International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 12, Issue 4, 2020

Original Article

PHYSICOCHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY OF THE NEW ANTIVIRAL SUBSTANCE

A. I. ODNOVOROV[1*], T. V. GREBENNIKOVA[1,2], T. V. PLETENEVA[1], T. M. GARAEV[2], E. V. USPENSKAYA[1], N. A. KHODOROVICH[1], O. V. LEVITSKAYA[1], A. M. KOLDINA[1]

[1]Department of Pharmaceutical and Toxicological Chemistry, Peoples Friendship University of Russia (RUDN University), 6, Miklukho-Maklaya Street, Moscow, 117198, Russian Federation, [2]N. F. Gamaleya Federal Research Centre of Epidemiology and Microbiology, 18, Gamalei Street, Moscow, 123098, Russian
Email: mister.odn2010@yandex.ru

Received: 02 May 2020, Revised and Accepted: 08 Jun 2020

ABSTRACT

Objective: To develop a set of quality control procedures for the promising antiviral pharmaceutical substance L-histidyl-1-adamantylethylamine dihydrochloride monohydrate, a derivative of rimantadine.

Methods: Substances and solvents: synthesized in laboratory L-histidyl-1-adamantylethylamine dihydrochloride monohydrate (H-His-Rim•2HCl•H_2O), rimantadine hydrochloride (Rim•HCl), 99%, ethanol 96%, N, N-dimethylformamide (DMF) anhydrous, 99.8% and n-hexane anhydrous, 95%, deionized high-resistance water (18.2 MΩ•cm at 25 °C, Milli-Q system), silver nitrate. Infrared (IR) Spectroscopy–Cary 630 Fourier Transform IR Spectrometer, elemental analysis–elemental composition analyzer CHNS-O EuroEA3000, ultraviolet (UV) spectrometry–Cary-60 spectrophotometer, polarimetry–POL-1/2 polarimeter with an external Peltier module, granulometric analysis by optical microscopy (Altami BIO 2 microscope) and low-angle laser light scattering (LALLS)–Master Sizer 3600, measurement of potential for hydrogen–potentiometer PB-11, Spirotox method–the study of temperature dependences of *Spirostomum ambiguum* lifetime to characterize the biological activity of the studied compounds.

Results: The substance H-His-Rim•2HCl•H_2O is an amorphous yellowish powder, slightly soluble in water, soluble in ethanol, freely soluble in N, N-dimethylformamide, and practically insoluble in n-hexane. A study of the elemental composition has confirmed the authenticity of H-His-Rim•2HCl•H_2O. Comparison of the spectral characteristics of H-His-Rim•2HCl•H_2O and Rim•HCl by IR spectroscopy and UV spectrometry confirmed the authenticity of the substance. The racemic form of the substance Rim•HCl with an insignificant amount of impurity of the levorotatory enantiomer was proved polarimetrically: α =-0.0126±0.0003 (1% aqueous solution, 20±0.5 °C). The specific optical rotation of 1% aqueous solution H-His-Rim•2HCl•H_2O $[\alpha]^{20} = +7.22 \pm 0.06$. In 1% ethanol solution $[\alpha]^{20}$ =-10.32±0.12. Using the method of laser light diffraction for a substance H-His-Rim•2HCl•H_2O, the dimensional spectra «fraction of particles, %-d, μm» were characterized, the maximum of which in hexane is in the region of 40–50 μm. Arrhenius's kinetics on the Spirotox model established statistically significant differences in ligand-receptor interactions, which are characterized by values of observed apparent activation energy $^{obs}E_a$, kJ/mol: 132.36±1.55 for H-His-Rim•2HCl•H_2O and 176.15±0.48 for Rim•HCl.

Conclusion: The developed set of methods for assessment of physical and chemical properties and biological activity of a new antiviral substance H-His-Rim•2HCl•H_2O is the basis for establish of regulatory documentation.

Keywords: Antiviral pharmaceutical substance, Rimantadine, L-histidyl-1-adamantylethylamine, Quality control

DOI: http://dx.doi.org/10.22159/ijap.2020v12i4.38136. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

The prevention and treatment of seasonal influenza and possible pandemic infections require the development of new antiviral drugs that are effective against a wide range of influenza strains [1].

