МОЛЕКУЛЯРНА БІОФІЗИКА

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INTERMOLECULAR INTERACTIONS OF DECAMETHOXINUM AND ACETYLSALICYLIC ACID IN SYSTEMS OF VARIOUS COMPLEXITY LEVELS

O.V. Vashchenko[1], N.A. Kasian[1], V.A. Pashynska[2], M.V. Kosevich[2], A.O. Sadchenko[1], D.N. Tishko[3], T.V. Tishko[3], V.P. Titar[3], L.N. Lisetski[1]

[1]*Institute for Scintillation Materials of the National Academy of Sciences of Ukraine, 60, Nauky ave., 61001 Kharkov, Ukraine*

[2]*B. Verkin Institute for Low Temperature Physics and Engineering of the National Academy of Sciences of Ukraine, 47, Nauky ave., 61103, Kharkov, Ukraine*

[3]*V.N. Karazin Kharkov National University, 4 Svoboda sq., 61022, Kharkov, Ukraine*

e-mail: olga_v@isma.kharkov.ua

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Intermolecular interactions between decamethoxinum (DEC) and acetylsalicylic acid (ASA) have been studied in the phospholipid-containing systems of escalating complexity levels. The host media for these substances were solvents, *L*-α-dipalmitoylphosphatidylcholine (DPPC) membranes, and samples of human erythrocytes. Peculiar effects caused by DEC-ASA interaction have been observed in each system using appropriate techniques: (a) DEC-ASA non-covalent complexes formation in DPPC-containing systems were revealed by mass spectrometry with electrospray ionization; (b) joint DEC-ASA action on DPPC model membranes led to increasing of membrane melting temperature T_m, whereas individual drugs caused pronounced T_m decreasing, which was demonstrated by differential scanning calorimetry; (c) deceleration of DEC-induced haemolysis of erythrocytes under joint DEC-ASA application was observed by optical microscopy.

Key words: intermolecular interactions, noncovalent complexes, decamethoxinum, acetylsalicylic acid, model lipid membranes, erythrocytes.

МЕЖМОЛЕКУЛЯРНЫЕ ВЗАИМОДЕЙСТВИЯ ДЕКАМЕТОКСИНА И АЦЕТИЛСАЛИЦИЛОВОЙ КИСЛОТЫ В СИСТЕМАХ РАЗЛИЧНОГО УРОВНЯ СЛОЖНОСТИ

О.В. Ващенко[1], Н.А. Касян[1], В.А. Пашинская[2], М.В. Косевич[2], А.О. Садченко[1], Д.Н. Тишко[3], Т.В. Тишко[3], В.П. Титарь[3], Л.Н. Лисецкий[1]

[1]*Институт сцинтилляционных материалов НТК «Институт монокристаллов НАН Украины», пр. Науки, 60, Харьков, 61001, Украина*

[2]*Физико-технический институт низких температур им. Б.И. Веркина, пр. Науки, 47, Харьков, 61103, Украина*

[3]*Харьковский национальный университет имени В.Н. Каразина, пл. Свободы, 4, Харьков, 61022, Украина*

Межмолекулярные взаимодействия между декаметоксином (ДЕК) и ацетилсалициловой кислотой (АСК) изучались в фосфолипид-содержащих системах различного уровня сложности: растворах, модельных фосфолипидных мембранах и образцах эритроцитов человека. В каждой системе соответствующими экспериментальными методами были зарегистрированы специфические эффекты, обусловленные взаимодействием ДЕК-АСК: а) методом масс-спектрометрии с ионизацией электрораспылением зарегистрировано образование нековалентных комплексов ДЕК-АСК в трёхкомпонентом растворе, содержащем также *L*-α-дипальмитоилфосфатидилохлин (ДПФХ); б) в модельных мембранах ДПФХ методом дифференциальной сканирующей калориметрии было показано, что совместное действие ДЕК и АСК приводит к повышению температуры плавления мембраны T_m, тогда как индивидуальное действие каждого препарата приводит к снижению T_m; в) с помощью оптической микроскопии показано замедление ДЕК-индуцированного гемолиза эритроцитов при совместном введении ДЕК и АСК.

Ключевые слова: межмолекулярные взаимодействия, нековалентные комплексы, декаметоксин, ацетилсалициловая кислота, модельные липидные мембраны, эритроциты.

