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MASS SPECTROMETRIC STUDY OF INTERMOLECULAR INTERACTIONS BETWEEN THE ARTEMISININ-TYPE AGENTS AND NUCLEOBASES

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Artemisinin-type agents are well known as effective antimalarial medicines and recently their anticancer activity was reported, however there is lack of investigations on the molecular mechanisms of antitumor activity of artemisinin and its derivatives. This study is aimed at the examining of mechanisms of artemisinin-type agents anticancer activity. DNA as carrier of genetic information is one of the major targets for antitumor drugs effect and nucleic acids are also suggested as molecular targets of artemisinin action in cancer cells. Biologically significant intermolecular interactions of artemisinin and dihydroartemisinin with some purine and pyrimidine nucleobases were studied by means of testing the drug-nitrogen base mixtures by electrospray ionization mass spectrometry. The peaks of stable noncovalent complexes of the artemisinin-type drug with Ade, Cyt, and mThy were registered in the mass spectra. Comparison of the relative abundances of the peaks of the drug-nucleobase complexes for purine and pyrimidine nitrogen bases was performed. The spectra analysis allowed us to conclude that an effectiveness of the complexation process depends on the structural peculiarities of the drugs and nucleobases molecules. The experimental data and features of the molecular structure of the drugs and nucleobases testify to the suggestion that noncovalent complexes of the artemisinin-type agents and nitrogen bases are stabilized by van der Waals forces and hydrogen bonds between the functional groups of the interacting molecules. Formation of the supramolecular complexes of the artemisinin-type drugs and nucleobases is considered as a possible molecular mechanism of anticancer activity of the studied antimalarial agents. The obtained results demonstrate the great potential of electrospray ionization mass spectrometry method in the study of the artemisinin-type agents and their intermolecular interactions related to the mechanisms of the drugs biological activity.

KEY WORDS: antimalarial agents, artemisinin, dihydroartemisinin, nucleobases, noncovalent complexes, anticancer activity, electrospray ionization mass spectrometry.

Antimalarial agent artemisinin and its derivatives, which have been used in Chinese traditional medicine since ancient times, are currently successfully used to treat severe or multidrug-resistant *Plasmodium falciparum* malaria [1, 2]. The molecular mechanisms of their action, however are still not properly understood and are the subjects of a number of investigations [3-7]. Our previous mass spectrometry study related to the molecular mechanisms of the artemisinin-type agents antimalarial activity have revealed the noncovalent complexes formation between the drugs and their suggested molecular target – heme - *in vitro* [8]. Recently in scientific literature it was also reported about anticancer activity of artemisinin and some its derivatives. In 1995 researchers H. Lai and S. Narenda of the University of Washington published paper in Cancer Letters journal [9] concerning the use of artemisinin against numerous cancer cell lines in vitro. In a subsequent article [10] of these scientists pointed out the selective toxicity of artemisinin and holotrasferrin towards human breast cancer cells. In that article, quite rapid and complete distruction of a radiation-resistant breast cancer cell line was achieved when the in vitro cell system was supported in iron uptake with holotrasferrin. In a paper by Efferth et al, published in Oncology journal in 2001 [11], it was stated that the antimalarial artesunate was also active against cancer. That article described dramatic cytotoxic activity of this artemisinin derivative against a wide variety of cancers including drug resistant cell lines of leukelia and colon cancer, melanomas, breast,

ovarian, prostate and renal cancer cell lines. In the review [12] it was also reported that artemisinin has been used for about 30 years in Vietnam and China for cancer treatment and the experience with artemisinin for this purpose is increasing.

While the molecular mechanisms of artemisinin-type antimalarial action are actively investigating by a number of scientific groups there is lack of investigations on the mechanisms of anticancer activity of artemisinin and its derivatives. This study is aimed at the understanding of possible molecular mechanisms of artemisinin-type agents anticancer activity. DNA as carrier of genetic information is one of the main targets for antitumor drugs effect because of the ability to interfere with transcription, gene expression, protein synthesis and DNA replication, major steps in cell growth and division. The latter is central for tumorgenesis and pathogenesis. Taking into account this known property shared by cancer cells related to their active growth and proliferation we decided to examine the interaction of artemisinin and its active metabolite dihydroartemisinin with some nucleobases in the current study. We consider that formation of noncovalent complexes of the drugs with the nitrogen bases in malignant cells can affect the functioning of their nucleic acids on the stages of transcription and DNA replication and selectively suppress the growth of the tumor cells as compared to non growing or slowly growing healthy tissue. Such effect can explain artemisinin-type drugs citotoxisity towards malignant cells.

