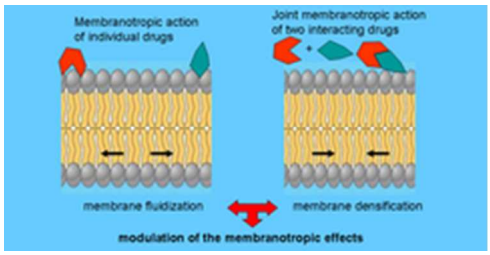
Probing of Combined Effect of Bisquaternary Ammonium Antimicrobial Agents and Acetylsalicylic Acid on Model Phospholipid Membranes: Differential Scanning Calorimetry and Mass Spectrometry Study

Journal:	*Molecular BioSystems*
Manuscript ID:	MB-ART-07-2014-000420.R1
Article Type:	Paper
Date Submitted by the Author:	22-Aug-2014

Complete List of Authors:	Kasian, Natalia; Institute for scintillation materials NAS of Ukraine, Molecular and hetero-structured materials Pashynska, Vlada; B. Verkin Institute for Low Temperature Physics and Engineering of the NAS of Ukraine, Department of Molecular Biophysics Vashchenko, Olga; Institute for scintillation materials NAS of Ukraine, Molecular and hetero-structured materials Krasnikova, Alina; Institute for scintillation materials NAS of Ukraine, Molecular and hetero-structured materials Gömöry, Agnes; Institute of Organic Chemistry Research Centre for Natural Sciences Hungarian Academy of Sciences, Kosevich, Marina; B. Verkin Institute for Low Temperature Physics and Engineering of the NAS of Ukraine, Department of Molecular Biophysics Lisetski, Longin; Institute for scintillation materials NAS of Ukraine, Molecular and hetero-structured materials



Qualitatively different membranotropic effects of the individual drugs and complexes formed in their combined use were demonstrated by differential scanning calorimetry
20x10mm (300 x 300 DPI)

Molecular Biosystems RSCPublishing

ARTICLE

Probing of Combined Effect of Bisquaternary Ammonium Antimicrobial Agents and Acetylsalicylic Acid on Model Phospholipid Membranes: Differential Scanning Calorimetry and Mass Spectrometry Study

Cite this: DOI: 10.1039/x0xx00000x

Received 00th July 2014,
Accepted 00th July 2014

DOI: 10.1039/x0xx00000x

www.rsc.org/

N. A. Kasian[a], V. A. Pashynska[b], O. V. Vashchenko[a], A. O. Krasnikova[a], A. Gömöry[c], M. V. Kosevich[b], and L. N. Lisetski[a]

A model molecular biosystem of hydrated dipalmitoylphosphatidylcholine (DPPC) bilayers that mimics cell biomembranes is used to probe combined membranotropic effects of drugs by instrumental techniques of molecular biophysics. Differential scanning calorimetry reveals that doping of the DPPC model membrane with individual bisquaternary ammonium compounds (BQAC) decamethoxinum, ethonium, thionium and acetylsalicylic acid (ASA) leads to lowering of the membrane melting temperature (T_m) pointing to membrane fluidization. Combined application of the basic BQAC and acidic ASA causes an opposite effect on T_m (increase), corresponding to the membrane densification. Thus, modulation of the membranotropic effects under combined use of the drugs studied can be revealed at the level of model membranes. Formation of noncovalent supramolecular complexes of the individual BQACs and ASA with DPPC molecules, which may be involved in the mechanism of the drug-membrane interaction at the molecular level, is demonstrated by electrospray ionization (ESI) mass spectrometry. In the ternary (DPPC + ASA + BQAC) model systems, the stable complexes of the BQAC dication with ASA anion, which may be responsible for modulation of the drugs membranotropic effects, were recorded by ESI mass spectrometry. The proposed approach can be further developed for preliminary evaluation of the drugs combined effects at the level of model lipid membranes prior to tests on living organisms.

Key words: bisquaternary ammonium compounds, acetylsalicylic acid, dipalmitoylphosphatidylcholine, model lipid membranes, differential scanning calorimetry, electrospray ionization mass spectrometry, combined drugs effects.

Introduction

The efficiency of combined multi-drug treatment of microbial infections can be enhanced not only by invention of new medicines, but by just avoiding possible antagonistic effects of several pharmaceuticals being applied simultaneously [1-3]. Efficient rapid evaluation of the drug activity can be achieved by means of experimental testing methods using model molecular and supramolecular systems, requiring shorter time and much smaller number of tests to be carried out on living organisms [4]. Recently, a growing interest has been observed in drug-lipid membrane interactions studies using model lipid membranes [e.g. 5-8, and references therein]. This is due to understanding of the relevance of drug-lipid interactions for pharmacokinetic properties of drugs (bioavailability, biodistribution, accumulation) and hence their pharmacological efficiency [6-8]. Another important aspect of drug-lipid interactions studies is a deeper insight into molecular mechanism of drug activity [5-9]. Such biomimetic media as model lipid membranes can be probed by experimental techniques of molecular physics and biophysics [5, 10, 11]. The majority of works in this field deal with effects of individual substances such as antibiotics, anticancer drugs, steroids, vitamins etc. on the physical parameters of model lipid membranes [5-8]. Evaluation of combined membranotropic

effect of several substances, to the best of our knowledge, is rather uncommon. Only a few works deal with joint membranotropic action: vitamin D_2/Ca^{2+} in dipalmitoylphosphatidylcholine (DPPC) membranes [12-13], vitamin E/Ca^{2+} in dimyristoylphosphatidylserine membranes [14], cholesterol/Ca^{2+} in phospatidylserines membranes [15], cholesterol/amphotericin B and ergosterol/amphotericin B in DPPC membranes [16], methanol/vitamin C and ethanol/vitamin C in DPPC mono-layers [17]. In all the works mentioned above, the simultaneous presence of two substances mutually altered their membranotropic behavior. Remarkably, no specific interactions between these substances were noted, hence these effects can be considered as membrane-mediated.