МІЖМОЛЕКУЛЯРНІ ВЗАЄМОДІЇ ДЕКАМЕТОКСИНУ ТА АЦЕТИЛСАЛІЦИЛОВОЇ КИСЛОТИ У СИСТЕМАХ РІЗНОГО РІВНЯ СКЛАДНОСТІ

О.В. Ващенко[1], Н.О. Касян[1], В.А. Пашинська[2], М.В. Косевич[2], А.О. Садченко[1], Д.М. Тішко[3], Т.В. Тішко[3], В.П. Титар[3], Л.М. Лисецький[1]

[1]*Інститут сцинтиляційних матеріалів НТК «Інститут монокристалів НАН України», пр. Науки, 60, Харків, 61001,Україна*

[2]*Фізико-технічний інститут низьких температур ім. Б.І. Вєркіна, пр. Науки, 47, Харків, 61103, Україна*
[3]*Харківський національний університет імені В.Н. Каразіна, м. Свободи, 4, Харків, 61022, Україна*

Міжмолекулярні взаємодії між декаметоксином (ДЕК) та ацетилсаліциловою кислотою (АСК) вивчались у фосфоліпід-вмісних системах різного рівня складності: розчинах, модельних фосфоліпід них мембранах та зразках еритроцитів людини. У кожній із систем відповідними експериментальними методами були зареєстровані специфічні ефекти, обумовлені взаємодією ДЕК-АСК: а) методом мас-спектрометрії з іонізацією електророзпиленням зареєстровано утворення нековалентних комплексів ДЕК-АСК у трьохкомпонентому розчині із вмістом *L*-α-діпальмітоїлфосфатидилхоліну (ДПФХ); б) в модельних мембранах ДПФХ методом диференціальної скануючої калориметрії було показано, що сумісна дія ДЕК та АСК призводить до підвищення температури топлення мембрани T_m, тоді як індивідуальна дія кожного препарату призводить до зниження T_m; в) за допомогою оптичної мікроскопії показано уповільнення ДЕК-індукованого гемолізу еритроцитів при сумісному уведенні ДЕК та АСК.
Ключові слова: міжмолекулярні взаємодії, нековалентні комплекси, декаметоксин, ацетилсаліцилова кислота, модельні ліпідні мембрани, еритроцити.

To clear up the action mechanism of a biologically active agent, its effects should be tested in systems of different complexity level, ranging from its intermolecular interactions with presumed molecular targets to its effects on model biomimetic systems and biological objects. Numerous studies of individual drugs effects in model systems have been reported (e.g., [1-6]). A novel step is related to studies of the combined drugs action [7-10], because joint application of drugs is expected to modify their inherent effects. Indeed, due to intermolecular interactions of different biologically active agents, their contributions could be non-additive. In our previous studies, some effects of joint membranotropic action were found in model systems [10-12].

In the present study, we consider possibilities and consequences of noncovalent intermolecular interaction of a pair of drugs of antiseptic and anti-inflammation action, namely, decamethoxinum (DEC) and acetylsalicylic acid (ASA), which potentially could be used simultaneously during treatment of throat infection.

Alongside the mechanisms of drugs action on molecular targets in microbial or human cells, an important aspect is the drug-membrane interaction which could essentially condition the drugs pharmacokinetics. In the present work, we focused on a particular mechanism connected with the effects of drugs on biomembranes under their individual and joint action.

Antimicrobial drug DEC is an amphiphilic substance that can be considered as a membranotropic compound. The main mechanism of DEC action is related to damaging of the membranes of microbial cells [11, 13-18].

ASA, known as aspirin, is a multifunctional drug with anti-inflammatory, antipyretic and analgetic activity [19]. Its fluidizing effect upon model membranes was reported in [20-24].

In the present work, joint DEC-ASA membranotropic action was studied in phospholipid-containing media of escalating complexity, namely, solutions (physico-chemical level), model lipid membranes (biophysical level), and membranes of native cells (biomedical level). The aim of this approach is to shed light on the effects and mechanisms of joint drugs action in biomembranes and to bridge the gap between intermolecular interactions of individual drug molecules and their effects on the native cells.

To carry out the above-stated chain of studies, appropriate experimental techniques were applied. Such, an efficient tool for detection of eventual formation of stable non-covalent intermolecular complexes between the drugs and between each drug and DPPC is mass spectrometry with electrospray ionization (ESI) [25, 32-35]. Differential scanning calorimetry (DSC) gives information on thermodynamic parameters of the membranotropic drugs action at the level of model phospholipid membranes [2-5]. Digital holographic interference microscopy (DHIM) [29-31] is suitable for monitoring of morphology of erythrocyte blood

cells treated by the drugs. The eventual drug-induced haemolysis of erythrocytes was observed by optical microscopy.

MATERIALS AND METHODS

Materials

L-α-dipalmitoylphosphatidylcholine (DPPC) was purchased from Alexis Biochemicals (Switzerland) and used without further purification. Acetylsalicylic acid (ASA) was obtained from the State Scientific Centre of Medications (Kharkov, Ukraine). Decamethoxinum (1,10-decamethylene-bis[N,N-dimethyl-menthoxycarbonylmethyl] ammonium dichloride, DEC) was produced by Biolek (Ukraine). Bidistilled water, purified methanol and physiological saline solution (PSS) with osmolarity of 308 mmol/l («Niko», Ukraine) were used for samples preparation.

