Absence in amphotericin B-spiked human plasma of the free monomeric drug, as detected by SERS

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Abstract Using surface enhanced Raman spectroscopy (SERS) which enables us to specifically detect traces of monomeric amphotericin B (AmB), we were able to show that in a 10^{-5} M AmB suspension, the concentration of free drug was below 10^{-8} M in the presence of low density lipoproteins (LDL) or plasma. The affinity constant of AmB for LDL determined from electronic absorption data, was found to be 4×10^{6} M^{-1}. Therefore, since AmB appears to be in the majority bound to lipoproteins under in vivo conditions, its toxicity should not result from the induction of host-cell transmembrane permeability but rather from the internalization of the AmB-LDL complex.

Key words: Amphotericin B; Lipoprotein; Low density lipoprotein; Plasma; Surface enhanced Raman spectroscopy

1. Introduction

Amphotericin B (AmB) is the drug of choice for many serious systemic fungal infections, which owing to AIDS and improved organ transplant immunosuppression drugs are becoming tragically frequent in immune-compromised individuals [1]. Unfortunately severe side-effects negate its usefulness and justify the numerous current studies aiming at increasing its therapeutic index. As far as the molecular mechanism of action of this drug is concerned, it has often been admitted that AmB binds to sterol-containing membranes and forms transmembrane pores through which components essential for the life of cells leak. The formation of these pores has been widely ascertained in serum-free media or in red blood cells, which are devoid of low density lipoprotein (LDL) receptors. However, AmB binds to serum proteins, in particular to LDL [2–4], which may affect its mechanism of action. Therefore, the following questions arise: whether the affinity of AmB for proteins is higher than that for cells and whether it is the AmB-protein complex which is active, or whether there is an exchange of the antibiotic between proteins and membrane; and whether the complex AmB-LDL is internalized by the LDL receptors of mammalian cells. Recent studies have shown that this latter possibility may occur [5] and that consequently, AmB is found in endosomes [6]. As a result, the undesirable toxicity of AmB for host cells may take its origin from a blockade of the endosome-lysosome fusion and not from transmembrane permeability changes.

Strategies for decreasing AmB toxicity and improving its therapeutic index, depend strongly on which of these mechanisms is operative in vivo, i.e. transmembrane permeability inducement or internalization of the AmB-LDL complex. Estimation of the amount of free, protein-bound and cell-bound AmB, in plasma would allow us to draw conclusions. Unfortunately, in vivo, HPLC or bioassays, currently used to determine the *total* AmB concentration in plasma, do not provide this essential information. One study has, however, shown that in the blood of rats treated with AmB, 80% of the amphotericin B is bound to plasma proteins [4]. In vitro, several studies have also shown that AmB binds to lipoproteins. The distribution of the drug among the different lipoproteins was also analyzed in detail [2–4]. Unfortunately, the procedure used did not allow a clear estimation of free AmB, the amount of which, above or below ca. 1 μM, would or would not allow host-cell membrane permeabilization. Spectroscopic studies (UV-visible absorption, circular dichroism) should do it but the characteristic bands of bound AmB and free AmB overlap partially. Under these conditions, traces of free AmB are difficult to detect. A recently developed method, surface enhanced Raman spectroscopy (SERS) could overcome this difficulty since it enables us to detect traces of organic molecules. A SERS effect can be observed provided that the molecules under study are adsorbed on a rough surface of certain metals such as silver, gold or copper. Silver, roughened electrodes and aggregated colloids are easily prepared and are the most used SERS active substrates. The very large intensity enhancement of the Raman spectrum mainly arises from the excitation, within the surface plasmon band of the rough silver, with the usual laser lines either in the green (514.5 nm), in the red (632.5 nm) or even in the near infrared (1064 nm). We have previously shown [7] that in silver colloids prepared according to the procedure of Creighton et al. [8], resonant SERS (SERRS) effect us allows to specifically detect monomeric AmB at very low concentrations (ca. 10^{-10} M), which is about 1000 times lower than those detectable by UV-visible absorption spectroscopy. The presence of AmB aggregates in the vicinity of the silver surface only manifests by a perturbing effect on the whole intensity SERRS spectra. For this reason we thought that it would be a good method to detect, or not, traces of free AmB in the presence of LDL.

2. Materials and methods

2.1. Materials

The silver colloids were prepared according to Creighton et al. [8], by reducing silver nitrate with an excess of $NaBH_4$ at room temper-

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ature. Solutions were mixed slowly, then vigorously stirred for 45–60 min.

AmB was a gift from Squibb (France) and used without further purification.