The first effective anti-influenza drugs were the amino derivatives of adamantane-rimantadine and amantadine [2, 3]. These compounds have been used for the treatment and prevention of influenza A since the 1980s. By their mechanism of action, these drugs belong to direct inhibitors of the reproduction of the influenza A virus, which disrupt the proton-conducting function of the M2 channel of the virus [4]. The economic and synthetic availability of adamantane derivatives has contributed to their widespread use in controlling seasonal influenza epidemics worldwide. However, this led to the occurrence of mutations in the M2-protein gene of the influenza virus and the loss of antiviral activity of amantadine and rimantadine [5]. According to the US Centers for Disease Control and Prevention, nearly 100% of influenza viruses are currently resistance to amantadine and rimantadine [6]. In this regard, there is an active search for new M2-channel inhibitors of the influenza virus, mainly adamantane derivatives [7-10].

As a result of the search for anti-influenza drugs of the adamantane series [11], L-histidyl-1-adamantylethylamine dihydrochloride monohydrate (H-His-Rim•2HCl•H_2O) was synthesized (fig. 1).

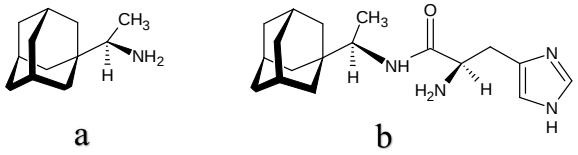

Fig. 1: Structural formula of L-histidyl-1-adamantylethylamine (b), a derivative of rimantadine (a)

Preclinical *in vitro* and *in vivo* studies demonstrated the pronounced activity of the new substance against rimantadine and amantadine resistant strains of influenza A virus in comparison with other analogs [12, 13], which served as the basis for further clinical trials of H-His-Rim•2HCl•H_2O. The identification of the new substance and the determination of its properties are crucial for further testing. Some characteristics of the compound (NMR spectrum, mass spectrum) were presented previously [14].

This article is devoted to assessing the properties of a new promising antiviral substance H-His-Rim•2HCl•H_2O in comparison with the known parent substance (Rim•HCl) using a complex of physicochemical and biological methods of analysis. The results can be used in the development of regulatory documentation for a promising pharmaceutical substance.

MATERIALS AND METHODS

Materials

The substance H-His-Rim•2HCl•H_2O, synthesized in laboratory conditions by the classical peptide synthesis method [14], rimantadine hydrochloride, 99% (Zhejiang Kangyu Pharmaceutical Co, China), ethanol 96% (purity category "Lux"); N, N-dimethylformamide anhydrous, 99.8% and n-hexane anhydrous, 95% (Sigma-Aldrich, USA); deionized high resistance water (18.2 MΩ•cm at 25 °C, Milli-Q system); silver nitrate (chemically pure, Himmed, Russia).

Methods

The solubility of substances was determined in accordance with the general monograph "Solubility" of the Russia State Pharmacopoeia XIV edition [15] in four solvents: deionized high-resistance water, ethanol 96%, n-hexane, DMF.

The analysis of substances in the mid-infrared region was carried out using a Cary 630 Fourier transform IR spectrometer (Agilent Technologies, USA) with an attenuated total reflection (ATR) attachment.

To analyze the elemental composition of H-His-Rim•2HCl•H_2O and Rim•HCl used the analyzer CHNS-O EuroEA3000 (Eurovector, S. p. A., Italy) [16].

The optical density of substance solutions in water, ethanol and DMF in the UV region was measured on a Cary-60 spectrophotometer (Agilent Technologies, USA).

The optical activity of the compounds was determined on a POL-1/2 polarimeter with an external Peltier module for temperature control (Atago Co., LTD, Japan). The solutions in water and ethanol (1%) were analyzed at 25 °C. The length of the polarimeter tube is 1 dm.

The size and shape of the substance particles were determined by optical microscopy method (microscope Altami BIO 2, Russia) with a 10-fold increase. The sample was applied to the slide and distributed evenly over the entire surface. The particles were observed in 10 separate fields of vision.

Granulometric analysis of the samples was carried out by the method of low-angle laser light scattering [17] with the Mastersizer 3600 (Malvern Panalytical, United Kingdom).

The potential for hydrogen of aqueous solutions of compounds was measured by the potentiometric method (Basic Meter PB-11, Sartorius, Germany) [15]. Buffer solutions with pH 4.0, 7.0 and 10.0 prepared from standard titer were used for calibration.