Human blood for the samples used in microscopic studies was collected from fingertips of healthy donors (4 authors of the present work) with their informal consent by standard clinical procedure. The blood was diluted by 0.1 % w/w solutions of the studied drugs in PSS, and by pure PSS for reference.

Mass-spectrometry

Electrospray ionization (ESI) mass spectrometry data were obtained in the positive ion mode using a triple quadruple (QqQ) Micromass Quattro Micro mass spectrometer (Waters, Manchester, UK) equipped with 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 spraying capillary was operated at 3.5 kV, and the desolvation potential (cone voltage, CV) value of 10 V was used. The analyte solutions (20 μL) were infused into the mass spectrometer at a constant flow rate of 0.2 mL/min of methanol solvent. ESI mass spectra were recorded in the mass range of 100-2000 Da. Data acquisition and processing were performed using MassLynx 4.1 software (Waters, Manchester, UK).

Stock solutions of DPPC, DEC and ASA (5 mM) in methanol were used for preparation of ternary DPPC-DEC-ASA mixture with 5:1:1 molar ratio. The excess of DPPC molecules in this system simulated quantitative predominance of phospholipids over drugs under physiological conditions of drug-membrane interactions. The mixtures were incubated at the room temperature for at least 10 minutes before the ESI analysis. The spraying procedure required dilution of the studied solutions by methanol to provide 250 μM (or less) final concentration of the components. It was confirmed that utilization of methanol as a solvent provided high quality of the ESI mass spectra and did not disturb essentially the composition of intermolecular complexes formed in water solutions [25, 26].

Differential scanning calorimetry (DSC)

DSC studies were carried out using a Mettler DSC 1 calorimeter. DSC profiles were obtained in cooling and heating modes at 2 K/min. Coincidence of two or more DSC profiles obtained in repeated scans was accepted as an equilibrium criterion. Phase transition parameters of model lipid membranes were determined using "Mettler Star^e 11.00" software.

Model lipid membranes of hydrated DPPC (1:1 w/w) were prepared according to the procedure described in [27]. To obtain DPPC membranes with DEC and ASA, the drugs solutions in double-distilled water were used. The resulting drug(s) concentration in all the systems were 1 % w/w with respect to dry DPPC. A number of ternary (DPPC membrane-DEC-ASA) systems was prepared with DEC to ASA molar ratios 2:1, 1:1, 1:2, 1:4, and 1:8. The samples quality was controlled by the correspondence of calorimetric parameters of undoped DPPC membrane to the literature data [28].

Digital holographic interference microscopy

Erythrocytes morphology studies were carried out using digital holographic interference microscope (DHIM) (Kharkov National University, Ukraine) [29].

For DHIM measurements, fresh donor's blood was mixed (1:3 v/v) with the drugs solutions in PSS or with pure PSS for reference. The procedure was performed directly on a glass slide. The samples were covered by a cover glass and examined just after preparation. Such method of samples preparation allowed us to observe separate erythrocytes in the field of view of the DHIM. Each drug(s) solution was probed with 8 to 10 fresh blood samples.

The prepared samples were placed into the DHIM for obtaining their real-time interferograms. The interferograms were digitally recorded and processed for obtaining 3D-images of the erythrocytes and for determination their sphericity coefficients as described by [30]. The sphericity coefficient $k = t_c/t_r$ is the ratio of the erythrocyte thickness at t_c to its thickness at half of its radius t_r (Fig. 1).

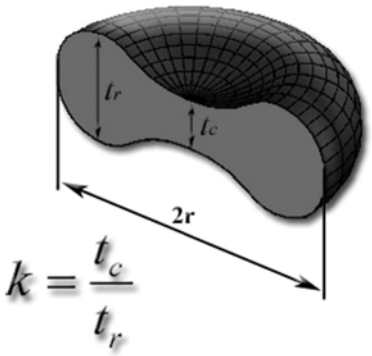


Fig. 1. Sphericity coefficient definition for erythrocytes.

Optical microscopy study of erythrocytes haemolysis

Erythrocytes haemolysis studies were carried out using a "Micromed Polar 3" optical microscope (Russia) equipped with digital camera "Canon EOS 1100D". To determine the response of erythrocytes to the drugs introduction, 0.05 % w/w solutions of DEC or ASA were prepared in PSS. The solution of both drugs (DEC 0.05 % w/w, ASA 0.026 % w/w) with DEC to ASA molar ratio 1:2 was prepared. Fresh human blood samples (~0.50 μl) were placed at a slide glass and mixed with the equal volume of the proper drug(s) solution with subsequent careful randomization on the glass surface during 10 s. The samples were covered with a cover glass and immediately placed onto the microscope stage. This moment was considered as a start of observation. Photos of the microscopic field were taken with 30 s intervals. Undamaged erythrocytes were clearly visible in the field of microscope, whereas the haemolysed ones (erythrocytes shadows) became almost invisible. The amount of visible (undamaged) cells was calculated using the microscopic photos. The initial amount of erythrocytes in each experiment was taken as 100 %. The average amount of erythrocytes (in percents) obtained in 3-4 independent experiments for each system was plotted as function of time. The Student's method was used to determine the confidence intervals for the reliability P = 0.95.

