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Spectroscopic study of amphotericin B - stigmasteryl trifluoromethylphenyl-carbamate interaction

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Amphotericin B, the most known polyene macrolide antibiotics, is still used as a life saving drug (golden standard or rather drug of choice) to treat systemic fungal infections. It is known that amphotericin B interacting with cellular membranes, causes an impairment of membrane function of sensitive cells, usually leakage of cellular constituents and eventually cell death. The binding of amhotericin B to stigmasteryl-trifluoromethylphenyl-carbamate was studied using UV-Vis absorption spectroscopy.

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1. Introduction

Polyene macrolide antibiotics are natural compounds and most of them are produced by soil bacteria *Actinomycetes*, mainly belonging to the genus *Streptomyces*. The polyene macrolides, in general, exhibit antifungal and antiprotozoal activity but little or no antibacterial activity. Due to this fact several of polyene macrolide antibiotics are used as antifungal drugs, e.g. amphotericin B, nystatin, pimaricin and candicidin. Amphotericin B was discovered in early 1950s and introduced as a drug in 1959 [1]. Several very interesting papers concerning just properties of amphotericin B (AmB) and its mode of action were published within recent years [2-5].

Cellular mechanism of action of AmB is very complex and still not known in details. Generally, it is accepted that AmB interact with plasma membranes of sensitive organism (containing sterols) causing an impairment of barrier function, leakage of cellular constituents, and ultimately cell death [3-10]. However, data collected from numerous laboratories suggest that lethality of sensitive cells is not a simple consequence of changes in permeability of cell membranes for AmB. It is known that AmB form membrane channels which cause leakage of monovalent ions (K^+, Na^+, H^+, Cl^-) and small organic molecules [8-11].

It is known that the detergent type of action or channel formation in cellular membrane is responsible for the disturbance of membrane barrier function. Nevertheless, the mechanism responsible for AmB chemotherapeutic selectivity (pathogen cells versus host cells) is still not well understood. Chemotherapeutic application of AmB is based on higher sensitivity of ergosterol - containing fungal cells to the antibiotic compared to cholesterol-containing mammalian cells. Therefore, it is postulated that sterol molecules are necessary for AmB channel formation and participate in the channel structure. Due to the complex mode of action of AmB on membranes of living organisms many simpler models were worked out, namely, lipid monolayers, bilayers, liposomes and, recently, *in silico* models [12,13].

In a monolayer model system, a molecular organization of AmB in the lipid environment, including formation of porous structures and an effect of the drug on structural properties of a lipid phase, have been studied. Molecular dynamic studies were performed to analyze both the interaction of single AmB molecule with the surface of lipid membrane and the membrane components and also the molecular properties of ionic channels built from AmB molecules and cholesterol or ergosterol.

The simulations applying Monte Carlo methods and Poisson-Boltzmann electrostatic model were used to study ion passage through AmB membrane channels as well as to study distribution of molecular electrostatic potential for AmB and their supramolecular complexes in the lipid bilayer [11-13].

Stigmasterol (Stg, 24-ethyl-cholesta-5,22-dien-3β-ol) is an unsaturated plant sterol occurring in the plant fats or oils of soybean, calabar bean and rape seed and in a number of medicinal herbs. In recent years, we have synthesized many cholesteryc liquid crystals derived from cholesterol and stigmasterol with a bulky substituent at C-3 sterolic [14,15] and trifluoromethylphenyl moiety [16]. It is known that in most mesogens having a terminal trifluoromethyl group, the trifluoromethyl groups are arranged around the smectic layer boundary. The inter-layer interaction around the trifluoromethyl groups facilitates and stabilities the layer arrangement of the molecules [16, 17]. In general, compounds containing a lateral floro substituent were synthesized in order to generate a high positive value of the dielectric anisotropy, a low melting point and no smectic mesophases.

