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The Disaccharide Anthracycline MEN 10755 Binds Human Serum Albumin to a Non-classical Drug Binding Site

Luigi Messori,[a,*] Francesca Piccioli,[a] Silvia Gabrielli,[a] Pierluigi Orioli,[a] Leonardo Angeloni[a] and Cristina Di Bugno[b]

[a]*Department of Chemistry, University of Florence, Via Gino Capponi 7, I-50121 Florence, Italy*
[b]*Menarini Ricerche, Gruppo Menarini, Via Vecchia Livornese 897, I-56010, S.Piero a Grado, Pisa, Italy*

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Abstract—The interaction of the novel disaccharide anthracycline MEN 10755 with human serum albumin (HSA) was investigated by visible absorption and fluorescence spectroscopies and by ultrafiltration. Notably, MEN 10755 binds serum albumin far stronger than doxorubicin. Albumin binding results into a drastic quenching of the intrinsic fluorescence of MEN 10755; a binding constant of 1.1×10^5 was determined from fluorescence data. To localize the HSA binding site of MEN 10755 competition experiments were carried out with ligands that are selective for the different drug binding sites of the protein. No relevant competition effects were seen in the case of warfarin, diazepam and hemin, known ligands of sites I, II and III, respectively. Modest effects were observed following addition of palmitic acid that targets the several fatty acid binding sites of the protein. In contrast, extensive displacement of the bound anthracycline was achieved upon addition of ethacrinic acid. On the basis of these results, it is proposed that MEN 10755 binds serum albumin tightly to a non-canonical surface binding site for which it competes specifically with ethacrinic acid.

Introduction

Anthracyclines are important antitumor agents that find large application in the clinics.[1,2] The reversible binding of anthracyclines to serum components was previously observed, and serum albumin was suggested to be the major ligand for this family of drugs.[3–5] Early binding studies with doxorubicin and daunorubicin were carried out by equilibrium dialysis. Later on, the interactions of 4-demethoxydaunorubicin, 4′deoxy 4′iododoxorubicin and *N*-acylated derivatives with serum albumin were characterised by spectrophotometric titrations.[3] Recently, the binding of anthracycline derivatives to HSA has been measured either by ultrafiltration or by gel filtration.[4] Overall, these studies demonstrated extensive binding of anthracyclines to albumin and led to the determination of the affinity constants of the individual anthracyclines for serum albumin under physiological conditions. In addition, some preliminary spectroscopic results on the consequences of doxorubicin binding to HSA were reported by Lemiesza et al.[5] In spite of these literature reports, the information obtained until now on the binding of anthracyclines to HSA is limited; the location of the anthracycline binding site on HSA is still to be determined; competition studies with other drugs are lacking.

Thus, we thought that new physico-chemical investigations should be carried out to characterise in more detail the anthracycline binding site of HSA. A great help to these studies comes from the large progresses recently achieved in the field of albumin chemistry and in the structural analysis of this protein.[6–8]

The structural and functional features of human serum albumin (HSA), by far the most abundant protein in the plasma, are now well documented. HSA is a monomeric 585 residue protein that contains three structurally similar alpha helical domains (I–III). Each domain can be divided into subdomains A and B which comprise six and four alpha helices, respectively. Structural studies mapped the locations of the fatty acid binding sites and of the primary drug binding sites. The fatty acid binding sites are distributed throughout the protein and involve all six subdomains; drug sites I and II are localized in subdomains IIA and IIIA. A third site, that is specific

*Corresponding author. Tel.: +39-055-275-7556; fax: +39-055-275-7555; e-mail: messori@cerm.unifi.it