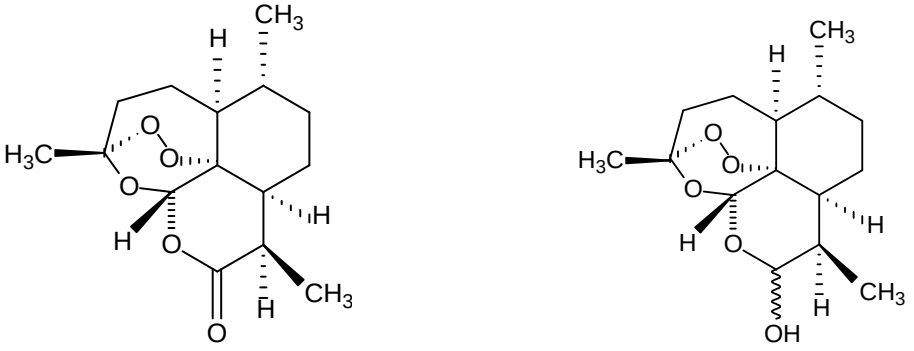
MATERIALS AND METHODS

Materials

Artemisinin (MW 282) and dihydroartemisisnin (MW 284) were provided by Dafra Pharma Company (Oud-Tunhout, Belgium) for investigations. The structures of Artemisinin and Dihydroartemisinin are presented on the Scheme 1a and 1b, respectively. The nucleobases Adenine (MW 135), Cytosine (MW 111), and Methylthymine (MW 140) were purchased from Aldrich Company. Nitrogen base Guanine was not tested in our experiments because its weak solubility. Methanol (super grade) was purchased from Lab-Scan (Dublin, Ireland). Stock solutions of artemisinin (5 mM), dihydroartemisinin (5 mM) and nucleobases (5 mM) were prepared in methanol. The analyte solutions of the compounds studied in methanol had final molar concentration of 250μM. Mixtures of the drugs with the nitrogen bases were also prepared in MeOH. The final analyte solutions of the drug-nucleobase mixtures had molar ratios of 1:1and molar concentration of 250 μM of drug and 250μM of the nucleobase.

Abbreviations: An, artemisinin; DHAn, dihydroartemisinin; Ade, adenine; Cyt, cytosine; mThy, methylthymine.

a) b)



Scheme 1. Structure of a) artemisinin and b) dihydroartemisinin.

Instrumentation

The mass spectral data were obtained in the positive ion mode, using an Atmospheric Pressure Ionization (API) Triple Quadrupole mass spectrometer API 2000 triple quadrupole HPLC-MS/MS (Perkin Elmer Sciex, Toronto, Canada) which was equipped with a Turbo IonSpray source. This source was operated in a standard electrospray ionization (ESI) mode. The analyte solutions (20 μL) were infused into the mass spectrometer by glass syringe at a constant flow rate of 0.2 mL/min of methanol solvent.

The electrospray ionization source temperature was set to 200^0C. Curtain gas (N_2) back pressure of 0.14 MPa (20 units), nebulizer gas (N_2) of 0.42 MPa (60 units) and turbo gas (N_2) of 0.21 MPa (30 units) were applied. Ion source capillary voltage was set to 4 kV. Typical declustering potential (also called skimmer potential) value was 35 V, focusing potential value was 200 V and entrance potential - 10 V.

ESI spectra were recorded in the mass range of *m/z* 100-4000.

Data acquisition and processing were performed using Analyst 1.4.1 software. All scans were acquired in the continuum mode.