RESULTS AND DISCUSSION

Model systems in solution (mass spectrometry investigation)

Initially, joint action of DEC and ASA on the DPPC membrane was probed at molecular level. Non-covalent intermolecular interactions of DEC and ASA with DPPC were evidenced by the obtained data of ESI mass spectrometry which is an efficient tool for detection of stable non-covalent intermolecular complexes [25, 32-35].

Ternary system containing DPPC-DEC-ASA mixture (5:1:1 molar ratio) dissolved in methanol was probed. The ESI mass spectrum of the system obtained in the positive ion mode (Fig. 2) contained peaks of the individual mixture components and their intermolecular complexes formed in the system. Ions ASA•Na^+ (*m/z* 203.0), 2ASA•Na^+ (*m/z* 383.1) were recorded for ASA. The intact dication Cat_{DEC}^{2+} (*m/z* 311.3) and Cat_{DEC}^{2+}·Cl^- (*m/z* 657.5) cluster were observed for DEC. The peaks of DPPC•H^+ (*m/z* 734.6), DPPC•Na^+ (*m/z* 756.6), and 2DPPC•H^+ (*m/z* 1468.1) ions corresponded to DPPC. Note that ionization of an analyte molecule by sodium cation attachment is characteristic of the ESI procedure and correlates with ion-molecule interactions under physiological conditions.

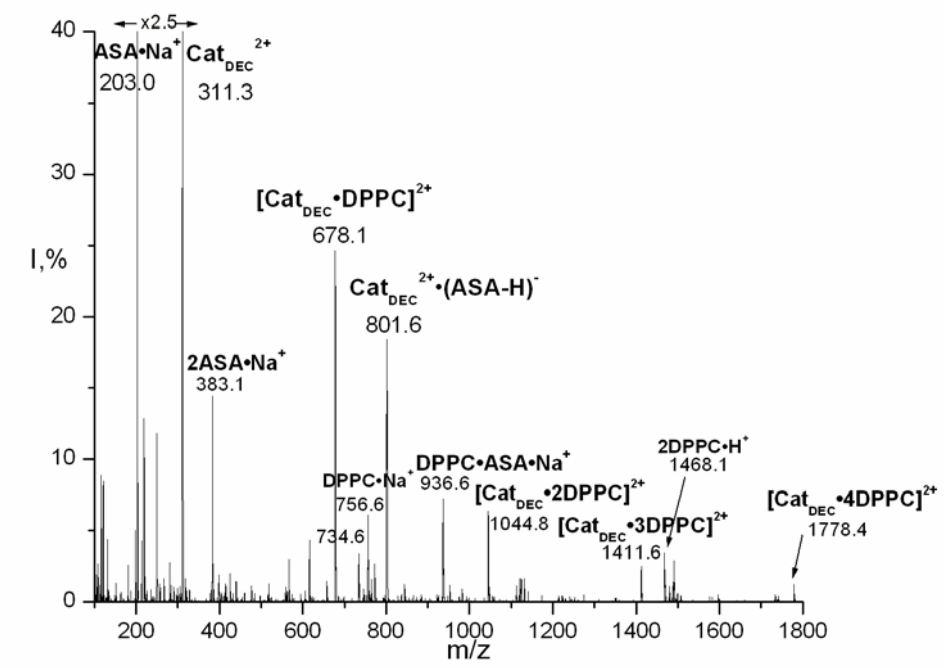


Fig. 2. ESI mass spectrum of DPPC-DEC-ASA (5:1:1 molar ratio) system.

The intermolecular complexes recorded in the mass spectrum reflected the complexity of specific non-covalent interactions in the model system. Firstly, supramolecular complexes of the DEC dication (Cat_{DEC}^{2+}) with the individual phospholipid molecule and phospholipid molecules associates containing up to 4 DPPC molecules $[Cat_{DEC}•nDPPC]^{2+}$ (n = 1 ÷ 4) were formed similarly to the drug-phospholipid clusters in their binary systems [18]: $[Cat_{DEC}•DPPC]^{2+}$ (*m/z* 678.1); $[Cat_{DEC}•2DPPC]^{2+}$ (*m/z* 1044.8); $[Cat_{DEC}•3DPPC]^{2+}$ (*m/z* 1411.6); $[Cat_{DEC}•4DPPC]^{2+}$ (*m/z* 1778.4). Secondly, ASA bound to single DPPC molecule similarly to its behaviour in the binary system forming the complex DPPC•ASA•Na^+ (*m/z* 936.6) [36]. Thirdly, dication-anion Cat_{DEC}^{2+}•$(ASA-H)^-$ complex (*m/z* 801.6) observed in the binary DEC-ASA mixtures by [36] was formed in the ternary system as well.

