Complexation of Lappaconitine with Glycyrrhizic Acid: Stability and Reactivity Studies

Nikolay E. Polyakov,*,† Vladimir K. Khan,† Marc B. Taraban,† Tatyana V. Leshina,† Nariman F. Salakhutdinov,‡ and Genrikh A. Tolstikov‡

Institute of Chemical Kinetics & Combustion, Institutskaya Str. 3, 630090, Novosibirsk, Russia, and Novosibirsk Institute of Organic Chemistry, Lavrentyev Ave. 9, 630090, Novosibirsk, Russia

Received: June 24, 2005; In Final Form: October 11, 2005

NMR and UV−vis spectroscopy have been used to study the complexation of antiarrhythmic alkaloid lappaconitine with an efficient complexing agent from licorice, glycyrrhizic acid, which is known to profoundly influence the therapeutic activity of the alkaloid in the complex. In MeOH, DMSO, or aqueous solutions, lappaconitine has been shown to form a stable complex with glycyrrhizic acid with 1:1 stoichiometry over a broad concentration range from 1 μM to 300 μM. The stability constant K_{11} equals 2.0×10^5 M^{-1} in aqueous solution. A similar complex of lappaconitine hydrobromide—the pharmaceutical formulation used in the treatment of arrhythmia—is 2 orders of magnitude less stable than pure lappaconitine. A notable decrease in the rate of the photoinduced electron-transfer reaction between lappaconitine in a complex with glycyrrhizic acid and tyrosine allows the suggestion of an explicit interrelation between the suppressed chemical reactivity of the bound alkaloid and the changes of its therapeutic efficiency.

Introduction

Physicochemical approaches are increasingly employed to explore the detailed mechanisms of the biologically relevant processes. Continuous interest to drug complexation is stipulated by the prospects of the application of the supramolecular complexes to increase the availability and stability of a drug and to decrease the required dosage. The most widely used complexing agents, cyclodextrins, have demonstrated a variety of advantages of drug−cyclodextrin complexes.[1] It has been shown that due to high stability constants these complexes could be effectively used for the purpose of drug delivery protecting the drug molecule from premature decay.[2,3] It is believed that complexation with cyclodextrin not only alters the physicochemical properties of the drug—for example, solubility—but also affects its pharmacokinetics[4] thus allowing to a certain extent regulation of the reactivity of the drug. Indeed, the photochemical studies have shown that the reactivity of a substance is suppressed significantly in the inclusion complex with cyclodextrin.[5] On the other hand, despite the clear evidences of the increase of the in vitro bioavailability of the complexed drug,[6] the mechanism of the complexation influence at the molecular level has not been elucidated in detail, therefore, it is still unclear whether the observed effects could be explained *solely* by the increase in drug solubility in the complex with cyclodextrin.[7]

Glycyrrhizic acid (Figure 1), triterpene alkaloid from licorice root, GA, is a well-known solubilizing agent[8] and, therefore, could be another potential efficient complexing agent for drug delivery. Although the complexes of different drugs with GA have not been widely used to date as compared to those with cyclodextrins, it is possible to cite a number of instances of the potent influence of GA on the efficiency of the drug. For example, it has been shown to decrease the required dosage of common antianginal nitrocompounds such as nitroglycerine;[9] studies of GA complexes with sulfanilamides and oxytetracycline derivatives have demonstrated a notable increase of resistance toward common bacterial infections in laboratory