RESULTS AND DISCUSSION

ESI mass spectrometry investigation of the individual components of the systems studied

In the first series of experiments the ESI mass spectra of individual components of the systems studied – artemisinin-type drugs and nucleobases - were recorded and the experimental conditions were optimized. In the spectrum of artemisinin (Fig. 1) characteristic molecular and quasimolecular ions can be noted: An•H^+(m/z 283, relative abundance (RA) 27%), An•Na^+ (m/z 305, RA 58%), An•K^+ (m/z 321, RA 10%), 2An•H^+(m/z 565,RA 6%), 2An•Na^+ (m/z 587, RA 100%), 2An•K^+ (m/z 603, RA 17%), 3An•Na^+ (m/z 869, RA 27%), 3An•K^+ (m/z 885, RA 2%). The most intensive quasimolecular peaks in the spectra are the peaks of artemisinin molecules cationized by Na^+ and K^+. Cationization as a way of ion formation is characteristic for the electrospray method of ionization [13] and peaks of cationized molecules of the artemisinin-type drugs were also registered in our previous ESI mass spectrometry study [8] of these agents. The high intensity of the protonation and cationization of artemisinin is obviously conditioned on the peculiarities of artemisinin structure (see Scheme 1) in particular the presence of endoperoxide bridge and carbonyl group in the drug structure provides the centers to connect protons and more heavy cations.

In the following experiment the artemisinin derivative dihydroartemisinin was examined. While in the ESI mass spectra of DHAn the ions of protonated drug molecules were not registered, the cationized adducts of DHAn drug monomer, dimer and trimer were recorded (Fig. 2): DHAn•Na^+ (m/z 307, RA 35%), DHAn•K^+ (m/z 323, RA 15%), 2DHAn•Na^+ (m/z 591, RA 30%), 2DHAn•K^+ (m/z 607, RA 3%), 3DHAn•Na^+ (m/z 875, RA 4%). The most intensive peak in the spectra is peak at m/z 261, which can be considered as a fragment adduct of DHAn molecule. The analysis of the spectra of An and DHAn also pointed to the fact that dihydroartemisinin as well as artemisinin tends to form ions of dimers and trimers under the ESI experimental conditions. Apparently the chemical structure of the artemisinin-type agents and in particular presence of the polar functional groups (CO, OH, endoperoxide bridge) in the structures can provide the dimmer and trimer noncovalent complexes formation by van

der Waals interactions, hydrogen bonds and other types of noncovalent interactions. Stacking interactions of An and DHAn molecules are less probable because of an absence of π-electron system in the heterocyclic structure of artemisini-type drugs and because of nonplanarity of the artemisinin-type molecules reported in [14]. Moreover the electrospray ion formation process including solvent drops spray with the following solvent evaporation also promotes the formation of ions of dimers and trimers of analyzed molecules.

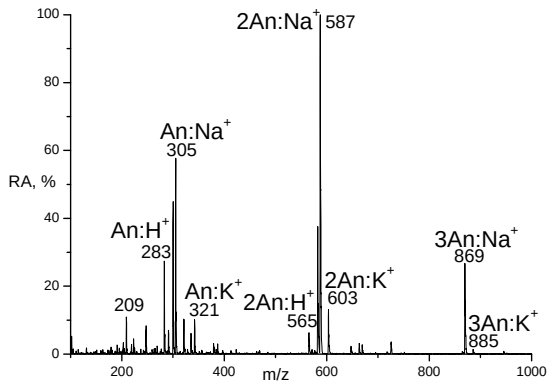


Fig. 1. Artemisinin ESI mass spectrum.

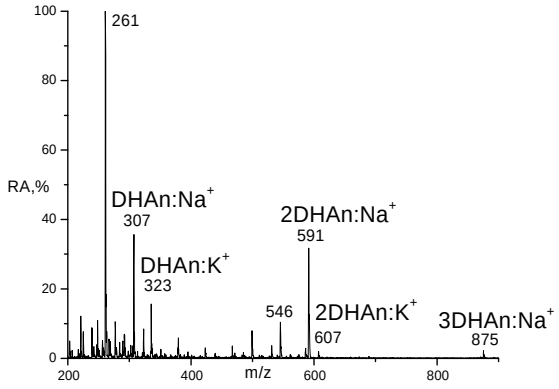


Fig. 2. Dihydroartemisinin ESI mass spectrum.

The peaks characteristic for An and DHAn observed in the present work are in a good agreement with the peaks registered for the drugs in our previous ESI investigation of these antimalarial agents [8] and can be used for the further analysis of the mixtures of the drugs with nucleobases.