Formation of the stable pair intermolecular complexes of DEC and ASA with DPPC proved the possibility of interactions of these drugs with phospholipid membranes. The result on formation of Cat_{DEC}^{2+}•$(ASA-H)^-$ complex demonstrated the possibility of intermolecular interactions between the components of the basic and acidic drugs. The peaks set in the mass spectrum of the ternary DPPC-DEC-ASA system pointed to the existence of a competition between DEC and ASA for binding to DPPC molecules in the ternary system, since abundances of the peaks of $[Cat_{DEC}•nDPPC]^{2+}$ complexes, Cat_{DEC}^{2+}•$(ASA-H)^-$ and DPPC•ASA•Na^+ associates were of comparable intensities. The competition of DEC and ASA for binding to DPPC molecules and the formation of dication-anion complexes, revealed

on the basis of the mass spectrometric data, can be considered as molecular mechanisms of the possible drugs activity modulation on their combined use.

Model lipid membranes (DSC investigation)

DSC-thermograms were obtained for model DPPC membranes containing DEC, ASA or DEC+ASA. Comparison of DSC data for binary (DPPC membrane-DEC, DPPC membrane-ASA) and ternary (DPPC membrane-DEC-ASA) systems allowed us to distinguish the effect resulting from the drugs complexes formation, described in the previous section.

For undoped DPPC membrane, the pre-transition temperature T_p = 35.2 ± 0.1 °C and the main phase transition temperature T_m = 41.8 ± 0.1 °C, which is in good agreement with literature data [28]. As one can see (Fig. 3), both DEC and ASA caused changes in the DSC profiles of DPPC membrane in qualitatively similar manner, i.e., the pre-transition disappeared, whereas the main phase transition peak smeared and shifted to lower temperatures. But joint DEC-ASA effect is just the opposite, resulting in T_m shifting to higher temperatures (Fig. 3). For all the systems studied, the enthalpy of the main phase transition remained almost unchanged within experimental error (data are not shown). Meanwhile, the half-width $\Delta T_m^{1/2}$ of the main phase transition exhibits clearly non-additive concentration dependence with maximum (Fig. 4, a). At the same time, the hysteresis h_m of the main phase transition shows less pronounced non-additivity (Fig. 4, b). Since these parameters are related to cooperativity of the melting process, one can conclude that DEC + ASA reduce the membrane cooperativity number more appreciably than DEC or ASA separately.

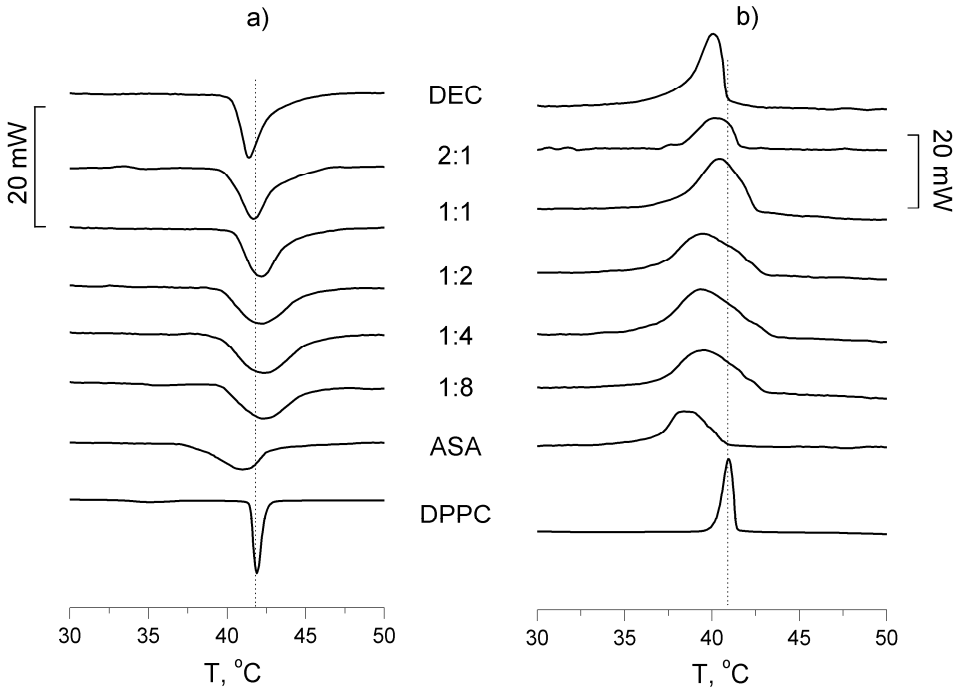


Fig. 3. DSC profiles of DPPC membrane -DEC-ASA systems: a – heating; b – cooling. Vertical dotted line marks T_m values of neat DPPC system. Designations: DPPC – neat lipid membrane; DEC – membrane containing DEC only; ASA – membrane containing ASA only; for ternary systems, DEC:ASA molar ratios are indicated.