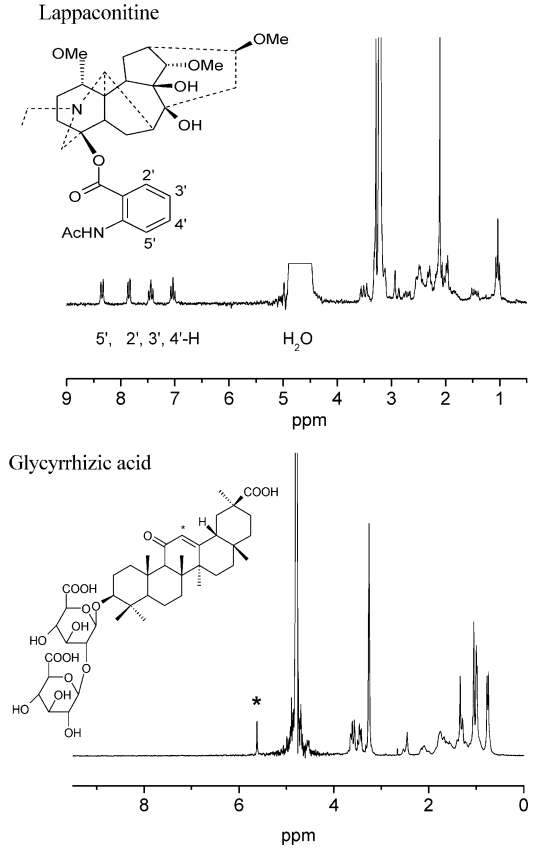


Figure 1. Structures and ^{1}H NMR spectra (200 MHz) of lappaconitine (top) and glycyrrhizic acid (bottom) in CD_3OD. Asterisk denotes the proton of glycyrrhizic acid demonstrating a maximum change of the chemical shift when the complexation was observed.

* To whom correspondence should be addressed. Fax: 7-383-3307350. Tel: 7-383-3332947. E-mail: polyakov@ns.kinetics.nsc.ru.

† Institute of Chemical Kinetics & Combustion.

‡ Novosibirsk Institute of Organic Chemistry.

10.1021/jp053434v CCC: $30.25
Published on Web 12/03/2005

animals.[10] Also, complexes with 5-fluorouracil and rubomycin have been shown to be less toxic than initial antitumor drugs, while at the same time retaining their antitumor activity.[11] Recent preclinical pharmacological studies have shown that the addition of GA substantially decreases[12] the required dosage of antiarrhythmic diterpenoid alkaloid[13,14] from monkshood−lappaconitine (Lap, Figure 1). The nature of this effect is, however, unclear. Given the above-mentioned data on the well-studied and widely employed drug−cyclodextrin complexes as well as those on drug−GA, it is reasonable to suggest that GA is also capable of forming a complex with Lap, thus affecting its properties and therapeutic activity.

It is common to attribute the antiarrhythmic effect of Lap to the binding to site 2 of the Na^+ channels[15] resulting in the blockade of sodium current and decreasing the maximum depolarization rate. The structure of all four binding sites of the channel is well characterized,[16] and the drug is believed to be bound by the peptide loop with a known sequence of amino acids.[17] Recently, we have proposed the approach to model the drug−receptor interactions based on the assumption that a chemical reaction between drug and reactive amino acids of the binding site is the underlying factor of the stability and decay of the drug−receptor complex.[18,19] In the case of the Ca^{2+} channel, it has been suggested that the dynamics of drug−receptor complex could be satisfactorily modeled by an electron-transfer reaction between, for example, known Ca^{2+}-antagonist nifedipine and tyrosine which is one of the six amino acid residues forming the binding site of the Ca^{2+} receptor.[18] A similar approach employing chemically induced dynamic nuclear polarization (CIDNP) was used to study the role of electron-transfer processes in the interactions of Lap with tyrosine and tryptophan−amino acids present in the peptide loop of site 2 of the Na^+ channel.[19] It has been demonstrated that electron transfer from amino acids to Lap results in the unstable radical anion species of the drug which could rapidly eliminate its active function, *N*-acetyl-anthranilic acid. This cleavage is suggested to model the underlying cause of the loss of Lap binding to an Na^+ channel as a result of chemical reaction.[19]

In light of the above suggestion of the existence of an Lap + GA complex with altered therapeutic activity, it is reasonable to expect that the effects of this complexation could be modeled[18,19] by the variations of reactivity of free and bound Lap in the reactions with an amino acid. Unfortunately, the reference data lacks the detailed information on the exact molecular structure of the GA complexes. The research is further complicated by the fact that GA is capable of forming micelles at critical concentrations above 10^{-3} M.[20] However, other aggregates have been shown to form at lower concentrations: "guest−host" complexes comprised one or two GA molecules per one guest molecule have been detected.[21]

Thus, the present work employs a two-pronged approach: First, to elucidate the stoichiometry and stability of Lap complexes with GA by means of UV and NMR spectroscopy using methods successfully employed to study similar complexes of cyclodextrins.[22,23,24] Second, to study the influence of complexation on the reactivity of Lap, it is suggested to trace the variations of photoinduced CIDNP effects in the reactions between Lap and tyrosine and to analyze the products of interaction between Lap and tyrosine in the presence and in the absence of GA.