In the present work, we describe an approach to study combined effect of drugs on model membranes based on multibilayers of hydrated DPPC. These drugs are bisquaternary ammonium compounds (BQAC), broad-spectrum antiseptics used for treatment of certain bacterial, mycotic and viral infections [18, 19], as well as a multi-functional drug - acetylsalicylic acid (ASA), with diverse therapeutic applications including symptomatic treatment of infections. The drugs are of basic and acidic nature which conditioned their potential intermolecular interaction. As known from the literature [18-21], membranes of bacterial cells are considered as the main targets of antiseptic quaternary and bisquaternary ammonium salts, so the membranotropic effect is believed to be an important constituent of their antimicrobial activity. Investigations of bactericidal effects of the BQACs showed that these drugs caused disintegration of the bacterial membrane structures leading to increase of their permeability and to alteration of the membrane enzymes functioning [18-21]. The membranotropic properties of ASA were revealed as well [22]. The idea to probe the combined effect of basic and acidic membranotropic agents emerged from our previous inter-disciplinary studies of molecular mechanisms of action of BQAC drugs decamethoxinum (DEC), ethonium (ETH) and thionium (THI) (Fig. 1). The main results of these investigations [23-31] may be summarized as follows:

1) Probing of DPPC model membranes doped by DEC, ETH, THI by differential scanning calorimetry (DSC) revealed significant lowering of membranes melting temperature (T_m) [23-25, 30], which is a marker of the membrane fluidization. This can be the reason of the BQACs pharmacological effect reported above.

2) Probing of the binary (DPPC + BQAC) systems by electrospray ionization (ESI) mass spectrometry (based on spraying of liquid solutions of biomolecules [32, 33]) revealed formation of stable supramolecular complexes of the BQAC dication (Cat^{2+}) with a number (up to nine) of DPPC molecules [29, 31].

3) By the evidence of matrix-assisted laser desorption/ionization mass spectrometry (MALDI), substitution of inorganic Cl^- counterion of BQAC salts by anions of organic 2,5-dihydroxybenzoic acid (DHB) was observed [27, 28]. It should be noted, that DHB used as MALDI matrix compound, is a metabolite of ASA [34].

On the basis of the above finding, it was suggested that under simultaneous application of the basic and acidic substances, their complexes can lead to modification of their membranotropic activity. This assumption was verified by the DSC method [25, 30]. For ternary (DPPC + BQAC + DHB) systems, it was demonstrated that the effect of the combined action of BQAC and DHB on T_m of DPPC membranes differed qualitatively from their individual action and consisted in increasing of T_m [30]. The data obtained stimulated us to study noncovalent interactions of BQAC with biologically significant organic acids [29].

So the aim of the present work was to investigate the combined effect of antimicrobial drugs DEC, ETH, THI with multi-functional ASA on DPPC model membrane using DSC and ESI mass spectrometric techniques. Such model studies of the combined drugs action performed on molecular biosystems could be a step to their pre-biological testing.

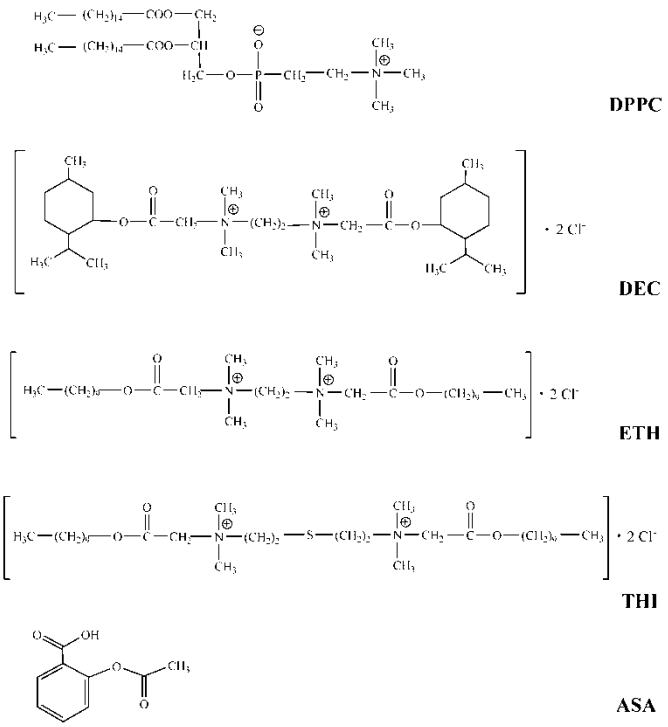


Fig. 1. Structural formulae of compounds under study: DPPC ($C_{40}H_{80}NO_8P$); DEC ($C_{38}H_{74}Cl_2N_2O_4$); ETH ($C_{30}H_{62}Cl_2N_2O_4$); THI ($C_{32}H_{66}Cl_2N_2O_4S$) and ASA ($C_9H_8O_4$)

Experimental

DEC, ETH, THI in the form of chloride salt (Fig. 1) were synthesized at the Institute of Organic Chemistry of the National Academy of Sciences of Ukraine (Kiev, Ukraine) and used without further purification. ASA was obtained from the State Scientific Centre of Medications (Kharkov, Ukraine). DPPC was purchased from "Alexis Biochemicals" (Switzerland).