Spirostomum ambiguum test culture was used to study the biological activity of substances by the Spirotox method [18]. Equipment: 5-well thermostatic plate, Alpha A6 thermostat (Lauda, Germany), MBS-10 binocular, daylight lamp (~ 10 W).

Statistical processing and graphical representation of the results were carried out using Origin Pro 9.1 software (OriginLab Corporation, USA). Statistical processing consisted in calculating the mean±SD.

RESULTS AND DISCUSSION

For substances synthesized in laboratory conditions, first of all, it is necessary to establish the chemical structure and prove its identity to the target compound. Earlier, for this purpose, we used NMR spectroscopy and mass spectrometry, which confirmed the structure of the synthesized compound H-His-Rim•2HCl•H_2O [13]. The presence of 2 moles of hydrochloric acid per 1 mole of the salt form was confirmed by the reaction to chloride ions with a solution of silver nitrate [15].

In addition, C, H, N ratios were determined (Dumas-Pregle method) for the parent substance Rim•HCl and the target rimantadine derivative H-His-Rim•2HCl•H_2O by the elemental analysis method (table 1).

The results confirm the authenticity of both substances and indicate an acceptable purity of the substances. In the case of H-His-Rim•2HCl•H_2O, a slight difference in the nitrogen and hydrogen contents from the calculated one may be due to the presence of concomitant impurities.

Solubility

The solubility of a pharmaceutical substance is an important characteristic that determines the purity, rate of release of the active pharmaceutical ingredient (API) from the finished dosage form, its absorption into the bloodstream, and the achievement of a therapeutic effect. The solubility of both substances was evaluated in different solvents (table 2), which made it possible to compare their lipophilicity.

Table 1: The results of elemental analysis of the substances H-His-Rim•2HCl•H_2O and Rim•HCl

Element	H-His-Rim•2HCl•H_2O		Rim•HCl	
	Calculated value, %	Obtained value, %	Calculated value, %	Obtained value, %
C	53.10	53.58±0.31	66.81	66.87±0.07
H	7.93	8.30±0.23	10.28	10.48±0.20
N	13.75	12.86±0.16	6.49	6.58±0.19

mean±SD, n= 3, P=0.90

Table 2: Solubility of H-His-Rim•2HCl•H_2O and Rim•HCl in various solvents

Solvent	The volume of solvent (ml) to dissolve 1 g of the substance	
	Rim•HCl	H-His-Rim•2HCl•H_2O
H_2O	20	200
C_2H_5OH	20	20
DMF	10	10
n-hexane	>1000	>1000

Thus, using conventional solubility terms, H-His-Rim•2HCl•H_2O can be characterized as a compound that is slightly soluble in water (1:200), soluble in ethanol 96% (1:20), freely soluble in DMF (1:10) and practically insoluble in n-hexane (>1:1000). The observed differences in water solubility compared to Rim•HCl indicate higher lipophilicity of the new substance due to the introduction of an additional fragment into the molecule (fig. 1).

Infrared spectroscopy

To determine the authenticity of substances, the attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) method was also used (fig. 2).

The analysis of the IR spectra of the two studied substances showed the presence of absorption bands corresponding to both the general structural fragments and the functional groups introduced into the H-His-Rim•2HCl•H_2O (table 3), e. g. the amide group (-NH-CO-). For both substances, there are several pronounced absorption bands in the region 2900–2800 cm^{-1}, which correspond to valence vibrations of the C-H bond in the-CH_3 and-CH_2 groups of the adamantane cycle and the linear carbon chain. Both substances also contain a primary aliphatic amino group in their structure. The presence of absorption bands in the spectrum of H-His-Rim•2HCl•H_2O in the region of 3300-3200 cm^{-1} indicates the presence of the-NH_2 free group. Its partial protonation is explained by the fact that there are three additional

nitrogen atoms in the molecule with free electron pairs. This leads to the appearance of absorption bands in the region of 2000-2500 cm^{-1}. In the case of Rim•HCl, there are no absorption bands typical for a free amino group, and the presence of absorption bands at 3036, 2507, 2030 cm^{-1} indicates the presence of a protonated amino group ($-NH_3^+$), which confirms the presence of Rim•HCl in the salt form.