Experimental Section

Chemicals. Lappaconitine (Lap) was isolated from air-dried roots of *Aconitum Septentrionale Koelle* in accordance with known procedure.[25] Glycyrrhizic acid (GA) was isolated from the root of *Glycyrrhiza uralensis Fisher*.[26] Deuterated solvents D_2O, CD_3OD (Aldrich), and *N*-acetyl tyrosine (Tyr) (Sigma) were used as supplied.

Calculation of Stoichiometry of the Complexes and Association Constants. The change in either the NMR chemical shift of protons or the parameters of absorption spectrum upon complexation is widely used to estimate the stoichiometry of supramolecular complexes and their association constants.[22−24] The formation of the complex between Lap and GA was traced through the dependence of the changes in the electronic absorption spectrum of Lap (optical absorption and λ_{max}) on GA concentration. The complex was prepared by adding the MeOH solution of GA to the H_2O/MeOH solution of Lap. The total methanol content was kept equal to 20%. The analysis was based on the shift of absorption maximum (around 300 nm) in the UV spectrum of Lap. The stoichiometry of the Lap + GA complex was determined using a continuous variation technique (Job's plot)[27,28] by measuring changes in the chemical shifts of the NMR spectrum of GA. To this end, solutions with different Lap/GA molar ratios but constant overall molar concentration of (Lap + GA) were prepared.

Reactivity Study. NMR analysis of reaction products was performed immediately after the photolysis directly in the probe of an NMR spectrometer Bruker DPX 200 (^1H operating frequency 200 MHz). In CIDNP experiments, the samples in standard 5 mm Pyrex NMR tubes were irradiated directly in the probe of the NMR spectrometer at room temperature. Solutions (reagent concentrations were in the range from 10^{-3} to 10^{-2} M) were deaerated by Ar bubbling. An EMG 101 MSC Lambda Physik excimer laser was used as the light source (λ = 308 nm, pulse duration 15 ns, average pulse energy 100 mJ). The quasi-steady-state (QSS) CIDNP experiments were performed using a special presaturation technique: saturation−180° pulse−a series of laser pulses−evolution time−detection pulse−free induction decay. Since, in this case, the background (equilibrium) NMR signals in CIDNP spectrum were suppressed, only the signals of the products demonstrating nuclear polarization could be observed.

Results and Discussion

Measurement of Association Constants and Stoichiometry of Supramolecular Complexes. NMR and optical spectroscopy methods often provide useful information when analyzing the structure and stability of supramolecular complexes. In particular, the advantages of the NMR method were demonstrated when studying the inclusion complexes of cyclodextrins.[22−24] Unfortunately, this method appeared to be of no avail for Lap + GA complexes due to a poor solubility of Lap in water. Therefore, in the present study, NMR spectroscopy was applied to the analysis of the complexation of Lap salt, lappaconitine hydrobromide (Lap-HBr), which demonstrates high solubility in water. In this case, the changes both in the aromatic portion of the Lap-HBr spectrum and of the chemical shifts of the proton at the double bond in the GA molecule (δ = 5.5 ppm, denoted by an asterisk in Figure 1) have been studied. The possibility to form a hydrogen bond between the NH group of Lap-HBr and CO group of GA presumably contributes to the stability of a given complex. An NMR method was used to measure the stoichiometry of the complex by means of Job's plot. In this experiment, the changes in the chemical shift of GA were measured as a function of Lap-HBr molar fraction with a constant sum [Lap-HBr] + [GA] = 5 mM (Figure 2). The position of the maximum at R = 0.5 in Figure 2 indicates the stoichiometry of the complex 1:1.

