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Formation of antibiotic cycloserine complexes with stearic acid and its calcium and magnesium salts: from quantum mechanical modeling to studies of membranotropic action

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Antibiotic cycloserine (CyS) is a highly efficient and widely used anti-tuberculosis drug, which is canonically formulated with such excipients as calcium stearate (CaSt) or magnesium stearate (MgSt). This makes interactions of CyS with CaSt, MgSt and structurally related stearic acid (StA), with eventual formation of intermolecular complexes in biological media, an important physico-chemical factor affecting pharmacological action of such drugs. A set of theoretical and experimental methods was applied to trace the complexes formation in vacuum and solvent media, as well as in model membranes of L-α-dipalmitoylphosphatidylcholine (DPPC). By means of quantum chemistry methods, a possibility of CyS complex formation with all the excipients has been shown. CyS–CaSt and CyS–MgSt complexes appeared to be much more stable than CyS–StA, both in vacuum and in water media due to involvement of the metal cations in the intermolecular interactions. The non-covalent complexes CyS–StA in polar solvent was experimentally observed by means of electrospray ionization mass spectrometry, which confirms the quantum chemical results on stability of CyS–StA complexes in different media. Meanwhile no evidence of CyS–StA clusters was observed in DPPC membranes. More stable complexes CyS–CaSt and CyS–MgSt were detectable in DPPC membranes *via* non-linearity of interlamellar repeat distance obtained in small-angle X-ray scattering (SAXS) experiments. A set of hydration parameters of the excipients elucidates

important discrimination between CaSt and MgSt. A possible implication of our findings might be related to competing interactions of CyS with the excipients and with its molecular targets in organism, which could influence both its cytotoxic and neurotoxic effects.

Keywords: cycloserine, magnesium stearate, non-covalent complexes, quantum chemistry modeling, model lipid membranes, SAXS.

Антибиотик циклосерин (CyS) — высокоэффективный противотуберкулёзный препарат, в состав которого в качестве вспомогательных веществ обычно входят стеараты кальция (CaSt) или магния (MgSt). Взаимодействия CyS с CaSt и MgSt, а также со сходной с ними по структуре стеариновой кислотой (StA), возможно, приводящие к образованию межмолекулярных комплексов в биологических средах, представляются важным физико-химическим фактором влияния на фармакологическую активность CyS. Комплексообразование CyS со вспомогательными веществами исследовано с помощью набора теоретических и экспериментальных методов в вакууме, водной среде, органическом растворителе, а также в модельной мембране L-α-дипальмитоилфосфатидилхолина (ДПФХ). Квантово-химическими методами показана возможность образования комплексов CyS со всеми рассматриваемыми вспомогательными веществами. При этом комплексы CyS–CaSt и CyS–MgSt оказались намного более стабильными, чем комплексы CyS–StA, в вакууме и водном окружении, очевидно, за счёт вовлечения в межмолекулярное взаимодействие катионов металлов. Образование стабильных комплексов CyS–StA в метаноле экспериментально показано методом масс-спектрометрии с ионизацией электрораспылением (ИЭР), тогда как в мембране ДПФХ доказательств их образования обнаружено не было. Вместе с тем, более стабильные комплексы CyS–CaSt и CyS–MgSt детектированы в мембране ДПФХ методом малоуглового рентгеновского рассеяния как нелинейность периода повторяемости бислоев по концентрации CyS в системе. Выявлены существенные различия в ряде параметров гидратации CyS–CaSt и CyS–MgSt, которые могут иметь значение для их комплексообразования. Полученные результаты указывают на возможность существования конкурентных взаимодействий CyS со вспомогательными веществами и со своими молекулярными мишенями в организме, что может иметь значение как для цитотоксического, так и для нейротоксического эффектов CyS.

Утворення комплексів антибіотика циклосерину зі стеариновою кислотою та стеаратами кальцію і магнію: від квантово-механічного моделювання до вивчення мембранотропної активності. *О.В.Ващенко, С.В.Шишкіна, Н.О.Касян, Л.В.Будянська, Л.А.Булавін, Д.В.Соловйов, В.А.Пашинська, А.Гоморі, Л.М.Лисецький*.