Differential scanning calorimetry

For DSC studies, DPPC was hydrated with 70% (w/w) of bidistilled water to provide formation of the lamellar phase. Then, the samples were incubated at room temperature for 4 to 6 days with repeated heating to the temperature of approx. 50° C (above T_m) with intense stirring. In preparation of the DPPC membrane doped with one of the drugs studied, the same

procedure was performed, using the aqueous stock solutions of the drugs instead of the bidistilled water. The drugs content relative to dry DPPC ranged from 1 to 15% (w/w). To study combined drugs membranotropic action, series of DPPC membranes with various content of two drugs were prepared. The total amount of (BQAC + ASA) was 1% (w/w) relatively to the weight of dry DPPC; molar ratios BQAC to ASA in the system were 1:8, 1:4, 1:2, 1:1 and 2:1. Further procedure of model membrane preparation was performed as described for pure DPPC.

Thermodynamic properties of the model membrane systems were studied by DSC technique using calorimeter Mettler DSC 1 (Mettler Toledo, Switzerland). Phase transitions were detected from low-temperature gel phase ($L_{\beta'}$) into the ripple ($P_{\beta'}$) phase (pre-transition) and then to the high-temperature liquid crystalline (L_α) phase (the main transition, or membrane melting) [10, 35]. The samples (approx. 20 mg) were placed into aluminum crucibles and sealed. The programmed scheme of the temperature scanning consisted of repeating heating-cooling cycles with rate 2 K/min. The parameters of the phase transitions were determined using the original Mettler DSC 1 software. The experimental error for T_m value was ±0.1° C, for ΔH_m value was ±1.5 kJ/kg, for *FWHM* ±0.2° C, and for asymmetry ±0.04.

ESI mass spectrometry

For ESI mass spectrometry investigations, stock solutions of BQACs, ASA and DPPC (5 mM) were prepared in methanol. Corresponding volumes of the stock solutions were mixed to provide the molar ratio of the drugs to DPPC 1:10 in binary systems. For ternary systems (DPPC + BQAC + ASA) molar ratio were 10:1:1. The mixtures were incubated at room temperature for at least 10 minutes before the ESI analysis. The ESI mass spectrometry spraying procedure required dilution of the solutions to provide 250 μM (or less) final concentration of the components.

ESI 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 spraying capillary was operated at 3.5 kV. The cone voltage (CV) value of 20 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 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)

DPPC membranes, reflecting integral changes in supramolecular ordering of lipids.

The original DSC thermograms (obtained in heating mode) of binary model systems (DPPC + drug 5% w/w) are represented in Fig. 2. It is clearly seen that all the drugs cause lowering of the membrane melting temperature T_m, smearing of the melting peak and disappearance of the pre-transition peak. Decreasing of T_m indicates membrane fluidization and so increasing of its permeability.

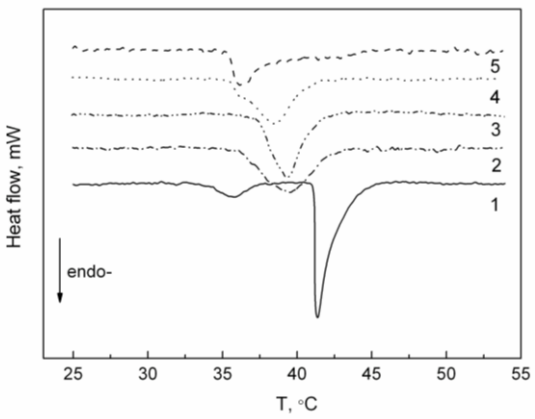


Fig. 2. DSC thermograms (heating mode) of (DPPC + drug 5% w/w): no admixture (1), THI (2), ETH (3), DEC (4), ASA (5)

The concentration dependence of T_m shift (ΔT_m) for (DPPC + DEC) system presented in Fig. 3 demonstrates a linear character up to 10% w/w of DEC. Further increasing of the DEC concentration does not result in significant changes of ΔT_m values. So, one can conclude that DEC incorporation in DPPC membrane is limited to approximately 10% w/w.

In order to compare the membranotropic effects of different drugs, mass and molar membranotropic activity coefficients (α_{mas} and α_{mol}) were determined. These coefficients represent the shift of the membrane melting temperature ΔT_m upon addition of 1% w/w (or 1 mol. %) of the admixture:

$$\alpha_{mas} = (T_m - T_m^o)/c_{mas}$$
$$\alpha_{mol} = (T_m - T_m^o)/c_{mol},$$

where T_m^o is the main phase transition temperature of the DPPC membrane without admixtures, T_m is the main phase transition temperature of the membrane with admixture, c_{mas} and c_{mol} are the admixture concentration in mass or molar percents.

The values of ΔT_m, α_{mas} and α_{mol} for the drugs studied are summarized in Table 1.

Results and Discussion

Membranotropic activity of individual drugs

The first step of our investigation included characterization of membranotropic effects of the individual drugs by means of DSC. DSC allows us to explore the effects of dopants on phase states of

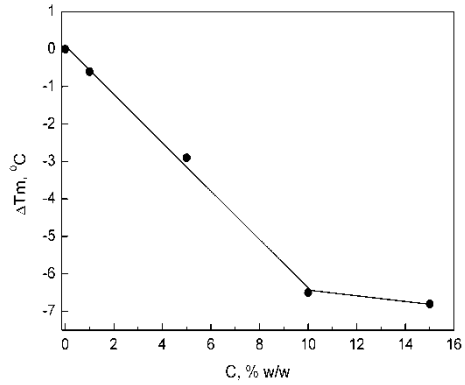


Fig. 3. Shift of the melting temperature of (DPPC + DEC) system *vs.* DEC concentration

Table 1. Characteristics of membranotropic effects of the drugs studied.