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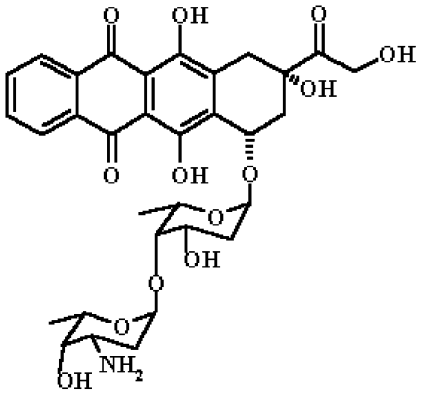
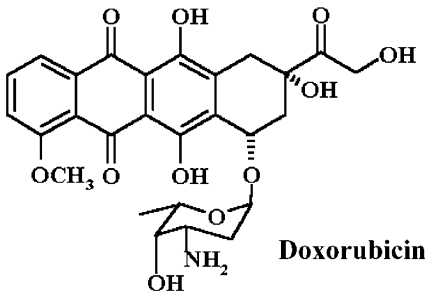


MEN 10755



Doxorubicin

Figure 1. Schematic representation of MEN 10755 and doxorubicin.

for hemin binding and is located in HSA domain I, has recently been characterised (site III) thanks to the utilization of recombinant protein fragments.[8]

MEN 10755 is a new anthracycline developed by the Gruppo Menarini, that has entered phase I clinical trials.[9–14] The presence of two sugar moieties and the absence of the methoxy group in position 4 confer to this anthracycline some significant pharmacological advantages with respect to doxorubicin (see Fig. 1). A comparative study on the binding of MEN 10755 and doxorubicin to *calf thymus* DNA has recently appeared demonstrating a substantially conserved pattern of interaction with DNA.[14] On this basis, it can be argued that the relevant differences in the biodistribution and pharmacokinetic profiles observed between MEN 10755 and doxorubicin arise from differences in their interactions with plasma proteins.

We report here on the interaction with HSA of the novel disaccharide anthracycline MEN 10755 probed by ultrafiltration and by absorption and fluorescence spectroscopies, in comparison to doxorubicin.

Results

Absorption spectra of MEN 10755 upon addition of HSA

The electronic spectrum of MEN 10755, dissolved in the physiological buffer, is characterized by two intense overlapping bands in the visible at 460 and 480 nm with a shoulder at 520 nm, as shown in Figure 2. Addition of HSA produces some slight modifications of the visible spectrum; in particular coalescence of the two peaks, at 460 and 480 nm, into a broader band centered at 475 nm is observed. These spectral changes can be reversed by ultrafiltration pointing out that the interaction of MEN 10755 for HSA is completely reversible. We noticed, however, that the spectral changes detected in the fluorescence mode are far larger than those revealed in the absorption mode implying that the former technique is more appropriate to investigate the MEN 10755/HSA interactions.

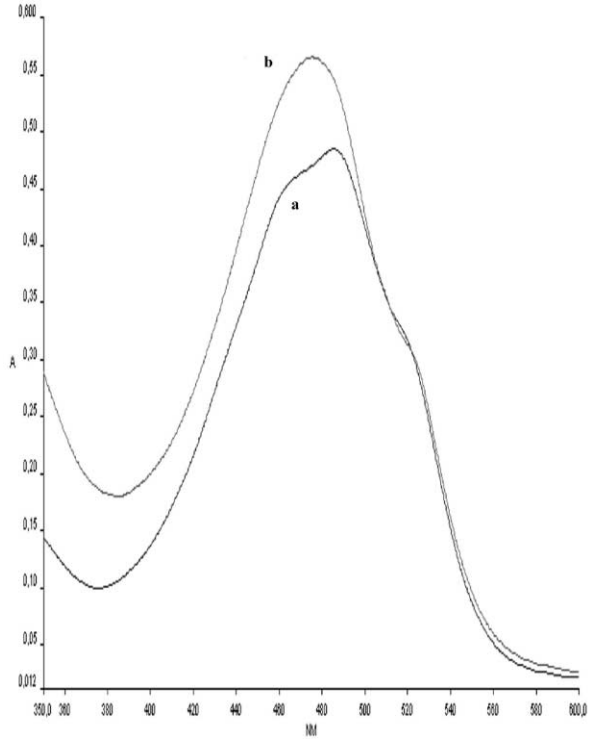


Figure 2. Electronic spectra of MEN 10755 (a) and of the adduct MEN 10755/HSA at 1:1 molar ratio. MEN 10755 concentration is 10^{-4} M in phosphate buffer pH 7.4.

