl derivatives, tilorone.*

Introduction

Although intense efforts in finding new antiviral drugs have been made the world over, several of these drugs still remain limited in their action against some infections. Moreover, there are no such drugs for separate infectious diseases. This is because of the peculiarities of the viruses' vital activity. Significantly, certain viruses exist, which are able to vary in their properties in their long persistent presence within the host's body (influenza virus, equine infectious anemia virus etc.). Some viruses counteract the antiviral host responses, by blocking the cytokine production and repressing the intracellular signal transduction. Also, they mimic cytokines and cytokine receptors [1, 2]. The intensive use of etiotropic antiviral direct-action drugs is seen to result in the emergence of virus-resistant strains [3-6]. Therefore, the search for new antiviral drugs with different action mechanisms and improved pharmaceutical properties is one of the most urgent needs of the day.

The basic requirements for new antiviral drugs include low cytotoxicity, ability to act directly on the virus

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and/or induce the body's own (endogenous) interferon (IFN). Of special interest are the interferon inducers, which are either natural or synthetic substances that can induce the production of endogenous interferon in the body. Using IFN-inducers reduces the basic side-effects, such as lymphopenia, flu-like symptoms (headache, fever, joints and muscle pain) produced by exogenous interferon, to zero. The IFN-inducers, contrary to recombinant IFN, do not induce the production of anti-interferon antibodies which neutralize the exogenous interferon [7, 8].

Among the low-molecular synthetic interferon inducers, tilorone dihydrochloride (known in the Ukraine, such as «Amixine IC») is the most effective. This compound exhibits various biological effects, such as interferon-inducing, immunomodulating and antitumor activities. Therefore, tilorone finds wide use in oncology, clinical immunology and for the treatment of infectious diseases of different etiologies [9-12]. However, there are some limitations on tilorone use. Introduction of the drug in large doses can trigger side-effects, such as a disruption of lipid and carbohydrate metabolism [13-15]. Tilorone, particularly, when used in large doses (152 and 189 g which correspond to 1216 and 1512 pills of Amixine IC, although the maximum recommended dose is 80-100 pills per year) in a group of patients, caused retinopathy and keratopathy which, however, gradually disappeared on withdrawal of the therapy [16, 17], without affecting visual acuity.

Therefore, the search for tilorone derivatives or analogues with lower toxicity and higher efficiency is a current research task being conducted in several laboratories across the world. In the experiments *in vivo* done earlier, the compounds – tilorone analogues – which were obtained due to substitution of fluorenone into diphenyl were found to be less toxic and induced a higher titer of interferon in the bodies of the experimental animals (18).

Considering the data mentioned above, the purpose of this study was to compare the toxicity, antiviral and interferon-inducing activities of diphenyl derivatives and tilorone for different cell cultures.

Methods

Reagents. In our experiments dihydrochloryde 2,7-bis-[2-(diethylamino) ethoxy] fluoren-9-one (tilorone) [19] 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**) [20] were used. To prepare the basic solution, the specified compounds were dissolved in distilled water to a concentration of 2.0 mg/ml.

Cell lines. The transformed cell lines used in the experiments include L929 (fibroblasts from the connective tissue of a C3H/An mouse, subline A) and Vero (African green monkey kidney, from 1962, vesicular stomatitis virus sensitive), obtained from the Bank of the Cell Lines of the R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NASU, as well as PST (primary swine testicle or EPT – embryonic pig testicles), obtained from the collection of the Institute of Veterinary Medicine, UAAS.

The cells were grown in a monolayer culture in a 38.5 cm^2 glass flask in the nutrient medium 199 with 0.68 mM L-glutamine («Bio-Test-Laboratory», Ukraine), which were supplemented with 10% fetal bovine serum (Sigma, USA), 25 mM HEPES (pH 7.4; «Serva», Germany) and 1.0 μg/ml kanamycin at 37 C with a controlled level of CO_2 (5%).

Cytotoxicity, IFN-inducing and antiviral activity were studied using cells that were in the logarithmic growth phase. The cells were detached from the flasks using 0.02% EDTA and suspended in the nutrient medium to a concentration of $5 \cdot 10^4$ cells/ml and then were seeded onto 96-well plates (Sarstedt, Germany) at 100.0 μl per well. The cells were incubated in a thermostat at 37°C with a controlled CO_2 level (5%) to form a complete monolayer. Within 24 hours, under such conditions, a monolayer was formed.