Low density lipoproteins from human plasma were purchased from Sigma. The initial solution at a 5 mg/ml protein concentration was dialysed against phosphate buffer saline (155 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4, adjusted to pH 7.4) in order to remove EDTA and proteins of molecular mass lower than 12 000 Da. The addition of the LDL solutions to the colloid suspension brings chloride anions, at a maximum final concentration of less than 2 mM, which has been shown not to significantly affect SERRS signals.

Plasma was separated from EDTA-treated blood (1 mg/ml) obtained from normal fasting donors, by two centrifugations at $3000 \times g$ for 5 min.

2.2. Spectroscopy measurements

The interaction of AmB with LDL was measured using a 10^{-3} M stock solution of AmB in DMSO and a 1 mg/ml protein stock solution of LDL in PBS. To 0.5 ml of water was added an aliquot of LDL solution in order to obtain the desired final concentration. Then, 20 μl of the AmB stock solution was added to water in order to obtain a 1 ml final volume. The mixture was incubated for 15 min at room temperature. Then this solution was diluted in 1 ml of water for absorption measurements or 1 ml of silver colloids preferably aggregated by 60 mM $NaNO_3$ for SERS studies. Spectra were immediately recorded. The presence of $NaNO_3$ induces changes in the size and shape of the metal particles, leading to a further increase of the SERS intensity of AmB adsorbed on the metal surface [9].

UV-visible absorption spectra were recorded on a Varian Cary 219 spectrophotometer, CD spectra on a Jobin-Yvon Mark V dichrograph.

SERS spectra were excited at two excitation wavelengths: the 514.5 nm line of a Spectra Physics ionized Argon laser (power 50 mW) which allowed excitation in resonance conditions, and the 1064 nm line of an YAG-Nd IR laser (power 200 mW) which was out of resonance. In the first case spectra were recorded on a multichannel Dilor XY modular spectrophotometer equipped with an intensified 1024-diode-array detector cooled by the Peltier effect; in the second case a Perkin-Elmer system 2000 NIR FT Raman spectrophotometer was used.

3. Results

3.1. UV-visible absorption and circular dichroism

AmB in an aqueous solution is present under monomeric and self-associated forms. UV-visible absorption and circular dichroism allow us to specifically detect them: a band at 409 nm is a specific of monomeric AmB, a band at 340 nm (a doublet for circular dichroism) is a specific of the aggregated form [10]. The addition of increasing concentrations of LDL to the 10^{-5} M AmB solution resulted in a change of the absorption spectra of AmB (Fig. 1). In the 400–430 nm region, the 409 nm band decreased, while a new band appeared at 415 nm. Its intensity increased up to an optical density of 0.47 (with a 1 cm cell path length), reached for a LDL concentration of 0.05 mg/ml in protein. In the 340 nm region, the absorption band decreased, as did the dichroic doublet [2]. The time dependence of the appearance of the band at 415 nm was also recorded for a mixture of 10^{-5} M AmB and 0.05 mg/ml of LDL. The time needed for reaching 50% of its maximum intensity was similar to that needed for the disappearance of 50% of the band intensity centered at 340 nm (data not shown).

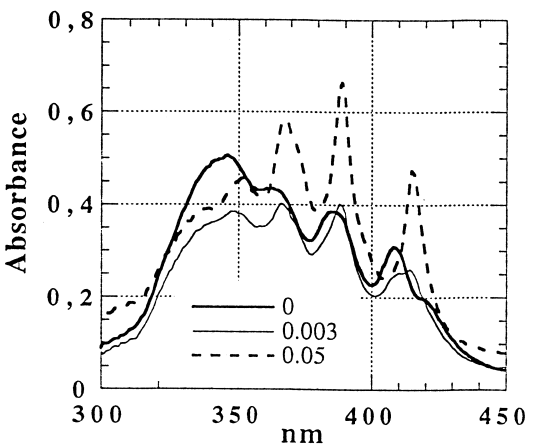

Fig. 1. Absorption spectra of 10^{-5} M AmB solutions in water in presence of LDL at various concentrations: (thick line) 0, (thin line) 0.003 and (dotted line) 0.05 mg/ml.