Fluorescence studies

As previously reported, MEN 10755, like the other anthracyclines, is characterised by an intense fluorescence in the visible, that is extremely sensitive to the interaction of the anthracycline chromophore with various biomolecules. Notably, addition of the protein to MEN 10755 aqueous solutions, at physiological pH, results into a net decrease of fluorescence intensity; progressive fluorescence quenching is observed as the HSA/MEN 10755 molar ratio increases from 0.5 to 5. At higher ratios, saturation is reached and the final fluorescence spectrum is assigned to the protein bound form of MEN 10755. Whereas the residual fluorescence intensity is only $\sim$15% of the original value the position of the maxima is not significantly modified implying

that the polarity of the anthracycline binding site is not very different from that of the solution. Remarkably, quenching of the fluorescence of MEN 10755 upon addition of HSA is much larger than that reported by Lemiesza et al. for the interaction of doxorubicin with human serum albumin.[5] For comparison purposes the effects of HSA on doxorubicin fluorescence were measured again and found to be far smaller than in the case of MEN 10755 (Fig. 3).

Analysis of the fluorescence decreases of MEN 10755 as a function of added HSA led us to determine an affinity constant value of $1.1(\pm 0.3)\times 10^5$. Notably, in the case of doxorubicin, an upper limit of $3.0(\pm 0.2)\times 10^3$ was estimated for the binding constant to HSA.

Ligand competition studies

The above results clearly point out that MEN 10755 binds specifically HSA to a high-affinity binding site; however, no definite information has been obtained so far concerning the nature and the location of this binding site. Thus, several competition experiments were carried out with ligands whose selectivity for the individual sites is clearly documented. Competition processes were monitored either by fluorescence spectroscopy or by ultrafiltration coupled to spectrophotometric determinations.

Competitive drug binding analyzed by fluorescence measurements

In a first series of experiments MEN 10755 samples saturated with HSA were treated with increasing amounts of the following ligands: warfarin, ethacrinic acid, diazepam, hemin, palmitic acid. We expected that possible displacement of MEN 10755 from its binding site would result into a measurable increase of anthracycline fluorescence. In the case of warfarin, diazepam and palmitic acid addition of even large amounts of these ligands failed to induce large displacement of the bound anthracycline. Just modest fluorescence increases were observed for (3:1) ligand to HSA molar ratios (increases in fluorescence intensity ranged from 10 to 25%). In the case of hemin or bilirubin no reliable results were obtained due to occurrence of relevant energy transfer effects and of nearly complete fluorescence quenching. At variance, a very large fluorescence recovery was observed following addition of ethacrinic acid as shown in Figure 4. It is notable that at 3:1 ethacrinic acid to HSA molar ratio, the fluorescence increase is more than 100%. At higher molar ratios almost complete recovery of the fluorescence of the free anthracycline is achieved. The fact that competition effects with ethacrinic acid are large suggests that MEN 10755 and ethacrinic acid share a common binding site on HSA.

Competition processes analyzed by ultrafiltration and absorption spectroscopy

To support the results obtained from competition experiments, displacement studies were repeated following a different procedure in which ultrafiltration and