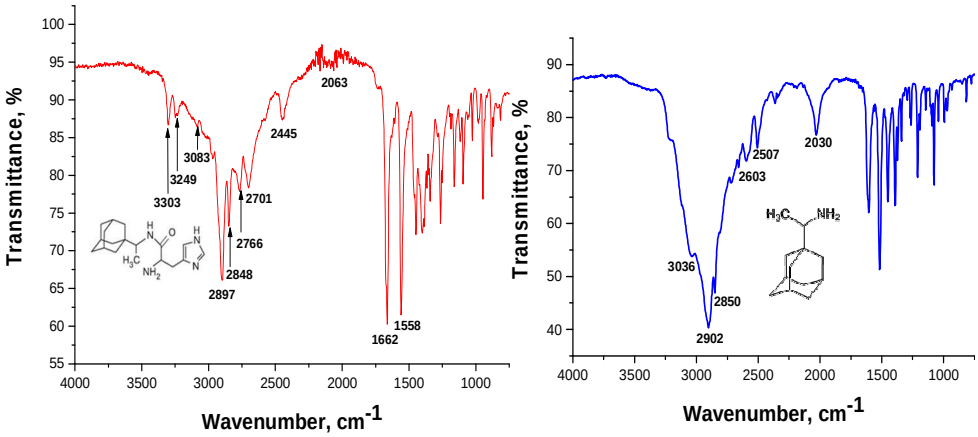

Fig. 2: ATR-FTIR spectra of H-His-Rim•2HCl•H_2O and Rim•HCl

Table 3: The main absorption bands in the ATR-FTIR spectrum of H-His-Rim•2HCl•H_2O (ν–valence vibration, δ–deformation vibration)

Wavenumber, cm^{-1}	Type of vibrations and functional group
3303, 3249	ν_{N-H} (-NH_2)
3083	ν_{N-H} (-NH-CO-)
2897	ν_{C-H} (-CH_3)
2848	ν_{C-H} (-CH_2)
2766, 2701	ν_{N-H} ($-NH_2^+$)
2445, 2063	δ_{N-H} ($-NH_3^+$)
1662	$\nu_{C=O}$
1558	δ_{N-H} (-NH-CO-)

Thus, the infrared spectroscopy method allowed to confirm the structural features of the compounds.

Ultraviolet-visible spectroscopy

Electronic spectra of substances solutions in 96% ethanol (fig. 3) were used for their identification.

In 0.5% Rim•HCl alcohol solution, there are two absorption bands with maximums at 230 nm and 256 nm wavelength and minimum at 246 nm. One absorption band with a maximum at 216 nm is observed on the spectrum of 0.01% alcohol solution of H-His-Rim•2HCl•H_2O.

The UV spectra of both substances in DMF are characterized by identical absorption bands with a maximum of about 270 nm, although they differ in their molar attenuation coefficients (fig. 4).

Using the formula of the Beer-Lambert–Bouguer law, the molar attenuation coefficient (ε) of solutions in DMF was determined. ε (Rim•HCl) = 18.12 $L•mol^{-1}•cm^{-1}$, ε (H-His-Rim•2HCl•H_2O) = 4073.70 $L•mol^{-1}•cm^{-1}$. An increase in the molar attenuation coefficient of H-His-Rim•2HCl•H_2O is associated with the appearance of new chromophore groups in the molecule.

Polarimetry

The presence of chiral carbon atoms in the test compounds indicates their optical activity. One asymmetric carbon atom is present in the Rim•HCl molecule, 2 chiral centers are characteristic of the H-His-Rim•2HCl•H_2O molecule. The location of chiral carbon atoms in the molecules of the studied substances is shown in fig. 5.

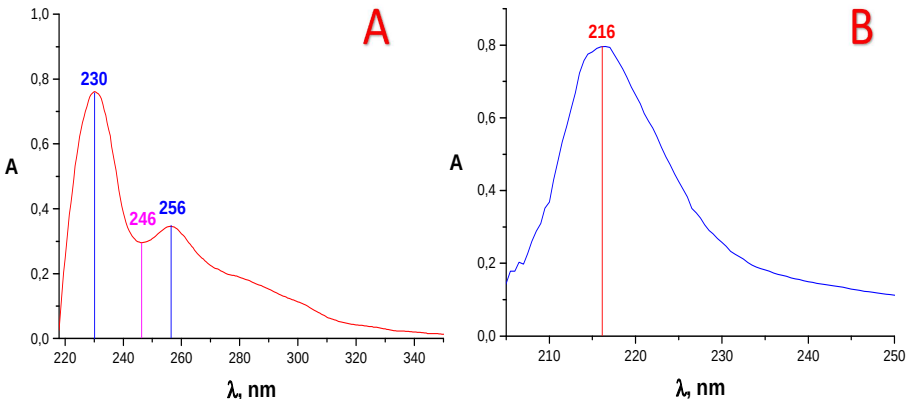

Fig. 3: UV spectra of alcohol solutions 0.5% Rim•HCl (A) and 0.01% H-His-Rim•2HCl•H_2O (B)