Антибіотик циклосерин (CyS) є високоефективним протитуберкульозним препаратом, до складу якого у якості допоміжних речовин зазвичай входять стеарати кальцію (CaSt) чи магнію (MgSt). Взаємодія CyS з CaSt та MgSt, а також зі структурною, близькою до них, стеариновою кислотою (StA), можливо, з утворенням міжмолекулярних комплексів у біологічних середовищах, уявляється важливим фізико-хімічним фактором впливу на фармакологічну активність CyS. В роботі комплексоутворення CyS з допоміжними речовинами досліджено за допомогою набору теоретичних та експериментальних методів у вакуумному та водному оточеннях, органічному розчиннику, а також у модельній мембрані L-α-дипальмітоїлфосфатидилхоліну (ДПФХ). Квантово-хімічними методами показано можливість утворення комплексів CyS з усіма розглянутими допоміжними речовинами. При цьому комплекси CyS–CaSt та CyS–MgSt виявилися набагато стабільнішими за комплекси CyS–StA як у вакуумному, так і у водному оточеннях, певно, за рахунок участі у міжмолекулярних взаємодіях катіонів металів. Утворення стабільних комплексів CyS–StA у метанолі експериментально показано методом мас-спектрометрії з іонізацією електророзпиленням, тоді як у мембрані ДПФХ доказів їх існування не виявлено. Разом із тим, більш стабільні комплекси CyS–CaSt та CyS–MgSt детектовано у мембрані ДПФХ методом малокутового рентгенівського розсіювання (МКРР) як нелінійність періоду повторюваності бішарів за концентрації CyS у системі. Встановлено суттєві відмінності у низці параметрів гідратації CyS–CaSt и CyS–MgSt, які можуть мати значення для комплексоутворення. Отримані результати вказують на можливість існування конкурентних взаємодій CyS з допоміжними речовинами та зі своїми молекулярними мішенями в організмі, що може мати значення як для цитотоксичного, так і для нейротоксичного ефектів CyS.

1. Introduction

In recent decades special emphasis is made on drugs interactions with artificial lipid membranes because pharmacological action of drugs is believed to be of a large extent depending upon drug-phospholipid interactions [1–4]. In particular, antibiotic interactions with cell membrane play a crucial role in understanding the bioavailability of drugs, their entry into the cellular compartments and drug induced toxicity [1, 2, 5, 6].

In this respect anti-tuberculosis drugs attire focused attention [7–10] due to their poor drug bioavailability and dose-related adverse effects [1, 3]. Antibiotic cycloserine (CyS) is a high-effective and widely used anti-tuberculosis drug with heavy neurotoxic adverse effects [11–14]. In pharmaceutical products, CyS is canonically formulated with calcium stearate (CaSt) or magnesium stearate (MgSt). Generally, it is postulated that excipients have to be chemically inert with regard to an active pharmaceutical ingredient; nevertheless, physical interactions (such as sorption, non-covalent complex formation, etc.) are possible anyway, particularly, with MgSt [15–18]. Therewith, a number of works demonstrate that complexes formation by drug molecules can affect their action at various levels, including their interactions with lipid membranes [19–21].

Formation of intermolecular complexes CyS–CaSt and CyS–MgSt was assumed in [22], where these substances were studied in model membranes of hydrated L-α-dipalmitoylphosphatidylcholine (DPPC). In these systems, excessive increase of the temperature of "gel to liquid crystal" phase transition of DPPC membrane (T_m) was established, i.e. synergism of membranotropic effects of CyS and the stearates, which was more pronounced for MgSt. However, such effect was not observed for another excipient, stearic acid (StA), which is a structural part of stearate molecules.

A natural question arises on the relation between chemical structure of the excipient and the effects observed. In accordance to their amphiphilic chemical structure, CaSt, MgSt and StA interact both with hydrophilic surface and hydrophobic interior of lipid membrane. Among the main membranotropic effects of amphiphilic substances there are changes in bilayer organization (effect on membrane permeability and functioning), formation of new lipid phases and disruption (solubilization) of membrane [23]. It was established that, in the order of their effect on T_m increasing, the excipients are ranged as CaSt < MgSt < StA [22].

Discrimination between the effects of CaSt and MgSt is obviously specified by the nature of the metal cation in their structure, which presets a number of its properties. In particular, Ca^{2+} ions were shown to have certain advantages as compared to Mg^{2+} due to their higher binding ability to phosphate and carboxyl lipid groups [24, 25] (though being parts of organic compounds the ions could change their specificity). Besides, specificity of Ca^{2+} and Mg^{2+} to lipid membrane is related in literature to membrane hydration [24]. Taking into account the location of amphiphilic substances in lipid membrane, with its metal moieties exposed to water interior, their hydration properties are considered to be of key importance.

In order to gain greater understanding, it seemed instructive to elucidate such issues as:

– the possibility of non-covalent complexes formation between CyS, StA and the stearates;

– experimental verification of complexes formation effects in various media;

– comparison of hydration properties of the stearates.

2. Materials and methods

Materials and membrane preparation. Calcium and magnesium stearates (CaSt and MgSt) were obtained from Magnesia GmbH, Germany. Stearic acid (StA) was obtained from NIOPIK, Russia, and cycloserine (CyS) — from Enamine, Ukraine.

Model lipid membranes (lipidmultibilayers with water content 65 % w/w) were prepared as desrcribed in [26] using L-α-dipalmitoylphoshpatidylcholine produced by Avanti Polar Lipids, USA. CaSt, MgSt and StA were introduced into the membrane during preparation in the form of chloroform solutions; chloroform was then evaporated carefully using a concentrator "Concentrator plus" (Eppendorf, USA). Then, CyS was added as a compound of water subphase. The membranes examined contained CaSt, MgSt or StA, either individually (2 % w/w) or coupled with CyS (1 % w/w of each compound). For SAXS experiments, DPPC membranes were used containing 93 % w/w of water with the same amount of the substances studied.