Drug	Monoisotopic/ average† molecular mass (Da)	ΔT_m††, °C	Membranotropic activity coefficients	
			α_{mas}, °C	α_{mol}, °C
DEC	692.5 / 693.9	-2.8	-0.6	-0.6
ETH	644.5 / 645.9	-2.0	-0.4	-0.4
THI	584.4 / 585.7	-2.0	-0.4	-0.3
ASA	180.0 / 180.2	-5.1	-1.0	-0.3

Among the drugs studied, the highest value of α_{mas} was obtained for ASA (-1.0° C), but the corresponding value accounting for the molecular mass, α_{mol}, appears close to that for ETH, THI. For DEC, both α_{mas} and α_{mol} appear to be the highest among all BQACs. Individual membranotropic action of BQACs and ASA was similar to the effects of most dopants in liquid crystalline media [36], i.e. decreasing of the membrane melting temperature and smearing of the DSC phase transition peaks.

Combined drugs membranotropic action

The next step was to study the drugs effects under their simultaneous introduction into DPPC membrane, i. e. combined membranotropic action. It was demonstrated that the combined membranotropic action of the (BQAC + ASA) is qualitatively different as compared to the effects of the individual substances.

Fig. 4 demonstrates that while the individual drugs (ETH and ASA) cause decrease of T_m, their joint membranotropic action becomes the opposite, i.e. T_m increases. The same effects were observed for pairs (DEC + ASA) and (THI + ASA).

To specify this effect, we used the approach and methodology of so-called "quasi-binary systems" developed by Fialkov [37] and applied to model phospholipid membranes in [25]. According to this approach, the lipid membrane is considered as a medium where certain interaction between the admixtures could take place. In the absence of specific intermolecular interactions, the system parameters are additive with concentration of the admixtures. On the contrary, if certain specific interactions between the admixtures take place, the parameters change in non-linear manner, and the admixtures ratio corresponding to the maximum deviation from additivity indicates the most probable stoichiometry of the intermolecular complex formed by the admixtures.

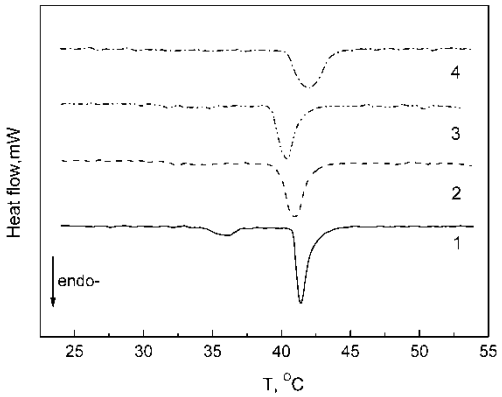


Fig. 4. DSC thermograms of the DPPC membrane (1) and the DPPC membrane containing 1% w/w of ETH (2), ASA (3) and (ETH + ASA) 1 to 2 mol/mol (4)

Based on the DSC thermograms of DPPC membrane with individual drug or drugs pairs (BQAC + ASA) with varying molar ratios, quasi-binary phase diagrams were plotted in coordinates "T_m vs. *n(BQAC)*" (Fig. 5). Here *n(BQAC)* is the BQAC molar fraction in the admixture, i. e. $n(BQAC) = \nu_{BQAC}/(\nu_{BQAC} + \nu_{ASA})$, where ν is the number of moles in the system. The total content of the admixture(s) was 1% w/w (relatively to dry DPPC) with molar ratios BQAC to ASA 2:1, 1:1, 1:2, 1:4, and 1:8. The horizontal line in the plot corresponds to T_m of the non-doped DPPC membrane (41.4º C). An imaginary straight lines connecting points 0.0 and 1.0 mole fractions of BQAC correspond to additive dependence of T_m on admixtures molar ratio.

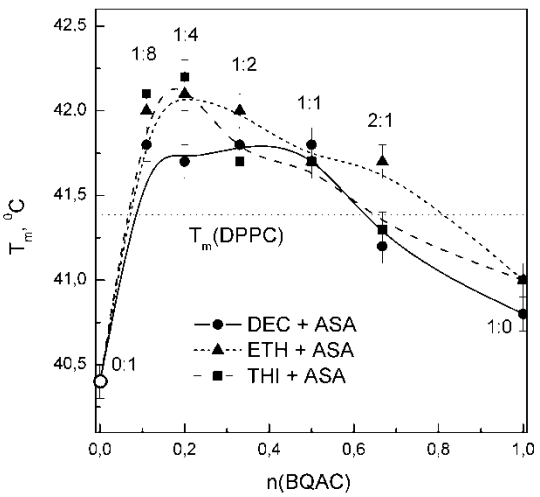


Fig. 5. Main phase transition temperature for DPPC membrane doped with 1% w/w (BQAC + ASA) vs. molar fraction of BQAC in the admixture. Molar ratios BQAC to ASA are marked above the experimental points.
○ – T_m for the system (DPPC + ASA)

As one can see from Fig. 5, the general trend of the plots for all three systems is similar. For all the pairs (DEC + ASA), (THI + ASA) and (ETH + ASA), the dependences of T_m on *n(BQAC)* are essentially non-linear, suggesting certain interaction between the admixtures resulting in complex formation.

For a wide range of *n(BQAC)* (from 0.1 to 0.7), increasing of T_m is observed. Such an effect is unusual and indicates densification of the bilayer due to increased ordering of the lipid molecules. Thus, (BQAC + ASA) complexes do interact with DPPC membrane, but in essentially different manner compared to the individual drugs.