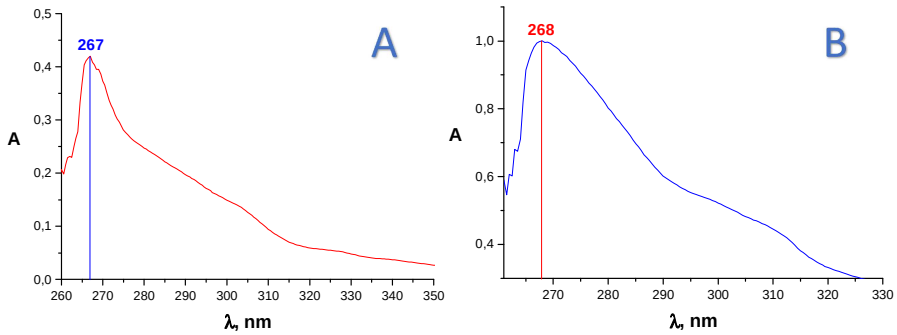

Fig. 4: UV spectra of solutions of 0.5% (0.023 mol/l) Rim•HCl (A) and 0.01% (0.245 mmol/l) H-His-Rim•2HCl•H₂O (B) in DMF

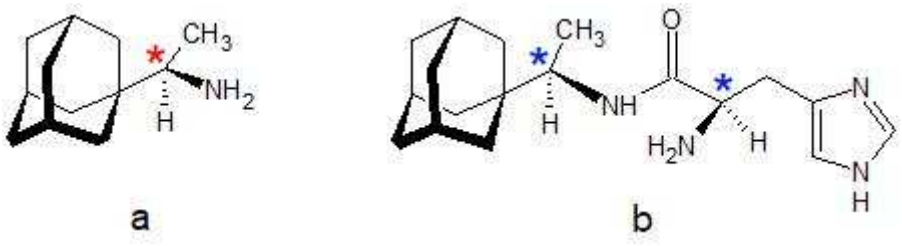

Fig. 5: Location of chiral centers in H-His-Rim•2HCl•H₂O (b) and Rim•HCl (a) molecules, the measured optical rotation (α) and the calculated values of the specific optical rotation ($[\alpha]_D^{20}$) for 1% solutions of substances are shown in table 4.

Table 4: Optical activity of 1% aqueous solutions of Rim•HCl and H-His-Rim•2HCl•H₂O

Solvent substance	H_2O		C_2H_5OH 96%	
	α	$[\alpha]_D^{20}$	α	$[\alpha]_D^{20}$
H-His-Rim•2HCl•H₂O	+0.0722±0.0006	+7.22±0.06	-0.1032±0,0012	-10.32±0.12
Rim•HCl	-0.0126±0.0003	-1.26±0.03	-0.0133±0.0007	-1.33±0.07

mean±SD, n= 5, P=0.90

Since the substance of rimantadine is a racemate, the optical rotation was close to zero in both solvents. The optical rotation of H-His-Rim•2HCl•H₂O depends on the solvent nature, both in absolute value and in sign.

Optical microscopy

The shape and size of the particles, as well as the state of their surface, have a significant impact on the development and production of drugs of proper quality, including the release of APIs from the dosage form [19]. Granulometric analysis of the substance (fig. 6) showed that particles of 20–40 μm (47%) and 40–60 μm (27%) prevailed in it.

The particles are irregularly shaped, roughened. The presence of many small particles indicates a low strength of large conglomerates. The predominance of small particles facilitates the release of API from the dosage form and increases bioavailability.

Low-angle laser light scattering

To determine the particle size of the substance, the laser obscuration parameter was used, which characterizes the loss of light intensity as a result of reflection, absorption, and diffraction processes.