Small-angle X-ray scattering (SAXS) experiments were performed on Rigaku instrument with high-intensity microfocus ro-

tating Cu anode X-ray generator in the Laboratory for advanced studies of membrane proteins (Moscow Institute of Physics and Technology, Dolgoprudniy, Russia), using a standard transmission configuration. We used X-ray wavelength $\lambda = 1.54$ Å, resulting a momentum transfer Q in the range of 0.007–0.2 $Å^{-1}$, where Q is the scattering angle. The samples were placed in borosilicate capillaries of 1.5 mm diameter and 0.01 mm wall thickness (W.Muller, Berlin, Germany). Water was used as a buffer sample. We used homemade temperature sample holder for changing temperature of lipid mixtures. Centering of beam line and conversation channel to the value of module Q-vector was done using silver behenate [27]. Experimental error remained within data points.

Electrospray ionization mass spectrometry (ESI MS). CyS and StA were used for preparation of the model systems for electrospray ionization (ESI) mass spectrometry (MS) investigations. Initial solutions of CyS and StA (5 mM) were prepared in methanol (polar solvent) and used for preparation the diluted solutions of CyS and StA and binary CyS:StA (1:10 molar ratio) model system for ESI MS probing. The mixture was kept at the room temperature for at least 10 min. before the ESI MS analysis. The spraying procedure requires dilution of the studied solutions to the final 250 mM or less concentration of the diluted components of the model systems in each solution. It was shown in a number of studies [28–31] that the use of methanol as a solvent significantly improves the quality of the ESI mass spectra while the composition of the intermolecular complexes formed in methanol is similar to those formed in the water solution [32].

Mass spectral data were obtained in the positive ion mode using triple quadruple (QqQ) Micromass Quattro Micro mass spectrometer (Waters, Manchester, UK) which was equipped with the electrospray ion source. This source was operated in the standard ESI mode. The ESI source temperature was set to 120°C and the desolvation temperature was 200°C. The capillary was operated at 3.5 kV. The cone voltage (CV) value of 10 V was used. The analyzed solution of the model systems under study (20 mL) was injected into the mass spectrometer at a constant flow rate of 0.2 mL/min of the methanol solvent. ESI spectra were recorded in the mass range of m/z 100–2000. Data acquisition and processing were performed using MassLynx 4.1 software (Waters, Manchester, UK).

Differential scanning calorimetry (DSC). In DSC measurements thermal scans were performed in heating mode for CaSt and MgSt in the range from 30 to 100°C at scanning rate 5 K/min (0.08 K/s). In such regime, dehydation of MgSt [11] and CaSt precedes their melting by a dozen of degrees, allowing one to obtain thermodynamic parameters of dehydration. The enthalpy of dehydration, ΔH_{dh} was obtained as the area of the normalized DSC peak. The dehydration entropy was calculated as $\Delta S_{dh} = \Delta H_{dh}/T_{dh}$, where T_{dh} is the absolute temperature of the DSC peak maximum.

Thermogravimetry analysis (TGA). TGA measurements were performed for CaSt and MgSt in the temperature range 30 to 100°C at several scanning rates (from 0.008 to 0.17 K/s). Basing on TGA-scans, the dehydration temperature was obtained, as well as the amount of crystalline water. Activation energy of dehydration, Ea, was determined using Kissinger plot approach [33, 34]:

$$\ln\frac{h}{T_p^2} = -\frac{E_a}{RT_p} + const,$$

where h is the scanning rate (in K/s), T_p is the temperature of dehydration, R is the universal gas constant.

The enthalpy of hygroscopic water sorption was obtained according to [35].

Isothermal sorption technique (IST). The method of isothermal water sorption is widely used (with certain variations) to hygroscopy determination in pharmaceutical substances [36]. In our experiments, dry samples of CaSt, MgSt and StA (approx. 4 mg) were placed in aluminum oxide pans without during lids and then were kept till equilibrium (20 to 40 h) under various temperatures in saturated water vapor. The maximal hygroscopic water amount was obtained as a function of temperature or corresponding water vapor pressure. The value of hygroscopic water was taken as equilibrium water uptake under water vapor pressure 2.8 kPa. The average values from 5 independent experiments are presented.