For quantitative comparison of membranotropic effects, we used the membranotropic activity coefficient α, which is defined as T_m shift per unit weight concentration of drug(s). In Fig. 5, α is presented as a function of DEC molar fraction in the DEC+ASA mixture. One can observe qualitative difference between α values for individual and joint DEC+ASA action. Namely, α values of individual DEC and ASA are both negative, whereas α values of

DEC+ASA mixtures are generally positive. Such difference, according to [37], is a manifestation of DEC–ASA complexes formation described above (see Fig. 2).

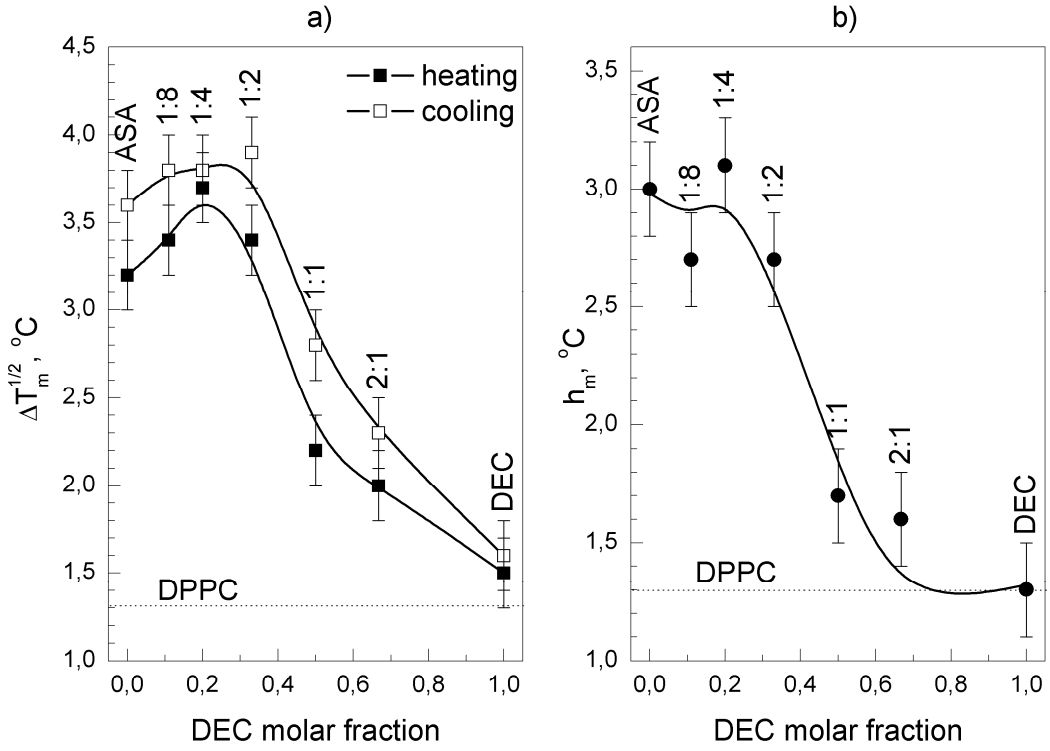


Fig. 4. Halfwidth (a) and hysteresis (b) of DPPC main phase transition as function of DEC molar fraction in the DEC+ASA mixture. Horizontal dotted lines mark corresponding values for neat DPPC membrane. Designations are the same as in Fig. 2. Standard deviations are given as error bars.

Neutralization of the positive charge of DEC dication by ASA anions in the complexes, apparently, plays an essential role in the change of membranotropic effect of the DEC–ASA complex relative to the individual drugs.

Negative α of DEC and ASA, as well as disappearance of the pretransition peak, indicates destabilization of the ordered $L_{\beta'}$- and $P_{\beta'}$-phases in favor of more fluid and disordered L_{α}-phase. It should be noted that the effects observed are in good agreement with membrane fluidizing ability of ASA [20, 21] and DEC [12]. Positive α indicates stabilization of the $L_{\beta'}$-phase. Changes of α under joint DEC-ASA introduction occurs not only for the electroneutral DEC–ASA complex (1:2), but in the broad range of DEC: ASA molar ratios (Fig. 5).

It is believable that the described effects could have biomedical implication. The fluidizing effect of DEC could make a contribution to the mechanism of its antiseptic action causing microbial cell disintegration [13-15]. Meanwhile, joint application of DEC and ASA may reduce DEC pharmacological efficiency.