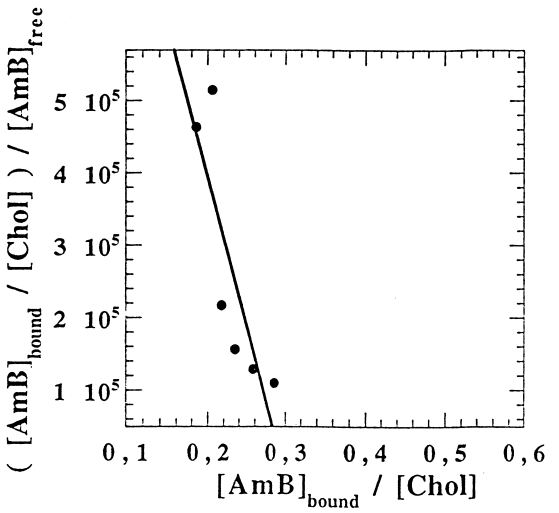

Fig. 2. Scatchard plot of the interaction of AmB with LDL: $r/L = K(n-r)$ with $r = [AmB]_{bound}/[cholesterol]$ and $L = [AmB]_{free}$.

Assuming that the new 415 nm band reflects the AmB bound to LDL, we used the already described algorithm [10] which enables us to quantify, from the absorption intensities at 409, 420 and 415 nm, the different forms of AmB, i.e. monomeric, self-associated and bound forms. From the concentrations of free AmB, a Scatchard plot was drawn as shown in Fig. 2, assuming that cholesterol is the binding site [2]. An association constant of $K=4\times10^6$ M^{-1}, with a stoichiometry of 1 molecule of amphotericin B for three molecules of cholesterol, was determined.

3.2. SERRS spectra

As previously described [7], the SERRS spectra of AmB consists essentially in two main lines at ca. 1556 and 1153 cm^{-1}. For increasing concentrations of LDL or plasma, a rapid intensity decrease of these Raman lines was observed. In order to quantify this effect, the ratio of the intensities of the 1556 cm^{-1} line and the 680 cm^{-1} line of DMSO was measured. As the amount of DMSO was maintained constant in all experiments (5% v/v) and this solvent does not give rise to a SERS spectrum under these conditions, the intensity of the line at 680 cm^{-1} can be used as an internal reference.

Two excitation wavelengths were used for these measurements. The excitation at 514.5 nm close to an electronic absorption band of AmB, allowed us to match the Raman resonance conditions with the concomitant strong enhancement

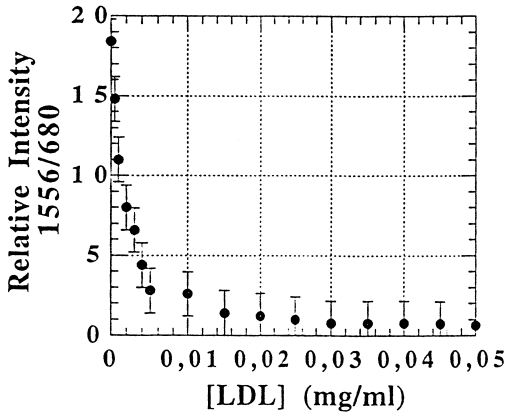

Fig. 3. Relative intensity of SERRS lines at 1556 cm^{-1} (AmB) and 680 cm^{-1} (DMSO) as a function of LDL concentration (10^{-5} M AmB in the presence of colloidal silver). Laser excitation: 514.5 nm; 50 mW.

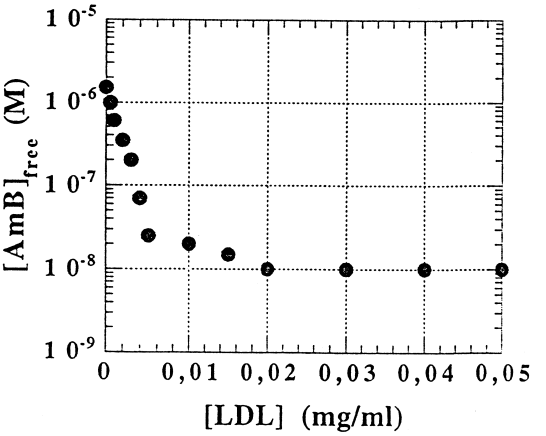

Fig. 5. Variation of the free AmB concentration in the presence of increasing concentration of LDL, as deduced from Figs. 3 and 4.

in SERS intensities (SERRS effect), whereas with the 1064 nm excitation, off resonance SERS spectra were obtained. The intensity ratios, measured at 514.5 nm excitation are shown in Fig. 3; it should be noted that they rapidly decrease from ca. 20 to 2, at a LDL concentration of 0.01 mg/ml in protein and they remain almost constant up to 0.05 mg/ml. Similar behavior was obtained for the intensity ratios measured with the 1064 nm excitation, but the measurements were less precise due to the poorest signal to noise ratios of SERS spectra excited at this wavelength (data not shown). However, this latter result gives evidence that no photochemical effect perturbed the measured ratios obtained under resonance conditions at 514.5 nm. A similar decrease was also observed with increasing plasma concentrations, but in this case SERS spectra could only be excited with a 1064 nm excitation wavelength since at 514.5 nm excitation wavelength spectra were obscured by a strong fluorescence background.