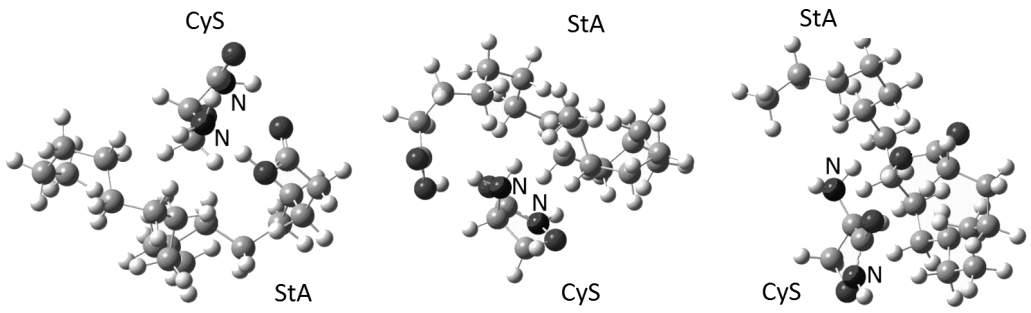

Fig. 1. The molecular structures of the most stable complexes of CyS–StA. Color labels of elements: H — white, C — light grey, O — dark grey; other elements are indicated.

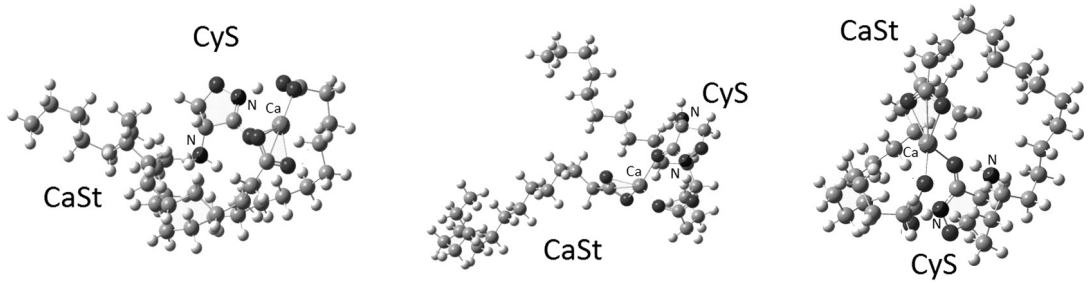

Fig. 2. The molecular structures of the most stable complexes of CyS–CaSt. Color labels of elements: H — white, C — light grey, O — dark grey; other elements are indicated.

3. Modeling of intermolecular interactions in pairs of cycloserine with stearic acid or its Ca/Mg salts by means of quantum chemistry methods.

The first step of the modeling of the possible complexes of cycloserine with stearic acid or its Ca/Mg salts was obtaining of the initial geometries of two-component complexes using molecular docking procedure within AutoDock Vina program [37]. For each system (CyS–StA, CyS–CaSt, CyS–MgSt), 20 structures with lowest energy were selected. The two-component complexes were optimized using m06-2x/cc-pvdz method [38, 39] *in vacuo* and taking into account polarizing effect of environment using PCM model (solvent is water) [40]. The interaction energy for each of these complexes was calculated and corrected for basis set superposition error using standard counterpoise procedure [41]. These calculations were performed using GAUSSIAN09 program [42].

The cycloserine molecule contains the proton donor groups as well as proton acceptor fragments. It creates the pre-conditions for hydrogen bonds formation with hydrophilic part of amphiphilic molecules. In addition, the interaction energy between molecules depends essentially on different types of non-specific interactions where the total dispersion is considered to be the strongest. As a result we should expect that the most stable complexes must be formed due to specific interactions between cycloserine with hydrophilic part and dispersive interactions with hydrophobic part simultaneously.

The comparison of the optimized complexes has shown that the most stable complexes are formed by intermolecular interactions of both types (Fig.1–3). The interaction energies for all optimized complexes have been calculated and their average magnitudes for each type of studied two-component complexes are shown in Table 1. It should be noted that the smallest average interaction energy corresponds to the CyS–StA complexes. The salt with bivalent metal contains two stearate anions what causes significant increasing of the interaction energies with CyS (Table 1). The difference in the average interaction energy between calcium and magnesium salts may be explained by the nature of cations.

To take into account the polarizing influence of environment we applied PCM approach with water as solvent. The modeling of the non-specific influence on the complexes results in some reducing of the aver-

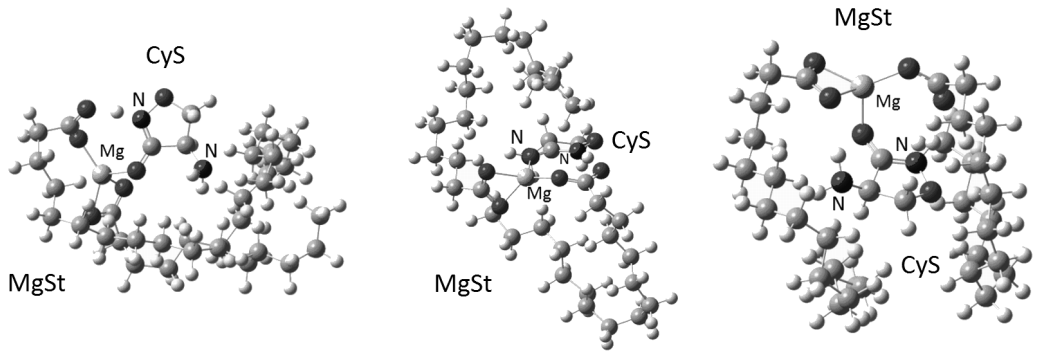

Fig. 3. The molecular structures of the most stable complexes of CyS–MgSt. Color labels of elements: H — white, C — light grey, O — dark grey; other elements are indicated.

age interaction energies and difference between them. But the total tendency keeps.