Erythrocytes (optical microscopy investigations)

The next step was to study the effects of DEC and ASA on erythrocytes. Studies at the level of native cell membranes are obviously more sophisticated due to variety of membrane components and substantially more complex structure of native membranes as compared to the model ones. Erythrocytes have no cell walls, and the lipid component of their membrane is directly exposed to the extracellular medium. Thus, they are widely used for studies of biomembranes [38, 39].

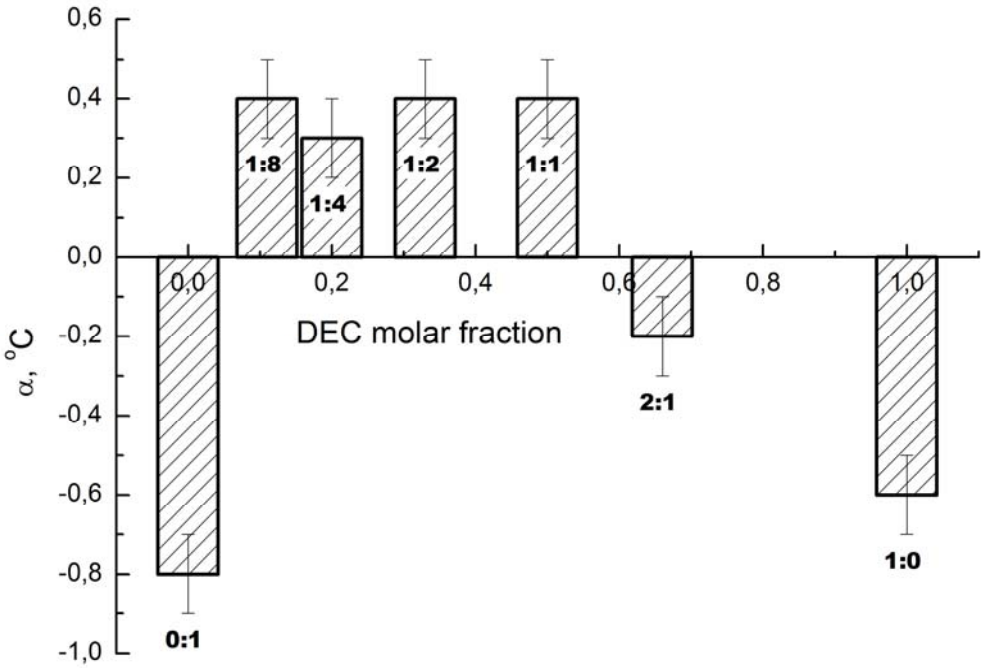


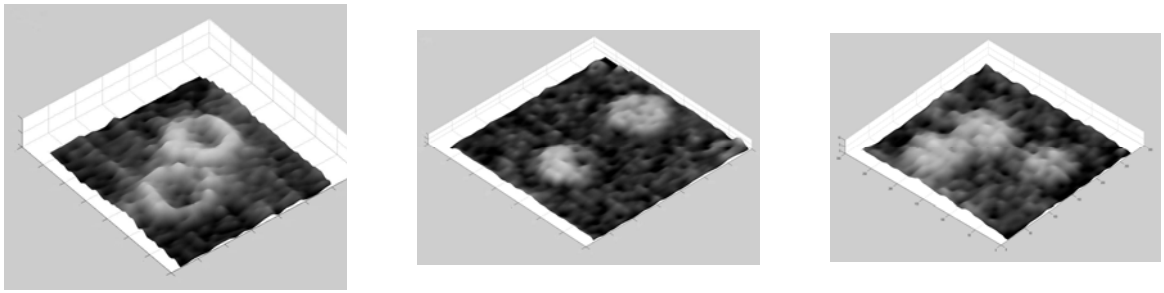
Fig. 5. Membranotropic activity coefficient of drugs as function of DEC molar fraction in the DEC+ASA mixture. Molar DEC to ASA ratios are marked as labels. Standard deviations are given as error bars.

The erythrocyte shape can be specified with sphericity coefficient k (see Fig. 1). The coefficient k is inversely proportional to the surface area of an erythrocyte, and can be considered as a characteristic of its morphological functionality [29]. For biconcave erythrocytes $k < 1$, for flat disk-shaped erythrocytes $k \sim 1$, and for spherical erythrocytes $k > 1$.

In our DHIM experiment, in the absence of drugs the biconcave erythrocyte shape was predominant; the mean k value was 0.45 ± 0.12 (Fig. 6, a). After addition of DEC or ASA solutions, the native erythrocytes morphology was changed, and the mean k value increased up to 1.1 ± 0.2 (Fig. 6 b, c). Such erythrocyte shape transformation is non-specific; it was previously reported for various substances (including drugs and cationic detergents) and external factors [30, 31, 40].