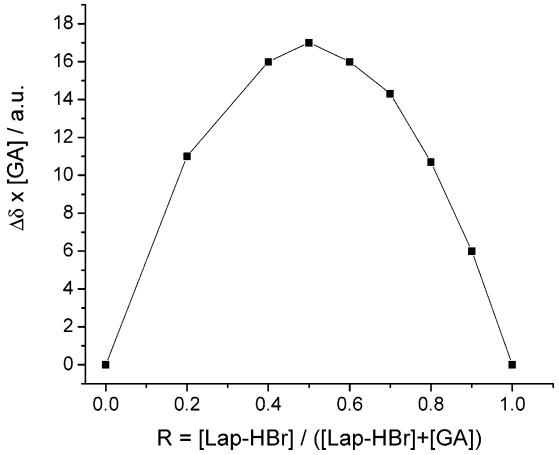


Figure 2. Job's plot of the NMR chemical shift changes of selected GA proton, $\delta = 5.6$ ppm for the Lap-HBr/GA complex in 50% D_2O/CD_3OD solution. [Lap-HBr] + [GA] = 5 mM.

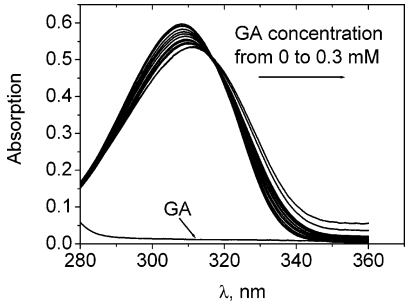


Figure 3. Changes of lappaconitine absorption spectrum in the presence of GA in CH_3OH, [Lap] = 0.1 mM.

The formation of the complex between pure lappaconitine and glycyrrhizic acid was studied by monitoring the dependence of the changes in the electronic absorption spectrum of Lap on GA concentration. Optical absorption spectra of Lap methanol solutions in the presence and absence of GA are shown in Figure 3. The UV−vis spectrum of Lap shows a maximum at 300 nm with molar extinction around 6000, while GA has no maxima in this range, and at 300 nm, the optical density of 0.1 mM GA solution is as low as 0.017. Our data show that the weak acidity of GA[29] ($pK_1 = 4.4$, $pK_2 = 5.3$, and $pK_3 = 6.9$) does not affect the absorption spectrum of Lap. The change in the parameters of the absorption spectrum upon complexation is widely used for calculation of the stoichiometry of supramolecular complexes and their association constants.[22−24,30] The reaction equilibrium of the *n*/*m* complex (C_{nm}) between GA and guest (G) molecules is represented by the equation

$$m\mathrm{G} + n\mathrm{GA} \rightleftarrows \mathrm{C}_{nm}$$

The association constant of this complex is described as

$$K_{nm} = \frac{[\mathrm{C}_{nm}]}{[\mathrm{G}]^m[\mathrm{GA}]^n} \quad (1)$$

In the case of a 1:1 complex, the value K_{11} can be obtained using eq 2 from the dependence of lappaconitine absorption maximum on the GA concentration[30]

$$\Delta\lambda_{obs} = \frac{\Delta\lambda_C}{2[\mathrm{G}]_0}\{[\mathrm{G}]_0 + [\mathrm{GA}]_0 + 1/K_{11} - (([\mathrm{G}]_0 + [\mathrm{GA}]_0 + 1/K_{11})^2 - 4[\mathrm{G}]_0[\mathrm{GA}]_0)^{1/2}\} \quad (2)$$

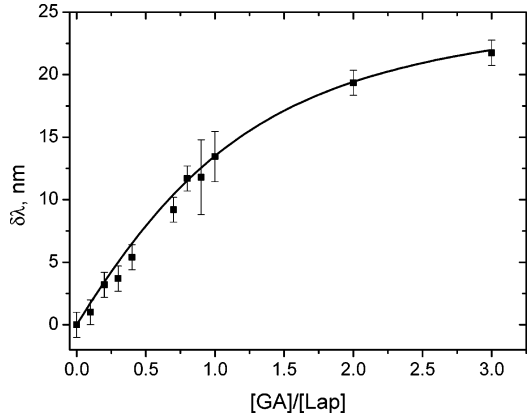


Figure 4. Experimental concentration dependence and its computer simulation of the shift of Lap absorption maximum ($\Delta\lambda$) in the presence of GA for the case of a 1:1 complex of Lap and GA. The experiment was carried out in a D_2O/CD_3OD mixture (20% CD_3OD) with [Lap] = 0.01 mM.