For each pair of substances, 20 most favorable complexes were calculated and characterized. Basing on these results, one can observe wide variety of possibilities of complex formation for CyS, which involves 4 different functional groups. Detailed analysis of the results allows one to conclude that proton-acceptor groups of CyS much more often participate in intermolecular interactions, than proton-donor ones. Meanwhile the most stable complexes are formed under participation of both types of groups.

4. Experimental

ESI mass spectrometry probing of intermolecular interactions of cycloserine and stearic acid

Soft-ionization mass spectrometry is a method that is widely used in molecular biology and pharmacology related investigations [43], and it is one of the most efficient tools to study intermolecular interactions in model systems in the gas phase as well as in solvents [28, 44, 45]. The electrospray ionization (ESI) mass spectrometry (MS) technique based on spraying solutions in polar solvents was successfully applied in our previous studies to investigate non-covalent intermolecular interactions in some mechanistic model studies of the interactions of biologically active compounds and medicines with potential targeting biomolecules [20, 21, 46, 47], including interactions of membranothropic antimicrobial bisquaternary agents with membrane phospholipid molecules [48, 49].

In the current study we applied the ESI MS approach developed in the previous investigations to examine the intermolecular interactions of CyS with StA in the model systems. ESI MS investigation of model systems contained MgSt and CaSt could not be performed, because of low solubility of these stearates in the polar solvents (methanol, ethanol, etc.) commonly used in ESI MS.

At the first stage of the experimental study the solution of CyS in methanol was investigated by ESI MS and characteristic mass spectrum of the preparation was obtained (Fig. 4) with intensive peaks of protonated molecules of $CyS[CyS{\cdot}H]^+$ at m/z 103.2 and cationized molecules $[CyS{\cdot}Na]^+$ at m/z 125.2 and peak of the CyS dimers $[2CyS{\cdot}H]^+$ at m/z 205.4. Such a high quality characteristic mass spectrum of the cycloserine drug with the low level of noise peaks and peaks of any admixtures confirms the applicability of the ESI MS method for CyS identification in different samples including biological and technological solutions.

Table 1. The average ($<E_{int}>$) and maximal (E_{int}^{max}) interaction energies of complexes of cycloserine with stearic acid or its Ca/Mg salts

System	in vacuo		PCM, water	
	$<E_{int}>$, kJ/mol	E_{int}^{max}, kJ/mol	$<E_{int}>$, kJ/mol	E_{int}^{max}, kJ/mol
CyS–StA	−51.9	−81.7	−49.0	−74.8
CyS–CaSt	−110.1	−239.3	−77.5	−188.8
CyS–MgSt	−134.4	−276.2	−98.0	−220.9

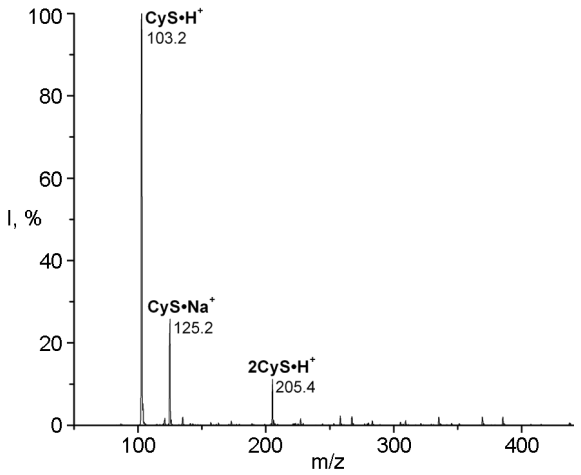

Fig. 4. ESI MS spectrum of CyS solution in methanol.

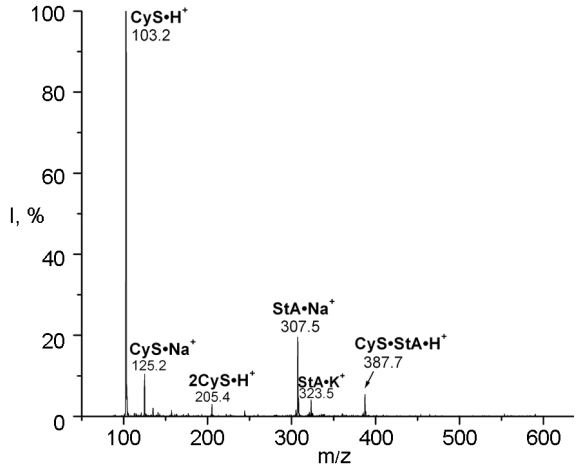

Fig. 5. ESI MS spectrum of the model system CyS:StA (1:10 molar ration) in methanol.