Also, there are distinctive features of DEC and ASA effects on erythrocytes. So, DEC induced instantaneous erythrocyte haemolysis at concentration above 0.05 % w/w (see Fig. 6, b), whereas in ASA-containing samples haemolysis was absent during observation time even under much higher concentrations (up to 1.5 % w/w), and echinocytosis was observed (see Fig. 6, c). The difference between DEC and ASA effects on erythrocytes could result from negative charge of the erythrocytes membrane surface. Indeed, binding of DEC dication to outer membrane surface is facilitated by this charge, whereas ASA anion binding becomes hindered, so ASA effect weakens. Generally, molecular mechanism of DEC-induced haemolysis should be a subject of thorough research.

Based on findings described above, DEC-induced haemolysis of erythrocytes was studied in more details. Kinetic of haemolysis under drug(s) action was monitored by calculation of the number of visible erythrocytes in the microscope field. Solutions of DEC, ASA and DEC+ASA 1:2 were used. This ratio was chosen basing on the DSC data presented above (see Fig. 5).



a) b) c)

Fig. 6. DHIM images of erythrocytes in donor's blood sample (a) and erythrocytes in the presence of DEC (b) or ASA (c).

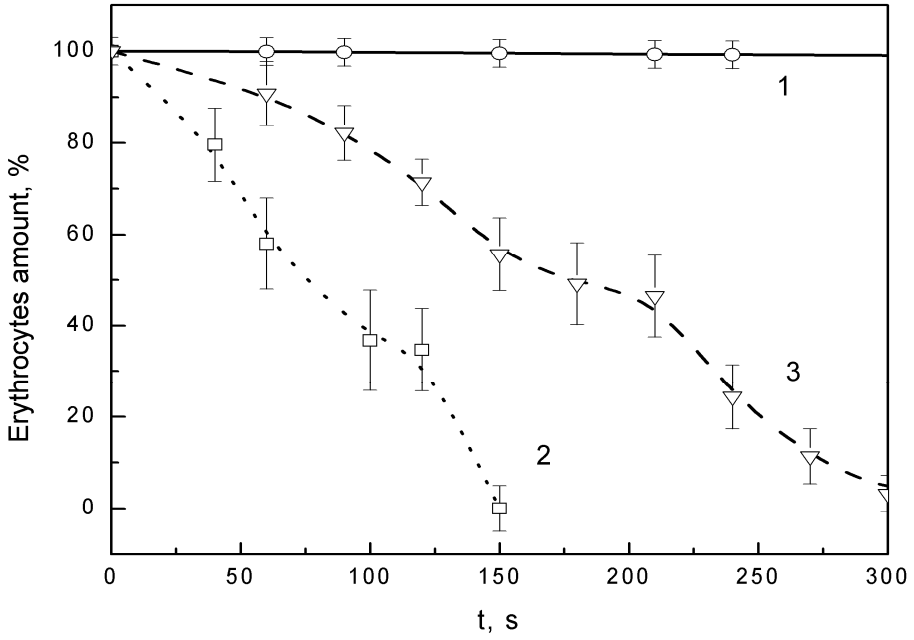


Fig. 7. Kinetic of erythrocytes haemolysis in the field of optical microscope in 0.05 % w/w solution of ASA (1), DEC (2) and of their mixture in molar ratio DEC to ASA 1 to 2 (3). The values of confidence intervals are given as error bars.

As one can see (Fig. 7), haemolysis goes rather quickly in DEC solutions, so in approximately 2 minutes the microscope field became virtually clear. On the contrary, haemolysis was practically absent in ASA solutions during 3 hours (time range is not shown completely in Fig. 7). Under joint DEC-ASA action, haemolysis deceleration was observed as compared to DEC-containing samples; the time of complete haemolysis became approximately 6 minutes. Thus, intermolecular interactions between DEC and ASA leads to deceleration of the DEC-induced erythrocytes haemolysis.

CONCLUSIONS

In phospholipid-containing systems of escalating complexity, various effects of joint DEC-ASA action were observed:

(a) In solutions containing DPPC, DEC and ASA probed by ESI mass spectrometry, the non-covalent DEC-ASA complexes formation was shown, as well as competition between the individual drugs for binding with the lipid component was registered;

(b) In model DPPC membranes, opposite individual and joint effects of DEC and ASA were revealed by DSC. While DEC and ASA individually caused pronounced T_m decreasing, their combined introduction led to T_m increasing;

(c) Being added to erythrocytes, both drugs caused erythrocyte spherocytosis, as revealed by DHIM technique. DEC induced erythrocyte haemolysis, which was decelerated by ASA under joint DEC-ASA application.

Thus, we have shown by different physical experimental techniques that formation of non-covalent complexes between two drugs could be a reason for their non-additive effects at supramolecular and cellular levels. Each of three systems probed can provide preliminary information on possible intermolecular interactions between different types of membranotropic agents prior to their biological testing. The used study approach is proposed as effective primary tool of investigation of drugs joint action prior to their pharmacological testing.

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