Figure 3 (a,b,c) shows the individual ESI mass spectra of each studied nucleobase, Ade, Cyt and mThy , respectively. In these mass spectra the peaks of protonated nitrogen bases are the most intensive: Ade•H^+ (m/z 136, RA 100%), Fig. 3a; Cyt•H^+ (m/z 112, RA 100%), Fig. 3b; and mThy•H^+ (m/z 141, RA 100%), Fig.3c. At the same time the peaks of cationized monomers, dimers and trimers of Ade, Cyt and mThy were registered too (Fig.3), however their intensity were lower than intensities of the protonated peaks: Ade•Na^+ (m/z 158, RA 43%), Ade•K^+ (m/z 174, RA 4%), 2Ade•Na^+ (m/z 293, RA 18%), 3Ade•Na^+ (m/z 428, RA 7 %), Fig.3a; Cyt •Na^+ (m/z 134, RA 25%), 2Cyt •Na^+ (m/z 245, RA 24%), 3Cyt •Na^+ (m/z 356, RA 10%), Fig. 3b; mThy•Na^+ (m/z 163, RA 53%), mThy•K^+ (m/z 179, RA 14%), 2mThy •Na^+ (m/z 303, RA 24%), 2mThy•K^+ (m/z 319, RA 10%), 3mThy•Na^+ (m/z 443, RA 4%), 3mThy•K^+ (m/z 459, RA 6%), Fig. 3c.

Some interesting peculiarities of ion formation process for studied compounds under ESI conditions can be revealed from the spectra (Fig. 1- 3) analysis. In particular, we can see that for the artemisinin-type drugs the ions of cationized molecules are prevailed over the protonated ones, while for the nucleobases a protonation of the bases molecules is more favorable process. Such peculiarities are obviously determined by the chemical structures of the molecules studied. The endoperoxide bridge is in existence in the artemisinin-type compounds structures (Scheme 1) and it is considered as a center with high density of negative charge in the molecules. Such center can provide more strong interaction of the drug molecules with cations Na^+ and K^+ in comparison with the interaction of nitrogen bases with these cations.