At the next stage, the model system of cycloserine with stearic acid (1:10 molar ratio) was examined. Along with the ions characteristic of the individual components of the mixture ($[CyS{\cdot}H]^+$ at m/z 103.2, $[CyS{\cdot}Na]^+$ at m/z 125.2, $[2CyS{\cdot}H]^+$ at m/z 205.4 — for CyS and $[StA{\cdot}Na]^+$ at m/z 307.5, $[StA{\cdot}KF255]^+$ at m/z 323.5 — for StA), the peak of protonated molecular clusters of cycloserine with stearic acid $[CyS{\cdot}StA{\cdot}H]^+$ at m/z 387.7 was observed in the mass spectrum (Fig. 5). Such clusters registration in the ESI mass spectra indicates the formation of stable complexes between CyS and StA in the studied model system and testifies to the effect that the complexes stability is sufficient to provide the clusters surveillance under the electrospray ionization processes. The results of the current ESI probing can be considered as experimental confirmations of the results of the described above quantum chemical modeling of the CyS–StA complexation.

The ESI MS findings point to the possibility of non-covalent complexation of CyS with StA and some its derivatives in the polar solvents. Such possibility to form the stable non-covalent complexes of CyS with the excipient should be taken into account under study of the drug behavior in different media, including model phospholipid membranes.

SAXS studies of model lipid membranes containing cycloserine, stearic acid and its Ca/Mg salts, both individually and in pairs

Amphiphilic molecules, with their hydrophobic moieties incorporated into lipid core and hydrophilic parts exposed to lipid/water interface, exert an effect on both the polar and non-polar lipid moieties, so their membranotropic action could be represented as a superposition of their interactions with polar and non-polar regions of the lipid membrane.

SAXS technique provides valuable and unambiguous information to lamellar repeat distance d_L. Representative SAXS diagrams are shown in Fig. 6. The most outstanding feature here is the anomalous broadening of diffraction peak for pair CyS–CaSt (the same pattern takes place for CyS–MgSt). To our mind, it probably reflects coexistence of a number membrane structures with similar values of d_L which could result from colliding with membrane a set of intermolecular complexes CaSt–CyS or MgSt–CyS with close values of E_{int}. In the temperature region examined (20 to 50°C) DPPC membranes undergo two mesomorphous phase transitions, namely, gel to ripple phase (at T_p ~ 37°C) and ripple to liquid crystalline phase (at T_m ~ 42°C) [50, 51]. Each of these phases has characteristic d_L value, which is the highest in the ripple phase (~ 72 Å), and slightly lower in the gel phase than in the liquid crystalline phase (~ 65 and 64 Å, correspondingly), in line with literature data [52, 53]. Temperature dependences of d_L for DPPC membranes containing CyS and StA are represented in Fig. 7. As one can see, individual effect of CyS is minor in all thermodynamic phases. Close inspection of Fig. 7 shows additivity of d_L for CyS–StA within experimental error (±0,04 Å).

As it follows from the data collected in Table 2, StA and the stearates increase the value of d_L in the low-temperature gel phase of DPPC in the order StA > MgSt > CaSt, which is the same for T_m increasing [22]. However, in high-temperature liquid

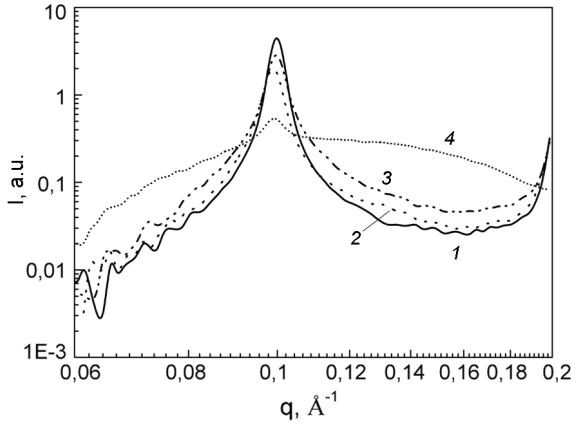

Fig. 6. SAXS profiles for DPPC membranes containing CyS and CaSt: neat DPPC membrane (*1*); with CaSt (*2*); with CyS (*3*); with CyS–CaSt (*4*).

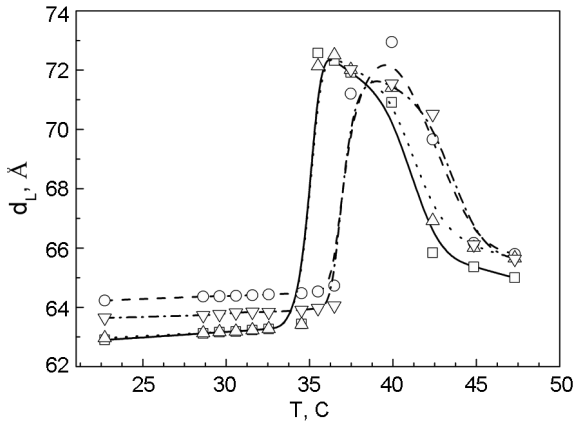

Fig. 7. Temperature dependences of d_L in the region of phase transitions of DPPC membrane with CyS and StA: neat DPPC membrane (□), with CyS (Δ), with StA (○) CyS–StA (▽).

crystalline phase the stearates decrease d_L value under individual application and increase it in pairs with CyS, which is a clear marker of non-additivity effects.