The particle size distribution of the substance H-His-Rim•2HCl•H₂O was carried out in n-hexane to exclude the process of sample dissolution. The substance is represented by a wide distribution band in the range from 10 to 120 μm with a maximum of the size group d = 50 μm and a shoulder at d = 70 μm (fig. 7).

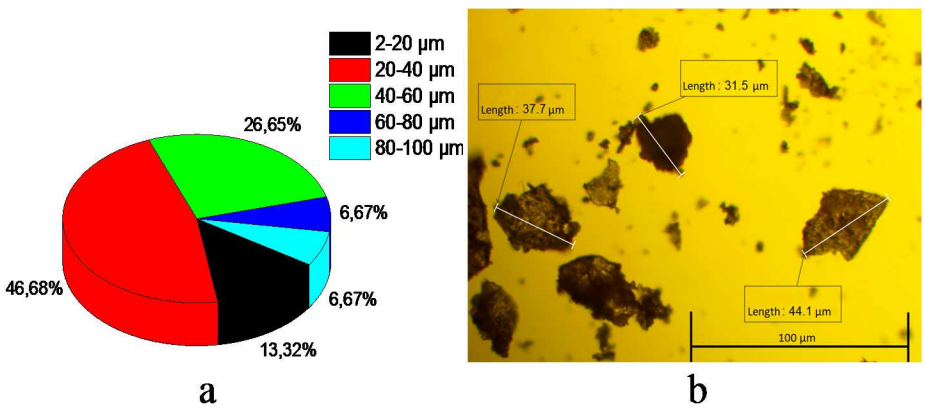

Fig. 6: Granulometric composition (a) and visualization of H-His-Rim•2HCl•H₂O (b) particle size and shape (b) by optical microscopy

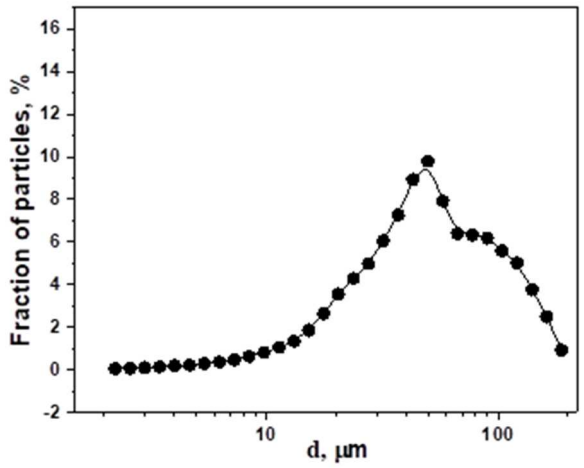

Fig. 7: Determination of H-His-Rim•2HCl•H_2O powder particle distribution by laser diffraction analysis in n-hexane

The size spectrum obtained by the LALLS method correlates with the results of optical microscopy (fig. 6).

Potentiometry

The pH was measured at 25 °C for 1% aqueous solutions of H-His-Rim•2HCl•H_2O and Rim•HCl in triplicate. pH (Rim•HCl) = 4.40±0.11, pH (H-His-Rim•2HCl•H_2O) = 3.15±0.09. The results obtained confirm that the compounds are the salt form of the strong acid HCl, while H-His-Rim•2HCl•H_2O is bound to 2 HCl molecules, which confirms a lower pH value.

Spirotox method

Protozoa, in particular, *Spirostomum ambiguum*, are widely used as test objects for toxicological and pharmacological studies of API [20, 21]. The death rate of a unicellular organism depends on the temperature of the environment, nature, and the concentration of the API.

To estimate the biological activity of H-His-Rim•2HCl•H_2O and Rim•HCl compounds, the temperature dependence of *S. ambiguum* death in the range of 299-305 K in increments of 2 K was analyzed (fig. 8, 9).