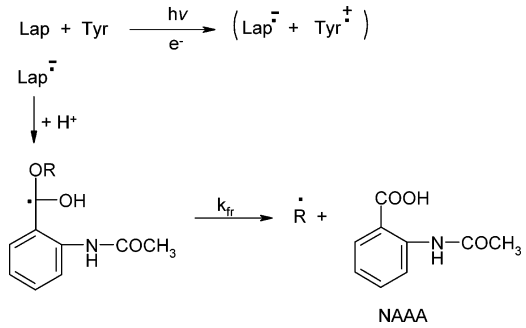
Here, $\Delta\lambda_C$ is the variation of absorption maximum of Lap totally bound in the complex relative to the free molecule. Experimental data, $\Delta\lambda_{obs}$, were measured at different GA concentrations and a constant concentration of lappaconitine in aqueous solution. The analysis was based on the shift of absorption maximum (at 300 nm) in the UV−vis spectrum of Lap. It has been found that an addition from 0 to 0.03 mM of GA to 0.01 mM aqueous solutions of Lap results in the shift of its absorption maximum from 296 to 319 nm. Computer simulation of the experimental concentration dependence using formula 2 for the 1:1 complex shows satisfactory agreement (Figure 4). This allows one both to draw conclusions on the complex formation with stoichiometry 1:1 and to calculate the stability constant of this complex, $K = 2.0 \times 10^5\ \mathrm{M}^{-1}$ ($\pm 0.13 \times 10^5\ \mathrm{M}^{-1}$). The conclusion on the stoichiometry of the complex obtained by the optical method is in agreement with the date for Lap-HBr obtained by NMR measurements using Job's plot technique.[27,28]

It is necessary to note that the resulting value indicates an extreme stability of the complex. For comparison, the stability constants of cyclodextrin complexes, widely used in pharmacology, are on the average around $10^3\ \mathrm{M}^{-1}$.[22,23] High stability constants were also observed for GA complexes with other drugs.[9] This makes the glycyrrhizic acid a rather prospective complexing agent for pharmacology, because, in this case, the complexation makes its possible to use very low concentrations of an active drug. The high stability of GA complexes is also stipulated by poor solubility of both GA and Lap in water. In aqueous solutions, a hydrophobic interaction can encourage the formation of supramolecular associates of complex structure.

Since Lap and its complex with GA are insoluble in pure water, in all experiments, water−methanol mixtures have been used as a solvent. To study the influence of a solvent on the GA−Lap complex stability, the same method was used to measure the stability constant of a given complex in pure methanol and DMSO aqueous solution. It has been found that, in the 20% DMSO aqueous solution, the complex demonstrates the same stability as in the 20% methanol solution. At the same time, in pure methanol, the stability constant decreases by an order of magnitude. This result indicates that hydrophobic interaction is the main driving force of GA complex formation.

As it has been already mentioned, in view of better water solubility, lappaconitine hydrobromide (Lap-HBr) is the preferred pharmaceutical formulation of Lap. From the point of practical use of Lap−GA complexes in pharmacology, it is

SCHEME 1: Radical Ion Stages of Photoinduced Electron Transfer Reaction of Lap and Tyr[19]



necessary to ascertain whether the complex of GA with Lap-HBr has the same high stability as that with the Lap itself. Similar to the above-described experiments, the stability constant of the complex Lap-HBr−GA was measured from the dependence of changes in the Lap-HBr absorption spectrum on GA concentration. The stability constant of the 1:1 complex Lap-HBr−GA is $K_{11} = 2.6 \times 10^3$ M^{-1}, which is 2 orders of magnitude lower than the corresponding value for pure Lap. From our viewpoint, this is due to a lower hydrophobicity of the salt.

It should be noted that we are well aware of the fact that all the above experiments have been performed for model systems in mixed water−alcohol solutions which in contrast to buffered aqueous solutions are not the real biological environment. Unfortunately, the low solubility of both Lap and GA in pure water does not allow us to study the concentration dependences of NMR chemical shifts and optical absorption of Lap. However, our previous experiments with GA and cyclodextrin complexes show that methanol does not affect the stoichiometry of the complex and is only capable to decrease its stability. Thus, it is reasonable to suggest the existence of the above complexes in buffered aqueous solutions.

Reactivity of Lappaconitine in the Presence of Glycyrrhizic Acid. To study the influence of GA on Lap reactivity, the reaction of photoinduced electron transfer between Lap and tyrosine (Tyr, amino acid present in the peptide loop of site 2 of the sodium channel) was used as a model reaction. The mechanism of this reaction has been recently studied by means of CIDNP.[19] The observation of CIDNP effects means unambiguously that the reaction proceeds via a free radical mechanism (Scheme 1).