Taking into account that in water medium BQACs dissociate forming dications Cat^{2+} , noncovalent complex of Cat^{2+} with two $[ASA\text{-}H]^-$ anions is electrically neutral (each of the two charged quaternary ammonium groups of a BQAC fits to one anion of ASA). Molar ratios of BQAC to ASA 1:4 and 1:8 correspond to negatively charged complexes, whereas at the ratio 1:1 and 2:1 the complexes formed appear to be positively charged. For (DEC + ASA) system, the plotted quasi-binary phase diagram has no sharp maximum, but a broad plato for 1:8, 1:4, 1:2, and 1:1 molar ratios (see Fig. 5). For the systems with ETH and THI, a maximum at 1:4 BQAC to ASA ratio can be noted. Though the mechanisms of interaction with membrane depend on the charge state of the complex, the wide concentration range where increased T_m are observed suggests qualitatively similar membranotropic effects for both neutral and charged complexes.

It is interesting that less than 0.1 molar fraction of BQAC is needed to eliminate T_m decrease induced by ASA, whereas elimination of T_m decrease induced by BQAC requires ~0.35 molar fraction of ASA. Thus, ASA causes weaker modulation effect on membranotropic activity of BQAC as compared with the effect of the same molar fraction of BQAC on activity of ASA.

Additional thermodynamic parameters of the systems studied are presented in Table 2. As one can see, the main phase transition enthalpy (ΔH_m) slightly decreases both in binary and in ternary systems, reflecting certain weakening in lipid-lipid interactions under doping the DPPC membrane with drugs. No significant difference between ΔH_m values for individual and joint drugs action is noticed. In contrast to ΔH_m, the full width at half-maximum (FWHM) of the main phase transition peak exhibits clear tendency to increase in the following sequence: undoped membrane < binary system < ternary system. So, one can conclude that under combined drugs action, the cooperativity of DPPC membrane phase transition (the parameter which is reciprocal to FWHM) decreases more significantly than under their individual action.

The asymmetry parameter (*A)* of the main phase transition peak was calculated according to [25] as a ratio of the right-hand shoulder of FWHM (with respect to T_m) to the FWHM value. In the case of the admixture affinity to $L_{\beta'}$ phase, one can observe extension of the left-hand shoulder of the melting peak; correspondingly, the right-hand shoulder extension is caused by the admixture affinity to L_α phase. For fully symmetric peaks A = 0.5, for low-temperature phase affinity this value decreases and for high-temperature phase affinity it increases. The values obtained indicate that all BQACs possess preferential affinity to $L_{\beta'}$ phase whereas ASA tends to stabilize L_α phase.

Based on the results of the present work, one could make the following recommendation from the viewpoint of medical application. BQAC antiseptics should be used separately from drugs of acidic nature (such as ASA) or acidic alimentary products (such as citrus fruits).

We suppose that the approach described can be used for pre-clinical estimation of combined drugs efficiency in the case when pharmacological effect of at least one of them is associated with the cell membrane.

Table 2. Parameters of main phase transition of DPPC membranes doped with the drugs in binary systems (DPPC + drug) and ternary systems (DPPC + BQAC + ASA) for BQAC to ASA 1:4.

System	ΔH_m, kJ/kg	FWHM, ºC	*A*
DPPC	23.5	1.0	0.6
DPPC + DEC	19.1	2.8	0.4
DPPC + DEC + ASA	23.2	3.7	0.5
DPPC + ETH	21.1	2.3	0.5
DPPC + ETH + ASA	20.3	3.5	0.5
DPPC + THI	20.2	2.7	0.4
DPPC + THI + ASA	22.4	3.2	0.5
DPPC + ASA	23.4	1.8	0.7

Experimental errors: ΔH_m ±1.5 kJ/kg, FWHM ± 0.2, ºC, A ± 0.04

Mass spectrometry studies of (DPPC + drug) systems

To study the above-described combined membranotropic effect of BQAC and ASA at the molecular level, model systems containing DPPC and the drugs were examined using ESI mass spectrometry approach, which was developed for such systems in [29].

First, binary (DPPC + drug) systems were tested. In Fig. 6 the ESI mass spectrum of (DPPC + ASA) binary mixture with 10:1 molar ratio is presented. Peaks of the individual components were found in the spectrum: $ASA{\bullet}Na^+$ (*m/z* 203.0), $2ASA{\bullet}Na^+$ (*m/z* 383.1) for ASA and $DPPC{\bullet}H^+$ (*m/z* 734.6), $DPPC{\bullet}Na^+$ (*m/z* 756.6), $2DPPC{\bullet}Na^+$ (*m/z* 1490.1) for DPPC. It should be noted that cationization by sodium ions is typical for ESI mass spectrometric technique and correlates with ion-molecule interactions under natural conditions of physiological solution. Further mass spectrum analysis reveals the presence of the peak of $ASA{\bullet}DPPC{\bullet}Na^+$ (*m/z* 936.6) ion that is a cationized noncovalent complex of ASA with phospholipid molecule formed in the system studied. The stable complexes recorded under ESI conditions prove noncovalent binding of ASA to DPPC; such a binding can be considered as a molecular mechanism of the mebranothropic action of ASA confirmed by the DSC study (Table 1).

Analyzing the data more deeply we have realized that ASA binds with a single DPPC molecule only. This result differs from the features of binding of some BQAC ($Cat^{2+}{\bullet}2Cl^-$) with DPPC in their binary mixtures studied in our previous works [29, 31]. It was found that the dication Cat^{2+} of the BQAC can bind up to 9 DPPC molecules for DEC [31] and up to 4 DPPC for ETH [29]. Such supramolecular complexes, $[Cat{\bullet}nDPPC]^{2+}$ can be supposed to be an adequate model of complexes of BQAC with phospholipid membrane assemblies. In the current ESI study we have probed the (DPPC + THI) binary system in 10:1 molar ratio. The spectrum of (DPPC + THI) system is shown in Fig. 7.