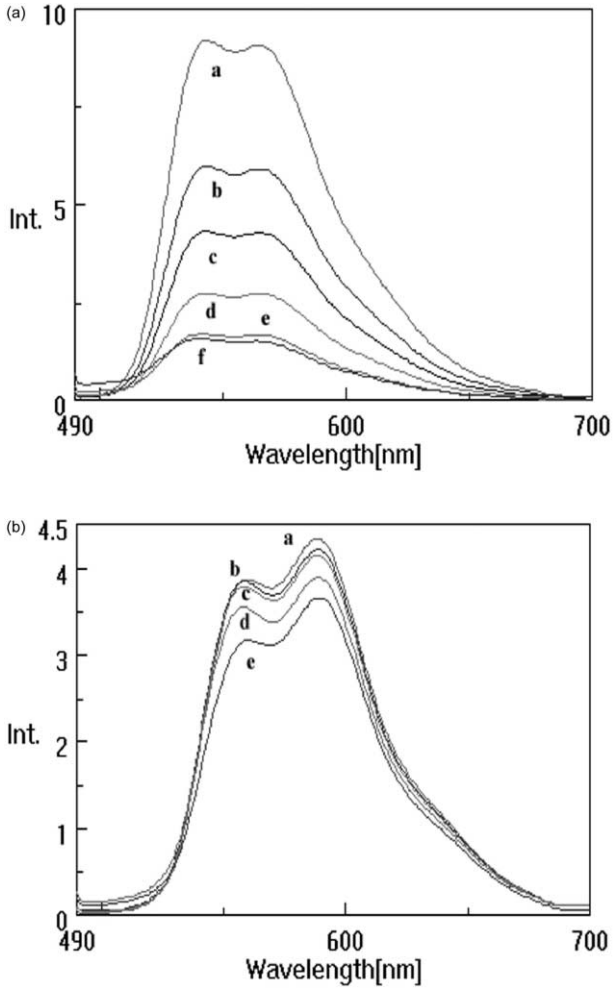


Figure 3. Fluorescence quenching of (a) MEN 10755 and (b) doxorubicin upon addition of increasing amounts of HSA, in phosphate buffer pH 7.4. In the course of the titrations *r* (number of the moles of HSA per mole of drug) varies from 0.5 to 10 (curves with the following *r* values are represented a = 0, b = 0.5, c = 1, d = 2, e = 6, f = 10).

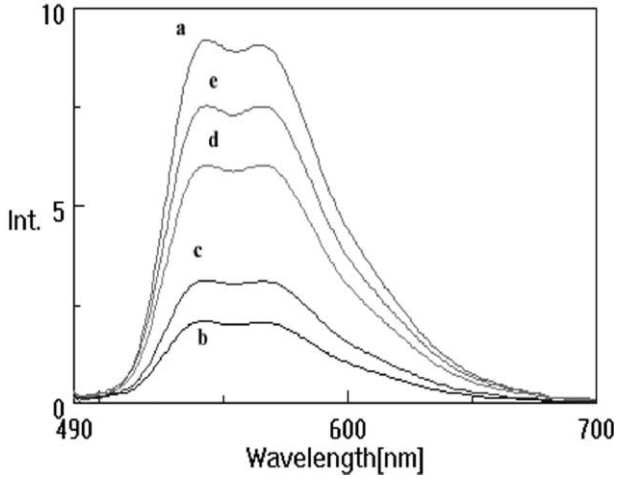


Figure 4. Fluorescence spectra of MEN 10755 (4×10^{-5} M) (a), and of the adduct MEN 10755/HSA (1:3) adduct (b) in the phosphate buffer, pH 7.4. Fluorescence increases observed upon addition of increasing amounts of ethacrinic acid are shown. ETA/HSA ratios are the following: 1.0 (c), 3.0 (d) and 5.0 (e).