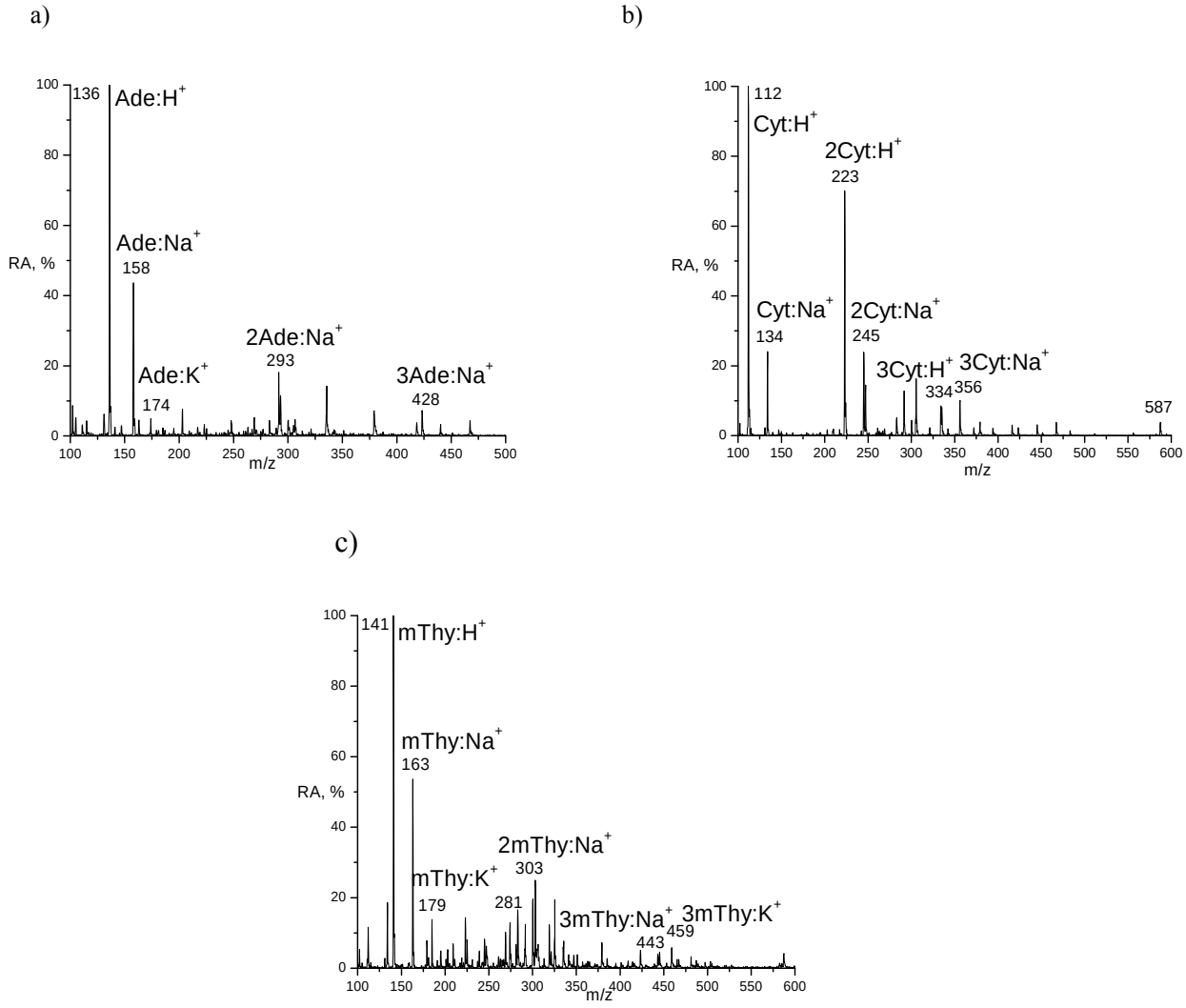
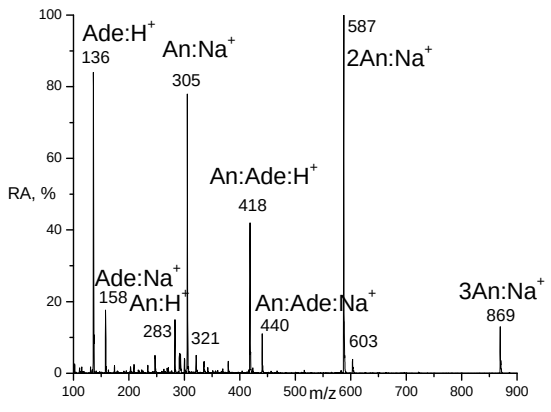


Fig.3. ESI mass spectra of nitrogen bases: a) adenine, b) cytosine, c) methylthymine.

ESI mass spectrometry study of the drug-nitrogen base systems

Artemisinin-Ade Mixture



The ESI mass spectrum obtained for the artemisinin-adenine (1:1) mixture is shown in Figure 4. There are peaks related to individual components of the mixture in the spectrum: An•H^+(m/z 283, RA 15%), An•Na^+ (m/z 305, RA 78%), An•K^+ (m/z 321, RA 5%), 2An•Na^+ (m/z 587, RA 100%), 2An•K^+ (m/z 603, RA 4%), 3An•Na^+ (m/z 869, RA 13%); Ade•H^+ (m/z 136, RA 84%), Ade•Na^+ (m/z 158, RA 17%). The most interesting result of this

experiment is that the formation of a artemisinin-adenine noncovalent complex in the mixture is evident from the intensive peaks $[An•Ade•H]^+$ (m/z 418, RA 42%) and $[An•Ade•Na]^+$ (m/z 440, RA 11%). Registration of quite intensive peaks of the cationized noncovalent complexes under ESI mass spectrometry conditions is nontrivial phenomenon and testifies to the high stability of such complexes. Analysis of the chemical structures of artemisinin (Scheme 1) and adenine allowed us to suggest that complex stability can be provided by van der Waals forces and hydrogen bonds between the endoperoxide bridge or CO groups of artemisinin and NH groups of adenine. Such interactions can supply with quit high stability of the supramolecular complexes and their survival under the mass spectrometry registration process. Stacking interactions between the heterocyclic systems of artemisinin and nitrogen base are less probable because mentioned above absence of the π-system in artemisinin structure and the drug molecule nonplanarity [14].

Fig.4. ESI spectrum of artemisinin-adenine system.