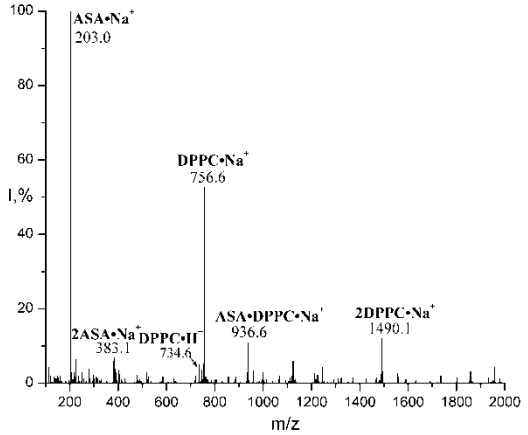


Fig. 6. ESI mass spectrum of (DPPC + ASA) system

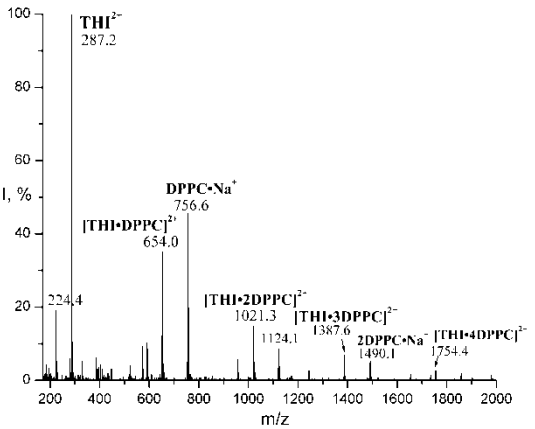


Fig. 7. ESI mass spectrum of (DPPC + THI) system

Along with the characteristic ions of DPPC listed above and that of thionium (THI^{2+}, *m/z* 286.2) the peaks of the complexes of THI with one, two, three and four molecules of DPPC have been recorded: $[THI•DPPC]^{2+}$, *m/z* 654.0; $[THI•2DPPC]^{2+}$, *m/z* 1021.3; $[THI•3DPPC]^{2+}$, *m/z* 1387.6; $[THI•4DPPC]^{2+}$, *m/z* 1754.4. The supramolecular complexes $[THI•nDPPC]^{2+}$ with larger amount of phospholipid molecules could not be detected in the current experiment because of the mass range limit (2000 a.u.) of the instrument used.

Distinctions in the types of the complexes formed by ASA and BQACs with DPPC are in correlation with the differences in mechanisms of their intermolecular interactions with phospholipids. While the dications of surface active BQACs are known to be incorporated as components into the phospholipid assemblies [7, 18], ASA molecule, as it is shown in Ref. [22], interacts with the glycerol moiety of a separate DPPC molecule. Thus, the results of the ESI mass spectrometric study of the binary systems (DPPC + ASA) and (DPPC + BQAC) show the stable drug-phospholipids noncovalent complexes formation confirming at the molecular level the drugs membranothropic activity. They also point at one of the possible mechanisms of the drugs activity modulation related to possible competition of the drugs for the binding with the membrane phospholipids.

At the next stage of the mass spectrometric study, the ESI mass spectra of equimolar mixtures of the BQAC and ASA were obtained. The spectrum of the (ASA + THI) system is shown in Fig. 8 as an example. Intensive peaks of noncovalent clusters of the Cat^{2+} •$(ASA-H)^-$ type were revealed for all mixtures: $[THI•(ASA-H)]^+$, *m/z* 753.6 for (ASA + THI) mixture (Fig. 8), as well $[DEC•(ASA-H)]^+$, *m/z* 801.6 and $[ETH•(ASA-H)]^+$, *m/z* 693.5 for (ASA + DEC) and (ASA + ETH) systems, respectively. The ions characteristic for individual components were recorded as well: Cat^{2+} and Cat^{2+} ·Cl^- for the BQAC, $ASA•Na^+$ (*m/z* 203.3) and $ASA•K^+$ (*m/z* 219.0) for ASA.

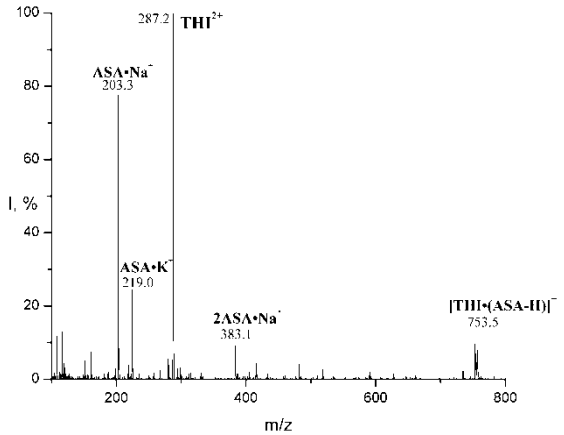


Fig. 8. ESI mass spectrum of (ASA + THI) system

The results of the (ASA + BQAC) systems study point to one more possible mechanism of the BQAC-based drugs action modulation by ASA based on the formation of stable pair complexes between the BQAC dication and the organic acid anion, which deactivates the ionic forms of the drugs. It should be noted that no definite information can be obtained on possible formation of triple complexes Cat^{2+} •$2(ASP-H)^-$, since they are neutral and thus undetectable by mass spectrometry. These ESI results also directly confirm the results of the DSC experiments for DPPC membrane doped with 1% (w/w) (BQAC + ASA) (Fig. 5) in which the increasing of T_m has been registered in contrast to the T_m decreasing under doping of DPPC membranes by individual drugs. This has been explained by formation of the drugs complexes in the system with combined doping by ASA and BQAC.