Cytotoxicity assay. The experimental compounds were then added to the monolayer of L929, PST and Vero cells with double serial dilution. Cells were incubated for 24 hours at 37°C. The plasma membrane integrity of the target cells was determined by the exclusion of 0.5% solution of Trypan Blue. The toxicity of the compound was estimated from the maximum tolerated dose (MTD), which was taken as the concentration for the compound (μg/ml) that led to the death of no more than 10% of the cells compared with the control. Cells not treated with the test compounds were used as the control group.

Induction of interferon. L929 and PST cells were cultured with tested compounds at different concentrations during 24 hours. Then samples of the cell culture medium were selected and stored up to 14 days at –20 C.

Determination of the interferon-inducing activity in samples of the cell culture medium was conducted by performing tests using the cell culture L929 against the vesicular stomatitis virus (VSV, Indiana serotype, Depositary of the D. K. Zabolotny Institute of Microbiology and Virology NASU) [21]. The interferon titer was expressed as $\log_2 (\text{dilution})^{-1}$.

Study of antiviral activity. The antiviral activity of the selected compounds was measured on cell lines L929, PST and Vero, using two models. Compounds were added on the cell monolayer 24 hours prior to (preventive model) and 30 min after (therapeutic model) the virus infection (100 $CCID_{50}$ in 50.0 μl of the nutrient medium 199). The test compounds were added to the cell culture medium with double serial dilution at concentrations not exceeding the MTD. The plates were placed into a thermostat and incubated at 37 °C until complete destruction of the cell monolayer occurred in the virus control wells. In the virus control wells cells were not treated with the test compounds and were infected with VSV. The number of living cells was recorded (to determine the degree of virus-specific cytopathic effect inhibition) by staining the unharmed cells with 0.2% Crystal Violet («Sigma», USA) in 2% ethanol (22). Cells optical density was measured on a spectrophotometer, Multiskan Ascent («Thermo Labsystems», Finland) at a wavelength of 540 nm.

To assess the antiviral activity of the test compounds effective concentrations of ED_{50} and ED_{100} were used. ED_{50} and ED_{100} values were estimated based on the concentration of the compound (μg/ml) at which the cytopathic effect of the virus was suppressed by 50% and 100%, respectively, compared with the complete destruction of the cell

monolayer that occurred in the virus control wells.

Mathematical treatment of the experimental results. Experimental data were presented as the median and interquartile range Me (LQ – UQ), where Me – median (50% percentile), LQ – 25% percentile, UQ – 75% percentile. In all the series, the number of experiments conducted was 3. The null hypothesis between the control and each experimental group was tested using the Mann-Whitney nonparametric test. The differences between the groups were statistically significant at $p<0.05$.

Results and Discussion

The integral indicator of the toxicity of the compounds gave the MTD value. The MTD values of tilorone and diphenyl derivatives are given in Table 1. The monkey kidney cells Vero showed the most sensitivity to the compounds; their MTD for compound **1** was more than 2-4 times lower, and for compound **2** it was 1.25-1.5 times lower compared with that of the cell lines PST and L929.

Table 1
Maximum tolerated dose of substances on different cell lines

Compound	MTD (μg/ml)		
	L929	***PST***	***Vero***
1	50.0	30.0	12.5
2	125.0	150.0	100.0
Tilorone	10.0	8.0	3.0

Notes: *Results represent the median of three experiments. Cells were incubated with tested compounds for 24 hours at 37°C.*

Compounds **1** and **2** were characterized by a much lower toxicity compared with tilorone ($p < 0.05$) for all the types of the cells studied. The hierarchy of MTD for tilorone and its structural analogues is: **2**>**1**>tilorone. Based on these results, in further experiments compounds at concentrations which not exceeding the MTD were studied. The interferon-inducing activity of the test compounds was examined using the L929 and PST cell lines which can produce IFN. This study showed no basal production of IFN by cells untreated with compounds. The induced interferon titers and corresponding concentrations of the test compounds are presented in Table 2. Analysis of experimental data suggests that compound **1**, such as tilorone, unlike compound **2**, is the inducer of IFN in L929 and PST cells. Compound **1**, unlike tilorone dihydrochloryde, which induces IFN production in the range of 3.1-6.2 μg/ml (higher concentrations are toxic in vitro), can instigate interferon genesis in a wide range of concentrations: 6.2-50.0 μg/ml in the L929 cell line and 12.5-25.0 μg/ml in the PST cell line.

Notably, when the interferon-inducing properties of compound **1** were compared with that of tilorone, the highest IFN production (4.0 $\log_2$ $(\text{dilution})^{-1}$) for both compounds was observed at the same concentration (6.2 μg/ml). This indicates that compound **1** does not yield tilorone dihydrochloryde in its interferon-inducing properties and its advantage is a much lower toxicity and a wider range of interferon-inducing concentrations.