Artemisinin-Cyt System

The major peaks of the ESI mass spectrum of the artemisinin-cytosine system (1:1) were also related to the individual components of the mixture studied, artemisinin and cytosine (Fig. 5): $An•H^+$(m/z 283, RA 19%), $An•Na^+$ (m/z 305, RA 100%), $An•K^+$ (m/z 321, RA 16%), $2An•Na^+$ (m/z 587, RA 83%), $2An•K^+$ (m/z 603, RA 16%), $3An•Na^+$ (m/z 869, RA 6%), $Cyt•H^+$ (m/z 112, RA 72%), Cyt $•Na^+$ (m/z 134, RA 25%), 2Cyt $•H^+$ (m/z 223, RA 54 %), 2Cyt $•Na^+$ (m/z 245, RA 24%). The group of peaks of nonocovalent complexes of artemisinin with cytosine (Fig. 5) can be registered in the spectrum too: $[An•Cyt•H]^+$ (m/z 394, RA 10%) and $[An•Cyt•Na]^+$ (m/z 416, RA 19%). The data obtained for this system demonstrate that cytosine just as adenine forms stable noncovalent complexes with artemisin in solution. Such complexes can also be provided by van der Waals interactions and hydrogen bonds between –O-O-, CO groups of artemisinin and NH groups of cytosine.

Artemisinin-mThy System

The same experiment as described above was performed with artemisinin-methylthymine mixture (Fig.6). Peaks of cationized noncovalent artemisinin-methylthymine complexes were detected in the spectrum (Fig.6) $[An•mThy•H]^+$ at m/z 423 with RA of 8% and $[An•mThy•Na]^+$ at m/z 445 with RA of 9.5%.The other intensive peaks in the spectrum could be attributed to the mixture components: $An•H^+$(m/z 283, RA 42%), $An•Na^+$ (m/z 305, RA 95%), $An•K^+$ (m/z 321, RA 23%), $2An•Na^+$ (m/z 587, RA 100%), $2An•K^+$ (m/z 603, RA 18%), $3An•Na^+$ (m/z 869, RA 12%), $mThy•H^+$ (m/z 141, RA 39%), $mThy•Na^+$ (m/z 163, RA 25%), 2mThy $•H^+$ (m/z 283, RA 42%). It is interesting to note that relative abundency of the peaks of noncovalent complexes of artemisinin with mThy in the spectrum (Fig. 6) are smaller than RA of the peaks for complexes artemisinin with Cyt (Fig. 5). This fact is probably connected with methylation of one of nitrogens in the mThy that results in decreasing the amount of polar NH groups in pyrimidine nucleobase and with presence in mThy one more carbonyl group in comparison with Cyt that can lead to less stability of noncovalent complexes of An-mThy comparing with An-Cyt.

The comparative analysis of the RA of the peaks of the complexes of artemisinin with purine base adenine (Fig.4) and the peaks related to the complexes of the drug with pyrimidine bases (Cyt and mThy) (Fig. 5 and 6) shows that artemisinin-base complexes with purine base are more relatively stable. Such result probably connected with the structural

compliance of artemisinin and adenine molecules to form complexes with several H-bonds. Moreover the bigger size of pyrine base molecule in comparison with pyrimidine one provides more strong van der Waals interactions with artemisinin molecule.

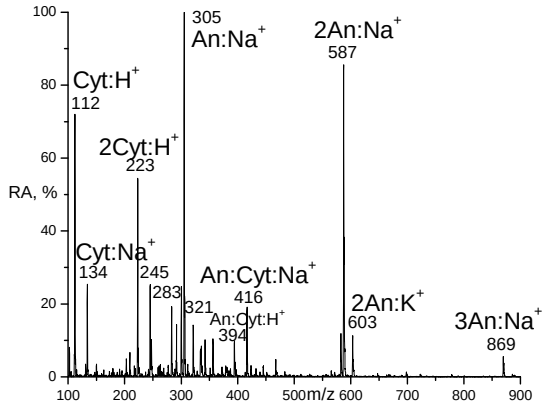


Fig.5. ESI mass spectrum of artemisinin-cytosine mixture.