Finally, the ternary systems (DPPC + ASA + BQAC) (10:1:1 molar ratio) have been probed. In Fig. 9 the spectrum of the (DPPC + ASA + THI) system is presented as an example. The spectra showed both complexation of ASA with BQAC and existence of a competition between the ASA and BQAC for binding with the DPPC molecules in the systems studied, predicted above. The two mechanisms are evidenced by comparable intensity of the peaks of the complexes of $[Cat•nDPPC]^{2+}$ (n=1÷4), $[Cat•(ASA-H)]^+$ and $ASA•DPPC•Na^+$ (*m/z* 936.6). The following peaks are recorded in the mass spectra: the characteristic peaks of ASA - $ASA•Na^+$ (*m/z* 203.0), $2ASA•Na^+$ (*m/z* 383.1); the peak of THI intact dication THI^{2+}, *m/z* 287.2; the characteristic peaks of phospholipids $DPPC•H^+$ (*m/z* 734.6), $DPPC•Na^+$ (*m/z* 756.6), $2DPPC•Na^+$ (*m/z*

1490.1) and the peaks of noncovalent complexes of ASA and THI with DPPC and pair drugs complexes - ASA•DPPC•Na^+ (*m/z* 936.6), $[THI•DPPC]^{2+}$, *m/z* 654.0; $[THI•2DPPC]^{2+}$, *m/z* 1021.3; $[THI•3DPPC]^{2+}$, *m/z* 1387.6; $[THI•4DPPC]^{+}$, *m/z* 1754.4, $[THI•(ASA-H)]^{+}$, *m/z* 753.5.

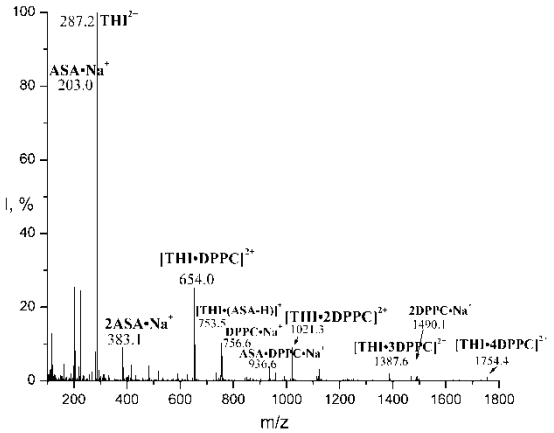


Fig. 9. ESI mass spectrum of (DPPC + ASA + THI) system

The intermolecular complexes recorded in the mass spectra reflected the complexity of specific noncovalent interactions in the model systems. Firstly, supramolecular complexes of the BQAC dication with up to 4 DPPC molecules were formed similarly to the drug-phospholipid associates in their binary systems [23, 29, 31]. Secondly, ASA bound to a single DPPC molecule similarly to its behaviour in the binary system (Fig. 8). Thirdly, dication-anion Cat^{2+} •$[ASA-H]^-$ complexes observed in the binary (BQAC + ASA) mixtures are formed in the ternary system as well. The peaks distribution in the mass spectra pointed to competition between BQACs and ASA for binding to DPPC molecules in the three systems, since abundances of the peaks of nDPPC•Cat^{2+} complexes, Cat^{2+} •$[ASA-H]^-$ and DPPC•ASA•Na^+ associates were of comparable intensities. The competition for binding of ionic forms of the drugs to DPPC molecules and the formation of dication-anion complexes, revealed on the basis of mass spectrometric data, can be considered as molecular mechanisms of the possible modulation of the drugs effects on the membrane.

Conclusions

An approach to preliminary tests of combined effect of drugs at the level of molecular biosystems, such as DPPC model membranes, is proposed.

The membranotropic effect of the drugs is detected by changes of the membrane melting temperature determined by DSC. It is shown that doping of membranes by individual drugs DEC, ETH, THI and ASA causes T_m decrease (membrane fluidization), while the combined use of BQAC and ASA causes T_m increase (membrane densification).

Interaction of the drugs at molecular level is probed by ESI mass spectrometry. Formation of noncovalent supramolecular complexes of the individual BQAC dications with DPPC molecules and assemblies as well as of ASA with one DPPC molecule demonstrated in the ESI mass spectrometry experiments may be involved in the molecular mechanisms of the drugs membranotropic activity. In the binary (BQAC + ASA) and ternary (DPPC + BQAC + ASA) systems, a new type of complexes is recorded by ESI mass spectrometry, namely the complexes of the BQAC dication with the deprotonated ASA anion.

Method of quasi-binary phase diagrams permits to determine complexes (BQAC + ASA) formation in lipid membrane medium. Formation of such complexes may be responsible for modulation of the drugs effects under combined application.

The approach proposed can be applied for preliminary evaluation of the combined effects of various drugs at the level of molecular biosystems prior to testing on living organisms. So in the case of combined application of BQACs with ASA, we can assume that their pharmacological effect could be reduced.

Acknowledgements

Authors acknowledge the Program of cooperation between Ukrainian and Hungarian Academies of Sciences for the financial support of visit of the scientists from B. Verkin Institute for Low Temperature Physics and Engineering of the National Academy of Sciences of Ukraine to the Institute of Organic Chemistry of Research Centre for Natural Sciences of the Hungarian Academy of Sciences, where the mass spectrometry experiments were carried out.

Notes and references

[a] Institute for Scintillation Materials of the National Academy of Sciences of Ukraine, 60, Lenin ave., 61001 Kharkov, Ukraine

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

[c] Institute of Organic Chemistry of Research Centre for Natural Sciences of the Hungarian Academy of Sciences, Pusztaszeri str., 59-67, Budapest, H-1025, Hungary.

† Monoisotopic mass is determined as the sum of masses of the first isotopes of all atoms of the molecule; average mass is calculated as the center of weight of the envelope of polyisotopic peaks, related to low resolution mass spectrometry.