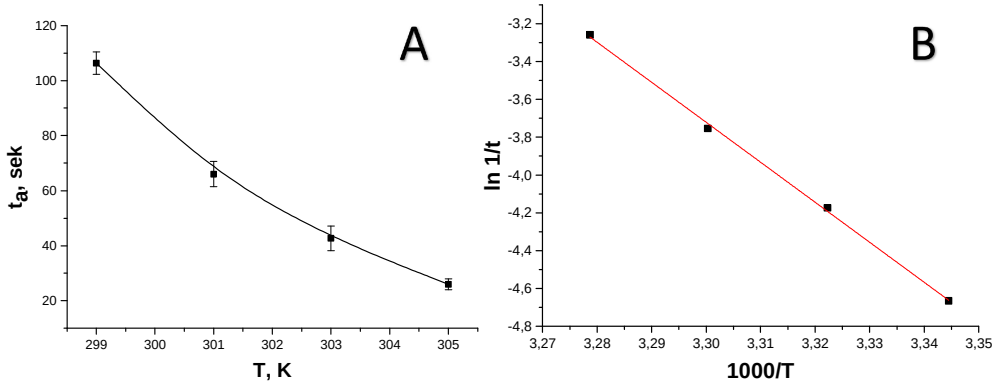

Fig. 8: Dependence of life span of *S. ambiguum* on temperature in 0.025% (1.16 mmol/l) aqueous solution of Rim•HCl in straight (A) and Arrhenius (B) coordinates (mean±SD, n=5, P=0.90)

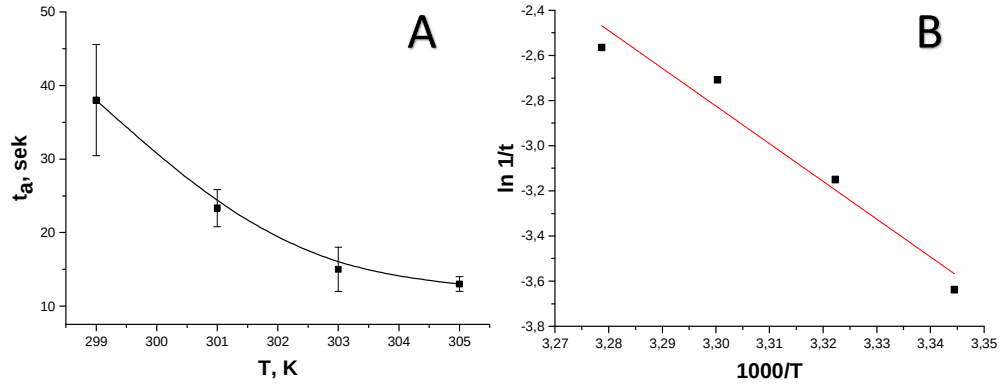

Fig. 9: Dependence of life span of *S. ambiguum* on temperature in 0.025% (0.61 mmol/l) aqueous solution of H-His-Rim•2HCl•H_2O in straight (A) and Arrhenius (B) coordinates (mean±SD, n=5, P=0.90)

The observed apparent activation energy ($^{obs}E_a$) values for the test compounds were found using Arrhenius coordinates (table 5).

Table 5: The calculated $^{obs}E_a$ values of ligand-induced *S. ambiguum* death process in aqueous solutions of H-His-Rim•2HCl•H_2O and Rim•HCl

API	$^{obs}E_a$, kJ/mol
H-His-Rim•2HCl•H_2O	132.36±1.55
Rim•HCl	176.15±0.48

mean±SD, n=5, P=0.90

The value of $^{obs}E_a$ reflects the ligand-receptor interaction accompanied by cell death. The lower activation energy, as well as the reduction of *S. ambiguum* lifetime by about 2 times at the same temperature for H-His-Rim•2HCl•H_2O compared to Rim•HCl, indicates a higher biological activity of this compound.

CONCLUSION

The compound H-His-Rim•2HCl•H_2O–is a promising anti-influenza drug for which the antiviral activity *in vitro* and *in vivo* has previously been confirmed in laboratory animals. For subsequent clinical trials, it is important to have regulatory documentation that allows quality control of the new promising substance. The results obtained in this study are the basis for the development of a pharmacopeia monograph for a new antiviral substance.

ACKNOWLEDGMENT

The publication has been prepared with the support of the "RUDN University Program 5-100".