The intensity ratio of the 1556 and 680 cm^{-1} lines was measured for increasing concentrations of AmB alone, from 10^{-8} to 10^{-4} M with the 514.5 nm excitation wavelength (Fig. 4). From the comparison between this plot and that obtained in the presence of LDL (Fig. 3), it was possible to estimate the concentration of free AmB in solution, in the presence of LDL, assuming as it was stated previously [7] that the AmB-LDL complex is not SERS active. These data are presented in Fig. 5 for different concentrations of LDL; it clearly appears that from 0.02 mg/ml of LDL and up to 0.05 mg/ml the amount of free AmB is ca. 10^{-8} M.

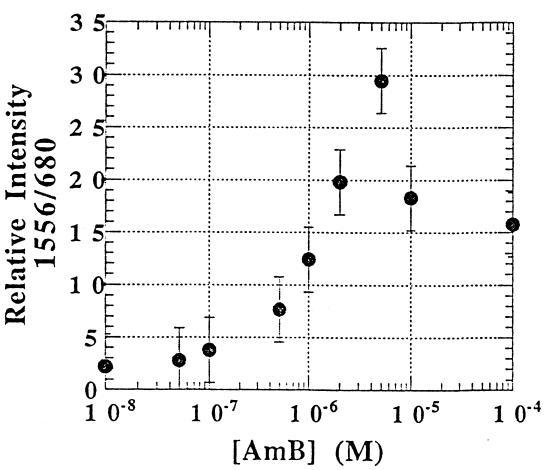

Fig. 4. Relative intensity SERRS lines at 1556 cm^{-1} (AmB) and 680 cm^{-1} (DMSO) as a function of AmB concentrations. Laser excitation: 514.5 nm; 50 mW.

4. Discussion

The most striking result of this study is that in the presence of LDL at a concentration higher than 0.02 mg/ml in protein, and 10^{-5} M total AmB, after a 15 min incubation, the concentration of free monomeric AmB detected by SERS was very low, significantly lower than 10^{-8} M. In blood, the LDL concentration being higher, the amount of bound AmB should also be higher and consequently the free AmB is negligible in these conditions ($< 10^{-8}$ M). Furthermore, in serum at 25°C, the amount of bound AmB is identical in LDL and HDL (high density lipoprotein) fractions [3], higher in VLDL and in non-lipoprotein fractions [4]. Under these conditions, it can be said that at the concentration of total AmB found in the sera of Fungizone-treated patients, that is around 5×10^{-6} M, the amount of free monomeric AmB in the blood is negligible. Therefore, under these conditions the formation of transmembrane channels is unlikely to occur.

It is interesting to compare the affinity constant of AmB for LDL obtained in this study from absorption data (4×10^{6} M^{-1}) with that obtained previously from circular dichroism measurements [2] (4×10^{5} M^{-1}). To explain this difference, it should be recalled that absorption mainly detects the monomeric form of AmB whereas circular dichroism detects self-associated species. Thus, the affinity of monomeric AmB for LDL would appear 10-fold higher than that of self-associated AmB (although the stoichiometry of the association is comparable, about three cholesterol molecules for one amphotericin B molecule). However, the self-associated species involve water-soluble oligomers and non-water-soluble aggregates: only water-soluble oligomers were shown to be toxic for human erythrocytes [11]. It may be assumed in the same way, that the monomers and the water-soluble oligomers only, interact with LDL and that the affinity constant of AmB for LDL is that obtained from absorption data. The absence of, or the weak binding of the non-water-soluble aggregates re-

sults in a lowered global affinity, as detected by circular dichroism.

Finally our results support the hypothesis that AmB toxicity for host cells does not result from transmembrane permeabilization by the free drug but implies a binding of the drug to LDL and LDL receptors. Thus, recent observations could be explained: the toxicity of AmB/LDL for rabbits is higher than that of AmB alone [12]; the inhibition of the AmB-lipoprotein interaction is correlated to the decrease of toxicity for mice of AmB bound to surfactants [13]; the toxicity of AmB for LLC PK1 renal cells is reduced when the cells do not express LDL receptors [14].

In conclusion, the importance of the modulation in plasma lipoprotein concentration and lipid composition upon the regulation of the activity of AmB should be emphasized [1,15].

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