Half-width of diffraction peak, $q_{1/2}$, which reflects structure non-homogeneity, sufficiently growths under joint StA and CyS application. It is in line with previous DSC data as to lowering of cooperativity and elevating of T_m hysteresis in this system [22] and apparently reflects coexistence of membrane regions which are bound with each of these substances. Similar cases are described in literature [54, 55].

Thus, SAXS data confirm complex formation in membrane interior for CyS–MgSt and CyS–CaSt, as distinct from CyS–StA.

Hydration properties of the Ca/Mg-*stearates — TGA, ISC and DSC studies*

Water functioning in living systems is uniquely complex, but it is commonly accepted that it plays a key role in cell structure and function and provides the driving force in the formation of the basic lipid bilayer structure of the cell membrane [56]. In particular, a strong correlation was established between the standard enthalpy of hydration of an electrolyte and the modification of the surface tension by that electrolyte [57 and refs. therein] and between interchain hydration and acyl chain order in model lipid membranes [56].

Hydration properties of drugs constituents, in particular, hygroscopicity, have sufficient influence on their practical utilization, so the water-solid interactions of pharmaceuticals are of great interest in drug development process. The ad-(ab)-sorption of water molecules by a pharmaceutical solid can largely affect its performance with regard to stability, flow, wetness, dissolution, compressibility or compatibility, etc. [15, 58, 59].

Table 2. Lamellar repeat distance (d_L) and half-width ($q_{1/2}$) of diffraction peak in gel and liquid crystalline phases of DPPC membrane containing stearic acid and its Ca/Mg salts

DPPC+,..	d_L, Å gel phase (22.7°C)	$q_{1/2}$, Å^{-1} liquid crystalline phase (47.3°C)	d_L, Å	$q_{1/2}$, Å^{-1}
—	62.8	0.0036	65.0	0.0036
StA	64.1	0.0035	65.8	0.0036
MgSt	63.9	0.0052	64.4	0.0067
CaSt	63.4	0.0048	64.4	0.0059
CyS	62.9	0.0046	65.7	0.0048
StA+CyS	63.6	0.0046	65.6	0.0075
MgSt+CyS	63.6	–	66.5	0.0042
CaSt+CyS	63.1	–	65.7	0.0052

Table 3. Hydration parameters of CaSt and MgSt obtained by various techniques.

Substance	Parameter	Mg-stearate	Ca-stearate
1.	Crystalline water, mol/mol	2.0±0.08	1.2±0.2
2.	Dehydration temperature, °C	76.3±0.2	90.2±0.2
3.	Enthalpy of dehydration, kJ/mol	48.2±4.2	79.7±7.5
4.	Enthropy of dehydration, J/mol·K	138±14	219±22
5.	Activation energy of dehydration, kJ/mol	268±5	359±36
6.	Hygroscopic water, mol/mol	4±0.5	11±1.4
7.	Enthalpy of hygroscopic water sorption, kJ/mol	–	32±4

As to the excipients under consideration, StA is classified as non-hygroscopic substance [60, 61]. MgSt and CaSt are designate as low hygroscopic, but condition-depending [36, 62]. However, it is pointed out that hygroscopicity of the stearates depends both on the manufacturer and on experimental conditions, therefore it should be specified for the given examples and techniques [15, 62], so the direct comparative characterization of the stearates seemed necessary. Besides, both CaSt and MgSt form of hydrates [63–65], which might to undergo exhaustive studies due to their topical importance for drug bioavailability [62, 66]. Moreover, the hydrate water molecules could participate in intermolecular interactions between MgSt and another substance [15].

A set of experimental techniques was used to establish hydration parameters of the substances under consideration. As one can see from Table 3, hydration properties of both stearates are quite different. As to crystalline water, binding stoichiometry for MgSt appears higher than for CaSt (2 water molecules *vs.* 1), but the other hydration parameters are much lower. In particular, hygroscopicity of CaSt is higher than of MgSt (row 6 of Table 3). As to StA, no crystalline water was observed and the amount of hygroscopic water was determined as 1 mol/mol, which is in line with literature data [60]. Qualitatively similar data on relative hygroscopicity of the excipients are reported in [61], together with the conclusion about variability of these parameters caused by manufacturing features. Though enthalpy of hygroscopic water sorption for CaSt (row 7 of Table 3) appeared to be 30 % lower than that for pure water, the corresponding value for MgSt appeared to be much smaller and could not be determined within the experimental conditions. Here, it should be noted that the obtained values of activation energy proved to be much higher then those reported in literature for hydrogen bond in water (ab. 13 kJ/mol [67, 68]), but typical for diffusion processes in crystals (~ 10^2 kJ/mol [69]). Thus, one can conclude that the limiting stage of the process of the stearate dehydration is water diffusion from the crystals after disruption of hydrogen bonds. Hence, one can assume the hydration shell of CaSt provides larger cross-sectional area of its polar moiety. So, even subtle changes in polar moiety of amphiphilic substance cause continuous changes in their hydration properties.