The purpose of the present work is to analyze the interaction between AmB and Stg-CF_3, by UV-Vis absorption spectroscopy, in order to identify the characteristics of the drug – sterol interaction and determine the binding parameters.

2. Experimental set-up and procedures, used substances

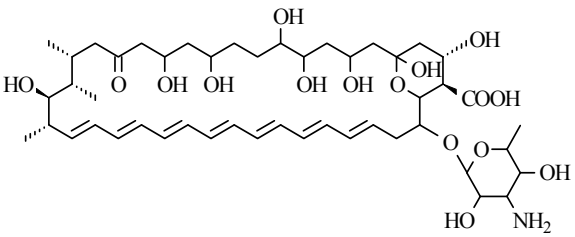
Amphotericin B (AmB) from *Streptomyces sp.* was obtained from Sigma-Aldrich. The antibiotic was stored in the dark at -20^0C. A stock solution of AmB in ethanol (spectroscopic grade) was also stored at -20^0C in the dark before being used. Its concentration was determined by UV spectroscopy using an molar absorption coefficient of $160000 M^{-1}cm^{-1}$ at 407 nm [19].

Stigmasteryl trifluoromethylphenyl-carbamate (Stg-CF_3) was obtained from stigmasterol (Stg) and ααα-trifluoro-*m*-tolyl isocyanate by reported method [16]. Stg was Merck product and ααα-trifluoro-*m*-tolyl isocyanate was Sigma Aldrich product. All solvents used in synthesis and recrystallizations of Stg-CF_3 were purified by distillation before use. The purity of Stg-CF_3 was checked by TLC silica gel plates 0.25 mm (Merck) using petroleum ether: ethyl ether 9:1 as eluent mixture. The stock solutions of Stg-CF_3 used in the spectral analyses were also prepared in anhydrous ethanol.

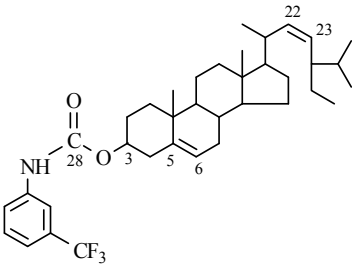
The absorption spectra were recorded in a Lambda 25 Perkin-Elmer spectrophotometer, with quartz cells, at room temperature.

3. Results and discussion

In Fig. 1 are shown the chemical structures of the compounds used. In AmB structure one may distinguish: (I) polar part containing polar head and polyol chain and (II) hydrophobic part containing chromophore region. Stg-CF_3 presents a bulky polarizable substituent on *metha* position of aromatic substituent at C-3 of steroidic ring.



(a)



(b)

Fig. 1. (a) Structure of AmB and (b) Stg-CF_3.

The stigmasterol derivative (Stg-CF_3) was prepared by direct reaction of stigmasterol with ααα-trifluoro-*m*-tolyl isocyanate in dry toluene. Pure crystals of the desired stigmasteryl ester were obtained after re-crystallization from amyl alcohol and a mixture of benzene and ethanol. The synthesis and purity of Stg-CF_3 was checked by IR and NMR spectra [16].

Due to amphiphilic and amphoteric properties, AmB is very poorly soluble in water but also in pure non-polar solvents. Solubility and the state of AmB in aqueous media are well characterized [18,19]. As we have shown AmB in polar organic solvents (e.g. methanol, ethanol, DMF, DMSO) exist as a monomolecular dispersion and its optical absorption spectra are not concentration dependent [19, 20].

UV-Vis absorption spectra of AmB presents a vibronic structure and four bands centred at 428, 407, 383 and 364 nm are observed [19]. It is known that the strong absorbance of AmB between 350-450 nm, determined by the heptaenes fragment existing on the apolar domain of the AmB structure, is heavily influenced by conformational changes provoked by its self-association in water media or by its interaction with other compounds, such as drug carriers [19,20].