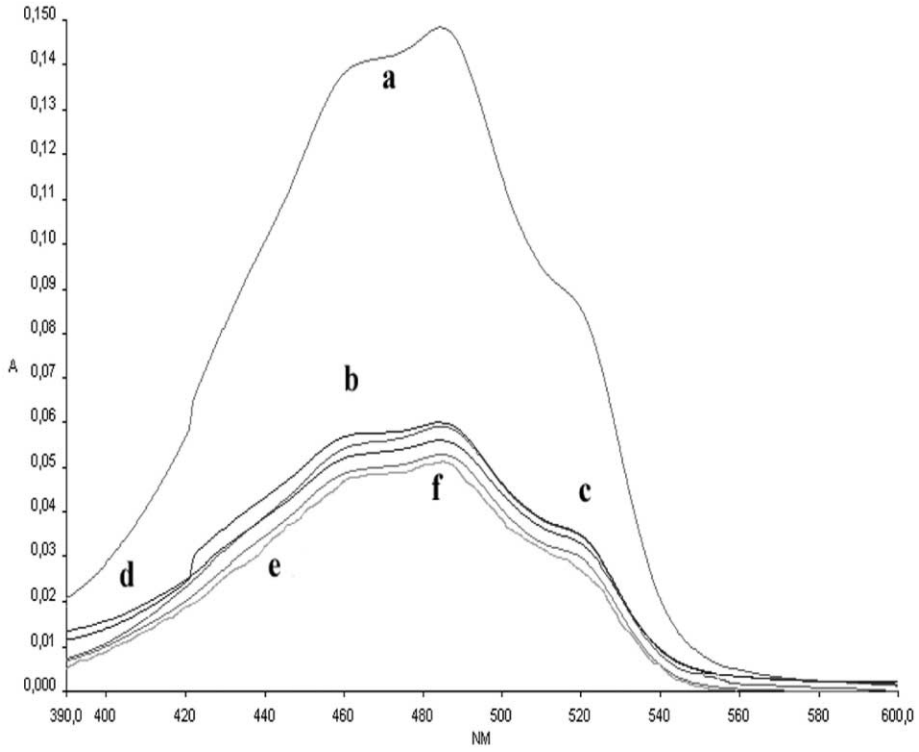


Figure 5. Visible spectra of the ultrafiltered solution for the following systems: MEN 10755 (10^{-4} M)/HSA (1:1) (d); MEN 10755 (10^{-4} M)/HSA (1:1) plus ethacrinic acid (a); MEN 10755 (10^{-4} M)/HSA (1:1) plus diazepam (b), MEN 10755 (10^{-4} M)/HSA (1:1) plus palmitic acid (c), MEN 10755 (10^{-4} M)/HSA (1:1) plus warfarin (e) and MEN 10755 (10^{-4} M)/HSA (1:1) plus hemin (f) All experiments were carried out in the reference phosphate buffer in the presence of an excess of each drug compared to MEN 10755.

spectrophotometric measurements were coupled. A series of MEN 10755 samples were treated with saturating amounts of HSA. Then, each of the following ligands (warfarin, diazepam, palmitic acid, hemin, and ethacrinic acid) was added at a 3:1 molar ratio and the samples were ultrafiltered. The amount of MEN 10755 in the lower fraction was analyzed spectrophotometrically with respect to a control; results are shown in Figure 5.

It is evident that ethacrinic acid is again very effective in promoting anthracycline release from albumin in line with fluorescence data; in contrast, diazepam, warfarin, palmitic acid and hemin do not induce significant MEN 10755 release from HSA, compared to the control.

If one considers that the tested ligands possess relatively large affinity constants for their specific HSA sites and that the displacement processes are generally fast, it follows that MEN 10755 is *not* a selective ligand for HSA site I, II or III nor for the fatty acid binding sites.

Discussion

Although the interactions of some representative drugs with albumin have been extensively studied and the respective mechanisms of binding clarified in detail, binding of anthracyclines to human serum albumin is still poorly investigated and understood. Thus, the novel disaccharide anthracycline MEN 10755 has offered us the chance to analyze in more detail the interactions of anthracyclines with serum albumin. At variance with doxorubicin the intrinsic fluorescence of MEN 10755 is drastically quenched by HSA within an accessible range of protein concentrations, and this quenching allowed us to monitor directly the protein binding and the drug displacement processes. Additional valuable information on the drug binding/displacement processes has been obtained by independent measurements based on absorption spectroscopy and ultrafiltration techniques.

The *first important result* of this study is the demonstration of tight binding of MEN 10755 to HSA under physiological conditions. Binding to HSA has been independently assessed by absorption spectroscopy, by ultrafiltration and by fluorescence quenching. The latter method turned out to be the most appropriate and most sensitive to reveal protein binding and to evaluate the affinity constant. The value of 1.1×10^5 calculated for MEN 10755 is far larger than the value of 3.0×10^3 estimated for doxorubicin. Such a result implies that the absence of the methoxy group and the presence of a second sugar moiety lead to a sizable stabilisation of the anthracyline/protein adduct.

Also, it is remarkable that the MEN 10755/HSA interaction is completely reversible as revealed by ultrafiltration experiments. Reversibility of protein binding is demonstrated by the observation that the modifications of the visible spectra induced by HSA are abolished by ultrafiltration. Binding reversibility implies that the

drug/protein interaction is not covalent in nature nor induces any chemical transformation of the drug.