6. Discussion

From consideration of the foregoing results, the difference in membranotropic effect of StA and its salt can be vividly understood. StA molecule possesses poorly hydrated terminal group and highly anisometric and fully saturated alkyl chain. As StA collide with the membrane, the population of gauche conformers in neighboring DPPC alkyl chains decreases, they become more neatly aligned parallel to each other and can pack in a more efficient way. Such increasing in packing density results in elevation of T_m and d_L.

In the stearates, this effect is moderated *via* the presence of metal group which determines minimal cross-area of the polar moiety of the molecule. Discrimination factor in membranotropic effects of MgSt and CaSt is cationic radius, which is 1.0 Å for Ca^{2+} and 0.7 Å for Mg^{2+}. As it was shown [70, 71], ΔT_m of membranes containing kosmotropic ions grows with decreasing of ion radius, so for Mg^{2+} it should be greater than for Ca^{2+}. Additional factor of the discrimination is hydration shell dimension which is evidently much greater for CaSt than for MgSt (see Table 3). So, T_m elevation of the stearates is in inverse correla-

tion with their hydration ability. In the framework of such mechanism, further dehydration of stearate could be supposed under their complexes formation with CyS.

Summarizing the above findings, one can conclude that hydration parameters of amphiphiles are able to be largely modified by changes of its polar moiety which is of crucial importance for their membranotropic action. Indeed, higher water binding ability corresponds to stronger and larger hydration shell. This, in turn, results in larger cross-section area of the polar moiety of the dopant molecule which leads to lowering of its membranotropic effect. It is believable that under complex formation further dehydration may take place, with proper decrease in cross-section area and corresponding increasing in T_m. Both enthalpy and entropy of dehydration for MgSt are less than for CaSt, so CyS–MgSt complex seems more favorable.

Moving to the significance of the results, one should note a substantial difference in properties of CaSt, MgSt and StA with respect to their intermolecular and membrane interactions, as well as to hydration properties. Unlike both stearates, StA is non-hygroscopic, it possesses low ability of complex formation and the highest T_m shift. In turn, CaSt is hygroscopic, possesses high ability of complex formation and lower membranotropic effect. MgSt is characterized by membranotropic action similar to StA, but moderate hygroscopicity and high ability of complex formation. So, this is indeed the case when excipient selection could be of extreme importance for drug action.

Finally, it should be noted that the mechanisms of CyS cytotoxicity (therapeutic effect) and neurotoxicity (adverse effect) are caused by its specific interactions [11–14, 72]. Thinking about that, a possible implication of our findings might be related to formation of complexes, which could prevent specific interactions of CyS. Keeping in mind the absence of straightforward explanation as to how each of CyS functional groups participate in specific interaction underlying its cyto- or neurotoxisity, it seems reasonable that further steps should be made in this direction.

7. Conclusions

Complexes formation between antibiotic CyS and a number of excipients (StA, CaSt and MgSt) was studied in vacuum, water, polar solvent methanol and lipid membrane interiors. Quantum chemistry methods have shown multiple ability of complex formation for CyS with all the excipients. However CyS–CaSt and CyS–MgSt complexes are much more stable, due to participation the proper metal cation in the intermolecular interactions. Similar results were obtained in vacuum and in water media.

The non-covalent complexes of CyS with StA formed in the polar solvent methanol were experimentally observed by means of ESI mass spectrometry. These findings confirm the theoretical data on CyS–StA complexes stability in different media obtained by quantum chemical calculations.

No evidences of CyS–StA complexes existence were observed as CyS and StA collide with the lipid membrane, neither by SAXS nor by our previous DSC experiments. At the same time, the complexes with higher interaction energies, CyS–CaSt and CyS–MgSt, are detectable in lipid membranes *via* non-linearity of interlamellar repeat distance, as judged from SAXS experiments.

A set of hydration parameters of the excipients elucidates important discrimination between CaSt and MgSt, which directly relates to its membranotroipic action. In particular, the former is much more hygroscopic and possesses higher energy of dehydration than the latter.

The present study of CyS-excipient complexes formation together with non-additive phase behavior reported earlier [22] can all cooperate to give a clear understanding of the importance of non-covalent interactions between drug constituents. Moreover, involving data obtained here for model membranes we may state that intermolecular complexes CyS–CaSt and CyS–MgSt are stable in water-lipid interior during several days as a minimum, which could be taken into account in drug formulation.

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