Here NAAA is the final reaction product, *N*-acetyl-anthranilic acid. In the present work, the influence of GA on Lap reactivity was detected from both the intensity of CIDNP effects reflecting a contribution of the radical reaction pathway and the product yield of the reaction with tyrosine. Figure 5 compares the QSS CIDNP and NMR spectra of the final products during photolysis in the presence and in the absence of GA. CIDNP spectrum exhibits the polarized lines of both the initial compounds of GA and Lap and the product, NAAA. Two additional singlet lines appear at 8.1 and 8.3 ppm in the spectrum due to a parallel process of self-phototransformation of Lap.[19]

The primary conclusion to be drawn from the comparison of spectra a and c is that the presence of GA decreases CIDNP significantly. Since CIDNP intensity is proportional to the radical pair concentration, the observed effect indicates that the complexation with GA almost fully blocks the radical reaction pathway. The polarization intensity of Tyr and the yield of the reaction product (NAAA) are observed to decrease by about a factor of 4 (cf. spectra b and d). Thus, the presence of GA substantially hampers the Lap transformation rate.

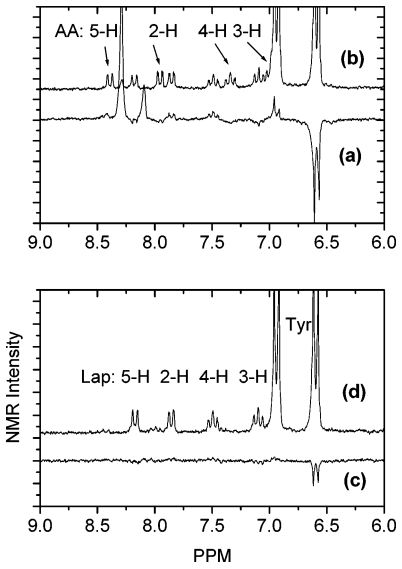


Figure 5. QSS ^{1}H CIDNP spectra observed after laser irradiation of Lap (3 mM) in the presence of Tyr (8 mM) in CD_3OD in the absence (a) and in the presence (c) of GA. ^{1}H NMR spectra (200 MHz) of the products measured after 800 laser pulses in the absence (b) and in the presence (d) of GA. Two additional singlet lines at 8.1 and 8.3 ppm appeared in the spectrum due to the parallel process of self-phototransformation of Lap.

Conclusion

Thus, in the present work, we have verified the formation of the complex of Lap with GA and measured the stoichiometry and stability constant of this complex. Note that, since Lap has the properties of a weak base and GA is a weak acid, one cannot exclude the possibility of the formation of a salt on their mixing. However, we suggest that, in this case, the influence of GA on the physical and chemical properties of Lap has another nature. A number of experimental findings contribute to this inference, in particular: (i) the addition of acetic acid with $pK_a \sim 4.6$ which is close to that of GA to Lap solution has no impact on the absorption spectra of Lap; (ii) the experiments with Lap salt, its hydrobromide, also demonstrate noticeable influence of GA on the absorption and NMR spectra of Lap; and (iii) the influence on the reactivity of Lap detected in the present paper for the complex with GA has not been observed for other organic acids. A high stability constant of this complex is in accordance with the data for a number of other drugs published earlier. It makes glycyrrhizic acid a more promising complexing agent as compared to cyclodextrins. Judging from the results of pharmacological studies demonstrating a repeated decrease in the therapeutic dose of a series of drugs in the presence of GA followed by a decrease in toxicity, GA seems to be a rather promising object for application in pharmacology.

The result obtained in the present work for the decrease of the Lap transformation rate in the presence of GA testifies in favor of the suggested mechanism of the influence of GA on the therapeutic activity of Lap and the role of chemical reactions in the dissociation of ligand−receptor complexes.[18,19]

Acknowledgment. This work was supported by RFBR Grant 04-03-32449 and by the funds of interdisciplinary integration project of the Siberian Branch of Russian Academy of Sciences No. 146, "Drugs and prophylactics design for medical purposes. Fundamentals and implementation".

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