In order to establish AmB affinity toward Stg-CF_3 molecules, the changes in the UV-Vis spectra of AmB occurring upon interaction with the steroid carbamate were determined. The percentage of steroid carbamate bound to antibiotic was calculated as the percentage of absorption at 407 nm decreases compared to free antibiotic spectra (Fig. 2). The amount of Stg-CF_3 bound AmB increased with increasing the ratio of concentrations of steroid carbamate to drug (*p*) (Fig. 2).

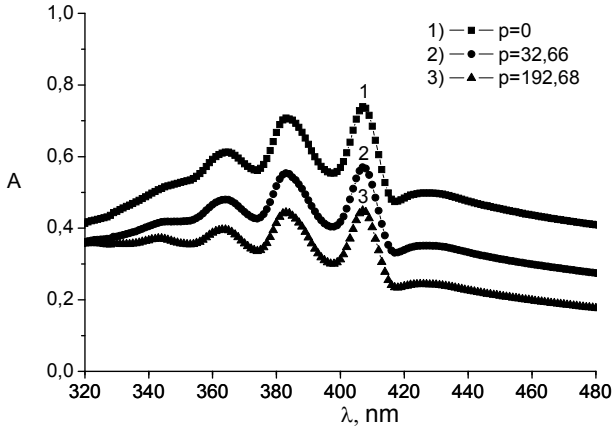
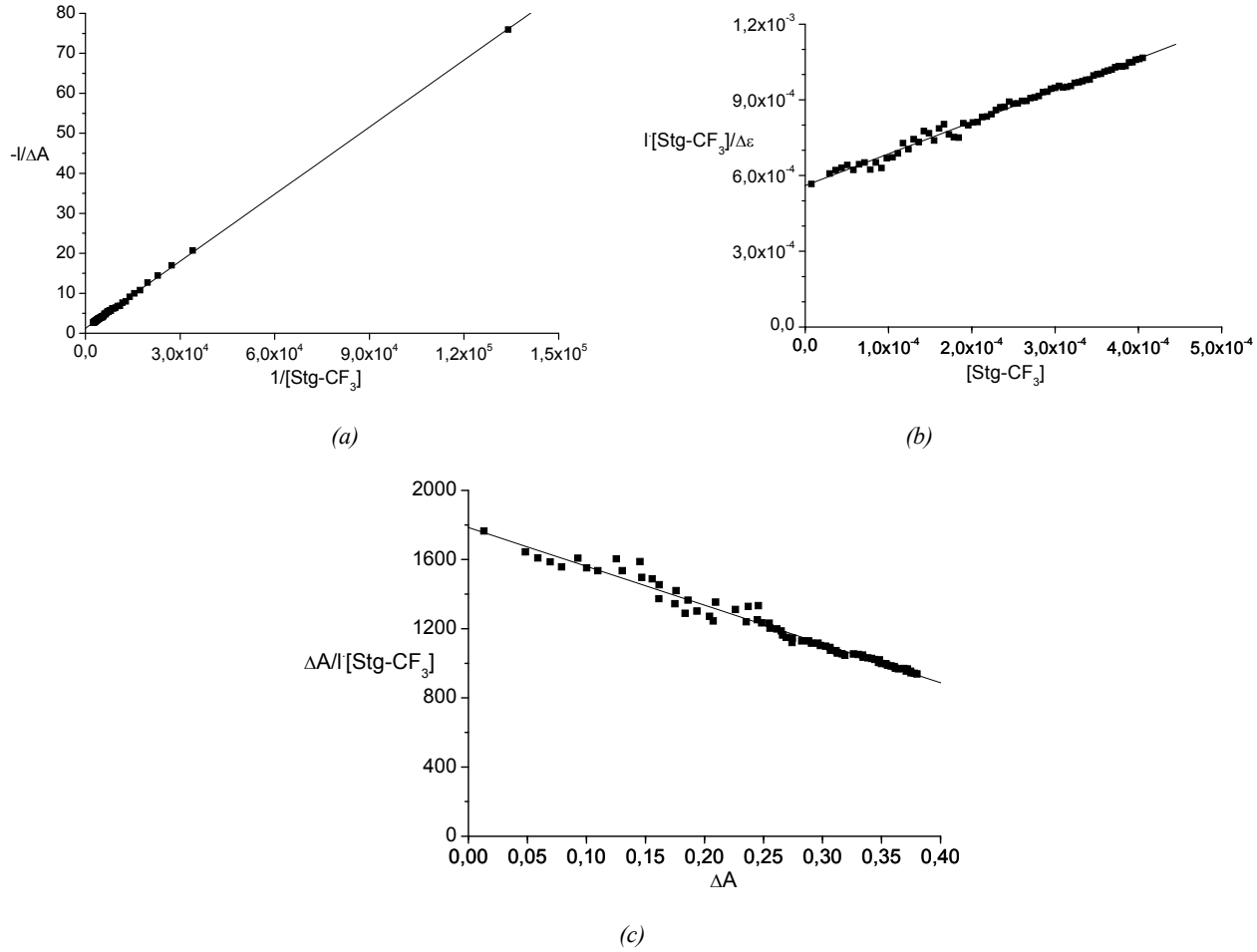