†† Data for 5% (w/w) of the drugs in DPPC membrane are presented

1 T. C. Chou, *Cancer Res*, 2010, **70**, N 2, 440.

2 J. R. Pritchard, P. M. Bruno, L. A. Gilbert, K. L. Capron, D. A. Lauffenburger, M. T. Hemann, *Proc. Natl. Acad. Sci. U S A*, 2013; **110**, 170.

3 A. K. Srivastava and Y. K. Gupta, *Indian J. Physiol.Pharmacol*, 2001, **45**, 475.

4 T. C. Chou, *Leuk. Lymphoma*. 2008, **49**, 2059.

5 T. Pignatello, L. Musumeci, C. Basile, C. Carbone, G. Puglisi, *J. Pharm. Biollied Sci.*, 2011, **3**, 4.

6 C. Peetla, A. Stine, V. Labhasetwar, *Mol. Pharm*., 2009, **6**, 1264.

7 J. K. Seydel, M. Wiese, Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling. Wiley-VCH Verlag GmbH, Weinheim, 2002, 349 p.

8 M. Lucio, J.L.F.C. Lima, S. Reis, *Current Medicinal Chemistry*, 2010, **17**, 1795
9 I. Fournier, J. Barwicz, M. Auger, P. Tancr`ede, *Chemistry and Physics of Lipids*, 2008, **151**, 41.
10 S. Tristram-Nagle, J. F. Nagle, *Chem Phys Lipids*, 2004, **127**, 3.
11 M. H. Chiu, E. J. Prenner, *J. Pharm. Bioallied. Sci*., 2011, **3**, 39.
12 N. Toyran, F. Severcan, Spectroscopy, 2002, **16**, 399.
13 N. Toyran, F. Severcan, *Chemistry and Physics of Lipids*, 2003, **123**, 165.
14 M. P. Sfinchez-Migallon, F. J. Aranda, J. C. Gomez-Fernamdez, *Biochimica et Biophysica Acta*, 1996, **1281**, 23.
15 S. Choi, W. Ware, S. R. Lauterbach, W. M. Phillips , *Biochemistry*, 1991, **30**, 8563.
16 I. Fournier, J. Barwicz, M. Auger, P. Tancr`ede, *Chemistry and Physics of Lipids*, 2008, **151**, 41.
17 M. Weis, M. Kopani, *Eur. Biophys. J*, 2008, **37**, 893.
18 A. N. Vievskij, *Tenside Surfactant, Detergents*, 1997, **34**, 18.
19 A. Vievskiy, Mechanisms of biological activity of cationic surface active compounds Kiev, 1991, 250 (in Russian).
20 P. Gilbert, L. E. Moor, *J Appl Microbiol*., 2005, **99**, 703.
21 N. N. D. Daoud, N. A. Dickinson, P. Gilbert, Microbios, 1983, **37**, 75.
22 L. Panicker, V. K. Sharma, G. Datta, K. Deniz, P.S. Parvathanathan, K.V. Ramanathan, C. L. Khetrapal, *Mol. Cryst. Liq. Cryst*., 1995, **260**, 611.
23 V. A. Pashinskaya, M. V. Kosevich, A. Gomory, O. V. Vashchenko, L. N. Lisetski, *Rapid Commun. Mass Spectrom*., 2002, **16**, 1706.
24 L. N. Lisetski, O. V. Vashchenko, A. V. Tolmachev, K. B. Vodolazhskiy, *Eur. Biophys. J*., 2002, **31**, 554.
25 O. Vashchenko, V. Pashynska, M. Kosevich, V. Panikarska, L. Lisetski, *Mol. Cryst. Liq. Cryst*., 2011, **507**, 155.
26 V.A. Pashynska, M.V. Kosevich, A. Gomory, K. Vekey, *Mass-Spectrometria*, 2012, **9**, 121.
27 V. A. Pokrovsky, M. V. Kosevich, V. L. Osaulenko, V. V. Chagovets, V. A. Pashynska, V. S. Shelkovsky, V. A. Karachevtsev, A. Yu. Naumov, *Mass-Spectrometria*, 2005, **2**, 183.
28 V. Pashynska, M. Kosevich, S. Stepanian and L. Adamowicz. *Journal of Molecular Structure: THEOCHEM*, 2007, **815**, 55.
29 V. A. Pashynska, M. V. Kosevich, A. Gomory, K. Vekey, *Biopolymers and Cell*, 2013, **29**, 157.
30 O. V. Vashchenko, V. A. Pashynska, M. V. Kosevich, V. D. Panikarska, L. N. Lisetski, *Biopolymers and Cell*, 2010, **26**, 472.
31 V.A.Pashynska, M.V.Kosevich, H.Van den Heuvel, F.Cuyckens, M. Claeys,*Biophysical Bulletin*, 2004, **1-2**, 123, (in Russian).
32 Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentation, Practicalities, and Biological Applications, Ed. C. R. Hoboken, New Jersey: John Wiley & Sons, Inc., 1008 p.
33 Th. Wyttenbach, M. T. Bowers *Annu. Rev. Phys. Chem*., 2007, **58**, 511.
34 K. Ashidate, M. Kawamura, D. Mimura, H. Tohda, S. Miyazaki, T. Teramoto, Y. Yamamoto, Y. Hirata, *Eur J Pharmacol*., 2005, **513** 173.
35 R. Koynova, M. Caffrey, *Biochim. Biophys. Acta*., 1998, **1376**, 91.
36 P. K. Mukherjee, *Liq. Cryst*., 1997, **22**, 239.
37 Yu. Ia. Fialkov, A. N. Zhitomirskij, Yu. A. Tarasenko, Physical Chemistry of Non-Aqueous Solutions, Leningrad: Khimija, 1973, 376 p (in Russian).