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

REFERENCES

1. Shie JJ, Fang JM. Development of effective anti-influenza drugs: congeners and conjugates-a review. J Biomed Sci 2019;26:84.
2. Haque SKM, Elzagheid M. Antiviral drugs for influenza viruses. Int J Pharm Pharm Sci 2019;11:1-10.
3. Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH. The molecular basis of the specific anti-influenza action of amantadine. EMBO J 1985;4:3021-4.
4. Pielak RM, Chou JJ. Flu channel drug resistance: a tale of two sites. Protein Cell 2010;1:246-58.
5. Bright RA, Medina MJ, Xu X, Perez Oronoz G, Wallis TR, Davis XM. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 2005;366:1175–81.
6. Fiore AE, Fry A, Shay D, Gubareva L, Bresee JS, Uyeki TM. Antiviral agents for the treatment and chemoprophylaxis of influenza: recommendations of the advisory committee on immunization practices (ACIP). MMWR Recomm Rep 2011;60:1–24.
7. Fytas C, Kolocouris A, Fytas G, Zoidis G, Valmas C, Basler CF. Influence of an additional amino group on the potency of aminoadamantanes against influenza virus A. II-Synthesis of spiropiperazines and *in vitro* activity against influenza a H3N2 virus. Bioorg Chem 2010;38:247-51.
8. Li F, Ma C, Hu Y, Wang Y, Wang J. Discovery of potent antivirals against amantadine-resistant influenza viruses by targeting the M2-S31N proton channel. ACS Infect Dis 2016;2:726-33.
9. Kuznetsov NY, Tikhov RM, Godovikov IA, Medvedev MG, Lyssenko KA, Burtseva EI, *et al.* Stereoselective synthesis of novel adamantane derivatives with high potency against rimantadine-resistant influenza a virus strains. Org Biomol Chem 2017;15:3152-7.
10. Hu Y, Musharrafieh R, Ma C, Zhang J, Smee DF, DeGrado WF, *et al.* An M2-V27A channel blocker demonstrates potent *in vitro* and *in vivo* antiviral activities against amantadine-sensitive and-resistant influenza viruses. Antiviral Res 2017;140:45-54.
11. Shibnev VA, Deryabin PG, Garaev TM, Finogenova MP, Botikov AG, Mishin DV. Peptide carbocyclic derivatives as inhibitors of the viroporin function of RNA-containing viruses. Russ J Bioorg Chem 2017;43:517-25.
12. Shibnev VA, Garaev TM, Finogenova MP, Shevchenko ES, Burtseva EI. New adamantane derivatives can overcome resistance of influenza A(H1N1)pdm2009 and A(H3N2) viruses to «remantadine». B Exp Biol Med 2012;153:200–2.
13. Shhelkanov MJu, Shibnev VA, Finogenova MP, Fedjakina IT, Garaev TM, Markova NV, *et al.* The antiviral activity of the adamantane derivatives against the influenza virus a (H1N1) pdm09 model *in vivo*. Voprosy Virusologii 2014;59:37–40.
14. Deryabin PG, Garaev TM, Finogenova MP, Odnovorov AI. Assessment of the antiviral activity of 2HCl•H-His-Rim compound compared to the anti-influenza drug arbidol for influenza caused by A/duck/Novosibirsk/56/05 (H5N1) (Influenza a virus, alphainfluenzavirus, orthomyxoviridae). Voprosy Virusologii 2019;64:268-73.
15. The State Pharmacopoeia of the Russian Federation 14 (XIV) Edition; 2018.
16. Zauer EA. Modern automatic CHNS/O/X organic compound analyzers. Anal Control 2018;22:6-19.
17. Uspenskaya EV, Anfimova EV, Syroeshkin AV, Pleteneva TV. Kinetics of pharmaceutical substance solubility in water with different hydrogen isotopes content. Indian J Pharm Sci 2018;80:318-24.
18. Goncharuk VV, Syroeshkin AV, Zlatskiy IA, Uspenskaya EV, Orekhova AV, Levitskaya OV, *et al.* Quasichemical description of the cell death kinetics of cellular biosensor spirostomum ambigua for testing the biological activity of aqueous solutions. J Water Chem Technol 2017;39:97-102.
19. Emshanova SV, Abramovich RA, Potanina OG. Influence of particle shape and size of substances on the quality of finished medicines. Drug Dev Registration 2014;2:50-63.
20. Nałecz Jawecki G. Spirotox spirostomum ambiguum acute toxicity test-10 y of experience. Environ Toxicol 2004;19:359-64.
21. Syroeshkin AV, Uspenskaya EV, Pleteneva TV, Morozova MA, Maksimova TV, Koldina AM, *et al.* Mechanochemical activation of pharmaceutical substances as a factor for modification of their physical, chemical and biological properties. Int J Appl Pharm 2019;11:118-23.