Replacing the fluorenone fragment in the tilorone molecule with diphenyl (in compound **1**) has no effect on the degree of the central part of the molecule planarity or on the level of the induced interferon, but leads to a significant reduction in toxicity. «Introduction» of the methoksykarbonyl group in position 2 of the diphenyl fragment reduces the probability of the planar structure for compound **2** compared with compound **1**, which is accompanied by a loss of the ability to induce IFN in L929 and PST cell lines (Table 2). However, an increase in the IFN production under the influence of compound **2** was seen in the mice serum [18, 20].

Table 2
Production of IFN on different cell lines

Concentration, μg/ml	Titer of IFN, $\log_2$ dilution^{-1}					
	1		**2**		**Tilorone**	
	L929	**PST**	**L929**	**PST**	**L929**	**PST**
0 (Control)	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
3.1	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0
6.2	4.0	2.0	< 2.0	< 2.0	4.0	4.0
12.5	4.0	4.0	< 2.0	< 2.0	n. d.	n. d.
25.0	4.0	4.0	< 2.0	< 2.0	n. d.	n. d.
50.0	3.0	n. d.	< 2.0	< 2.0	n. d.	n. d.
100.0	n. d.	n. d.	< 2.0	< 2.0	n. d.	n. d.

Notes: *Results represent the median of three experiments.*
n. d. – not-determined because cytotoxic.

Probably, this was due to triggering the cascade of reactions in the bodies of the experimental animals in response to the injection of the compounds studied.

As shown in Table 3, compound **1** and tilorone inhibit the VSV cytopathic effect for preventive and therapeutic models in cell lines L929 and PST. Notably, the antiviral effect of tilorone was observed at a lower concentration than for compound **1,** for both types of cell lines, in both models. The antiviral activity of tilorone was found to be similar in the therapeutic and preventive models

(the ED_{50} and ED_{100} values were 3.1 and 6.2 μg/ml, respectively). The lack of correlation between the level of induced IFN by tilorone dihydrochloryde and the degree of antiviral protection of the cells suggests that its antiviral effect is realized not only through induction of IFN. These data concur with earlier publications on the polimodal antiviral action of tilorone dihydrochloryde (preparation Amixine IC) [12, 24].

For compound **1**, the ED_{50} value was equal, for both models (6.2 μg/ml). The ED_{100} value for the therapeutic model was greater than for the preventive (25.0 and 12.5 μg/ml, respectively). These results suggest that compound **1** inhibits the development of the viral cytopathic effect by several mechanisms, simultaneously: by the induction of IFN and using other intracellular pathways. Favoring the assumption of polimodal antiviral actions of tilorone and compound **1** indicates that these compounds inhibit the development of the VSV cytopathic effect in the cell line Vero which cannot produce interferon (Table 3). The existence of other antiviral defense mechanisms confirms the fact that in the presence of compound 2 which does not induce IFN in L929 and PST cells only 50% inhibition of VSV cytopathic effect (Table 3) was observed. It is possible that tilorone and diphenyl derivatives may alter the membrane properties of cells that inhibit virus adsorption [25-27]. These action mechanisms of the test compounds definitely warrant further study, which will be a subject of future work following this study.

Table 3
Antiviral activity of substances on different cell lines against 100 $CCID_{50}$ VSV

Compound	Concentration, μg/ml											
	L929				PST				Vero			
	PM		TM		PM		TM		PM		TM	
	ED_{100}	ED_{50}	ED_{100}	ED_{50}	ED_{100}	ED_{50}	ED_{100}	ED_{50}	ED_{100}	ED_{50}	ED_{100}	ED_{50}
1	12.5	6.2	25.0	6.2	12.5	6.2	25.0	6.2	–	12.5	–	12.5
2	– [a]	100.0	–	100.0	–	100.0	–	100.0	–	100.0	–	100.0
Tilorone	6.2	3.1	6.2	3.1	6.2	3.1	6.2	3.1	–	3.1	–	3.1

Notes: *PM – protective effect in preventive model.*
TM – protective effect in therapeutic model.
[a] *– protective effect was not observed.*

Thus, the structural analogues of tilorone – diphenyls – have been shown to have a high degree of biological activity. The results of these studies suggest that toxicity, IFN-inducing and antiviral properties of diphenyls depend on their chemical structure. Antiviral activity of these compounds is mediated by IFN induction and by other intracellular routes that warrant further investigation. Diphenyl derivatives are less toxic than tilorone and may be hailed as the new inducers of IFN and as compounds which show great promise in producing new antiviral drugs.

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