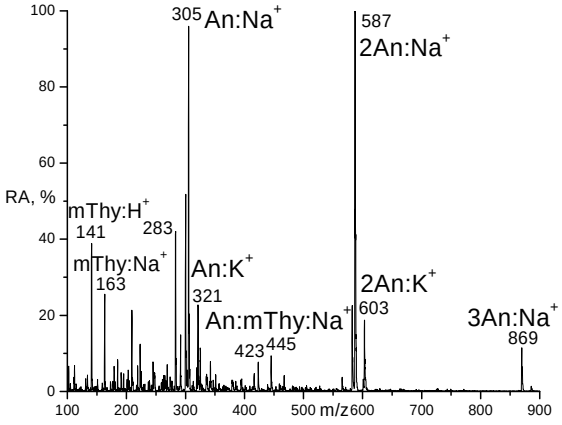
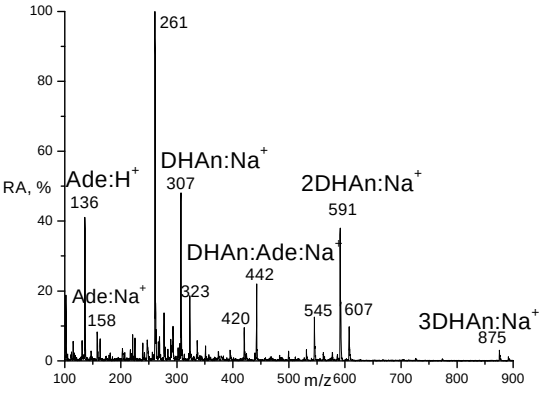


Fig.6. ESI mass spectrum of artemisinin-methylthymine system.

Dihydroartemisinin-Ade Mixture

Dihydroartemisinin is of particular interest for the understanding of artemisinin-type drugs action because DHAn is known as active metabolite of some derivatives of artemisinin in vivo [1, 15]. The mixtures of dihydroartemisinin with the nucleobases were studied at the same ESI mass spectrometry conditions. The ESI mass spectrum dihydroartemisinin –adenine system (1:1) is presented on the Figure 7.



The spectrum contains ions characteristic of the individual components of the mixtures, namely, DHAn•Na^+ (m/z 307, RA 49%), DHAn•K^+ (m/z 323, RA 19%), 2DHAn•Na^+ (m/z 591, RA 38%), 2DHAn•K^+ (m/z 607, RA 9%); Ade•H^+ (m/z 136, RA 42%), Ade•Na^+ (m/z 158, RA 8%). Registration in the spectrum the peaks of $[DHAn•Ade•H]^+$ (m/z 420, RA 10%) and $[DHAn•Ade•Na]^+$ (m/z 424, RA 22%) demonstrates that dihydroartemisinin like artemisinin forms a noncovalent complexes with adenine in the solution.

Fig.7. ESI mass spectrum of dihydroartemisinin-adenine system.

Dihydroartemisinin-Cyt System

Interaction of dihydroartemisinin with pyrimidine nitrogen bases was tested in the ESI experiment on the DHAn-Cyt mixture (1:1). In the Fig. 8 presenting the mass spectrum of this system we can find just one peak which can be related to the noncovalent complex of the drug with Cyt, namely $[DHAn•Cyt•Na]^+$ peak at m/z 418 with RA 6%. In contrast to the spectrum of An-Cyt system in the case of DHAn the protonated peak of the noncovalent complex of the

drug with the nucleobase was not registered. The intensive peaks of the spectrum of DHAn-Cyt mixture can be attributed to the individual components of the mixture: DHAn•Na^+ (m/z 307, RA 41%), 2DHAn•Na^+ (m/z 591, RA 4%), Cyt•H^+ (m/z 112, RA 87%), Cyt •Na^+ (m/z 134, RA 48%), 2Cyt •H^+ (m/z 223, RA 46 %), 2Cyt •Na^+ (m/z 245, RA 40%). RA of the peak of DHAn-Cyt complex (Fig.8) is smaller than that for An-Cyt (Fig. 5) that can be explained by the peculiarity of DHAn structure in which hydroxyl group is situated on the place of carbonyl group in An.