Fig. 2. UV-Vis spectra of AmB - Stg-CF_3 system, at various Stg-CF_3 to AmB molar ratios (p): (1) 0; (2) 32.66; (3) 192.68.

The binding of AmB to Stg-CF_3 induced a hypochromic effect in the bands at 407, 383 and 364 nm. It may be observed that at small and medium *p* ratios, the changes of the absorption spectrum of the drugs are similar to those observed on increasing concentration of the drug.

Supposing that the interaction of AmB with Stg-CF_3 is in system 1:1, the binding parameters were evaluated from the methods proposed by Benesi-Hildebrand, Scott and Scatchard [21-23].

In Fig. 3 are presented Benesi-Hildebrand, Scott and Scatchard plots for the studied system. The equations and the results obtained for the interaction of AmB with Stg-CF_3 being summarized in Table 1. The binding constant deduced from three methods is situated in a narrow interval and it is possible to take the mean value 0.423 as the most probable one.



(a) *(b)* *(c)*

Fig. 3. Benesi-Hildebrand (a), Scott (b) and Scatchard (c) plots of AmB - Stg-CF_3 system

Table 1. The binding constants of AmB – Stg-CF_3 interaction.

Methods	Equations	K, M^{-1}
Benesi-Hildebrand	$\frac{1}{\Delta A} = \frac{1}{C^0 \cdot K \cdot \Delta\varepsilon} \cdot \frac{1}{[Stg - CF_3]} + \frac{1}{C^0 \cdot \Delta\varepsilon}$	$0.49(\pm 0.05) \cdot 10^4$
Scott	$\frac{1 \cdot [Stg - CF_3]}{\Delta A} = \frac{1}{C^0 \cdot \Delta\varepsilon} \cdot [Stg - CF_3] + \frac{1}{C^0 \cdot K \cdot \Delta\varepsilon}$	$0.36(\pm 0.04) \cdot 10^4$
Scatchard	$\frac{\Delta A}{1 \cdot [Stg - CF_3]} = -\frac{K}{1} \cdot \Delta A + C^0 \cdot K \cdot \Delta\varepsilon$	$0.42(\pm 0.04) \cdot 10^4$

In Table 1 $\Delta\varepsilon_{app}=\varepsilon_{app}-\varepsilon_F$, $\Delta\varepsilon=\varepsilon_B-\varepsilon_F$, ε_{app}, ε_F and ε_B are the apparent, free and bound drug absorption coefficients, l is the path length, ΔA is the observed absorbance change, C^0 is the total concentration of drug, [Stg-CF_3] is sterol concentration (concentration in moles per unit volume).

As we can notice, the values for the binding constant of AmB to Stg-CF_3 obtained by the three methods do not differ too much.

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