Dihydroartemisinin-mThy System

The mixture of dihydroartemisinin with other pyrimidine base methylthymine was also studied in the experiment. In the spectrum of this system (Fig.9) the ions originated from DHAn are more intensive than the ions from the nucleobase: peak at m/z 261 (RA 100%) from DHAn, DHAn•Na^+ (m/z 307, RA 70%), DHAn•K^+ (m/z 323, RA 13%), 2DHAn•Na^+ (m/z 591, RA 58%), 2DHAn•K^+ (m/z 607, RA 9%); m Thy•H^+ (m/z 141, RA14.5%), mThy•Na^+ (m/z 163, RA 45%), mThy•K^+ (m/z 179, RA 3%), 2mThy •Na^+ (m/z 303, RA 16%). The peak of ion [DHAn•mThy•Na]$^+$ at m/z 447 with RA 17 % was registered in the spectrum that testify to the formation of stable supramolecular complex DHAn with mThy in the solution. RA of DHAn-mThy complex is higher than the RA of DHAn-Cyt complex. This fact is probably connected with the structural peculiarities of the nucleobases: in Cyt there is one CO group (which can interact with OH group of DHAn forming H bond) in comparison with two groups in mThy.

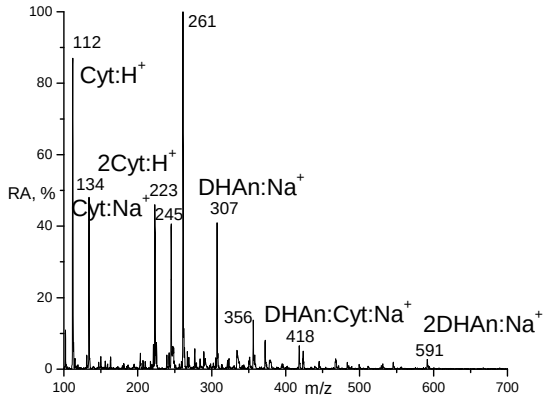


Fig.8. ESI mass spectrum of dihydroartemisinin-cytosine system.

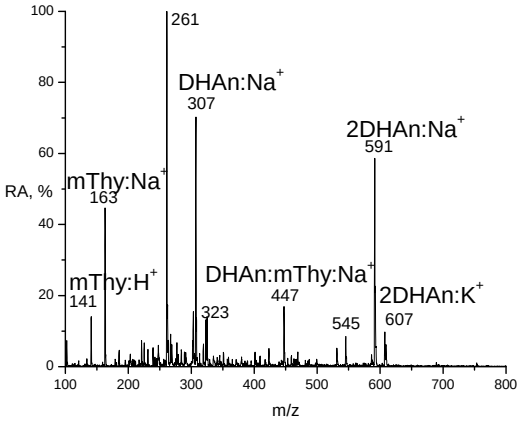


Fig.9. ESI mass spectrum of dihydroartemisinin-methylthymine system.

Analysis of the spectra of systems containing the artemisinin-type drugs and nucleobases confirms the formation of stable supramolecular complexes of the drugs and nitrogen bases in solution and shows that the RA of the peaks related to the noncovalent complexes of An and DHAn with purine nucleabase Ade is higher than that for the peaks of the complexes of the drugs with pyrimidine nucleabases Cyt and mThy.

CONCLUSIONS

Cationized noncovalent complexes of artemisinin and dihydroartemisinin with nucleobases Ade, Cyt and mThy, which model drug-DNA interaction in biological systems, were registered by ESI mass spectrometry. The spectra analysis allowed us to conclude that an effectiveness of the complexation process depends on the structural peculiarities of the drugs and nucleobases molecules: the relative abundances of the peaks of the complexes of artemisinin and dihydroartemisinin with purine base are higher than those for the peaks corresponding to the drug-pyrimidine base complexes. The experimental data and structural features of the drugs and nucleobases testify to the suggestion that noncovalent complexes of the artemisinin-type agents and nitrogen bases are stabilized by van der Waals forces and hydrogen bonds between the polar functional groups of the molecules, such as endoperoxide bridge or CO groups of the drugs and NH groups of the bases. Formation of the drug-nucleobase surpamolecular complexes in cells is suggested as a possible molecular mechanism of anticancer activity of the artemisinin-type drugs. The present investigation also demonstrates the great potential of electrospray ionization mass spectrometry method in the study of the artemisinin-type agents and their biologically significant intermolecular interactions related to the mechanisms of the drugs biological activity.

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