The *second issue* that we have specifically addressed in this study is the identification, characterization and localization of the specific HSA binding site for MEN 10755 and, more in general, for anthracyclines. The information presently available in the literature on this issue is fragmentary and somehow controversial.[3–5] We have performed a number of HSA binding competition experiments in the presence of either MEN 10755 or doxorubicin, using classical ligands whose selectivity for the HSA drug binding sites is documented. We specifically employed warfarin, diazepam, hemin, palmitic acid; ethacrinic acid was also chosen owing to its ability to promote large displacement of bound anthracyclines.

Our binding competition experiments have provided significant and consistent results. Notably, we have observed that ligands that are selective for sites I, II and III (warfarin, diazepam and hemin), produce modest release of the bound anthracyclines even when added at a 3:1 molar excess. These results rule out that MEN 10755 is a selective ligand for sites I, II or III. Similar results were obtained in the case of palmitic acid suggesting that competition of MEN 10755 for the numerous fatty acid binding sites of HSA is marginal.

In contrast, large competition effects were detected with ethacrinic acid. This latter drug exhibits some selectivity for Sudlow site II (the site of diazepam) but is also able to bind to additional drug binding sites of HAS.[15] The fact that diazepam, at variance with ethacrinic acid, does not induce any significant release of MEN 10755 tends to exclude site II as the possible binding site.

On the ground of these results we propose that MEN 10755 and ethacrinic acid share a common binding site that does not correspond to any of the classical drug binding sites of HSA. Competition studies also suggest that binding of ethacrinic acid to this site is stronger than binding of MEN 10755. This finding might be of clinical interest. Indeed, ethacrinic acid, a common diuretic agent, might be potentially dangerous for its ability to increase the plasma concentration of free anthracyclines.

Studies are in progress to better characterize this novel, non canonical, binding site of HSA, that most likely represents the common binding site of anthracyclines.

Conclusions

The interaction of the disaccharide anthracycline MEN 10755 with HSA has been monitored by various physicochemical techniques. It has been shown that MEN 10755 binds tightly but reversibly HSA with an affinity constant of 1.1×10^5. Notably binding of MEN 10755 to HSA is stronger than that of doxorubicin. Competition studies show that MEN 10755 does not interact significantly with the classical drug binding sites of HSA. In contrast, large displacement effects were observed upon addition of ethacrinic acid. These effects are interpreted in terms of competitive binding of MEN 10755 and ethacrinic acid to a non-canonical drug binding site of HSA.

Materials and Methods

Materials

MEN 10755 was prepared by Laboratori Guidotti, Gruppo Menarini, Pisa, according to the reported method.[9] Doxorubicin, Warfarin, Hemin, Ethacrinic Acid, Diazepam, Palmitic Acid and Human Serum Albumin were purchased from SIGMA Chemical Company. Where not differently stated, experiments were performed in phosphate buffer containing NaH_2PO_4 (50 mM), NaCl (100 mM), (pH 7.4).

Spectroscopic measurements

Visible absorption spectra were carried out with a Perkin-Elmer Lambda Bio 20 instrument. The measurements were done at room temperature (25 °C). Fluorescence spectra were performed with a Jasco FP-750 spectrofluorimeter in room temperature with $\lambda_{ex}=480$ nm.

Ultrafiltration experiments

The anthracycline/HSA adducts were ultradialyzed after 24 h incubation at room temperature, using Centricon YM-10 (Amicon Bioseparations, Millipore Corporation, USA). The absorption spectra of the upper and the lower portions were recorded to determine anthracycline concentration.

Calculation of the apparent equilibrium binding constant

The apparent equilibrium binding constant, K'_f, was calculated using the familiar mass action equation, in which the numerator contains the apparent concentration of albumin–anthracycline complexes, while the denominator is the product of the apparent unbound anthracycline concentration $[D_f]$ and the concentration of unoccupied albumin binding sites ($[HSA]-[D_t]+[D_f]$). $[D_f]$ is the measured total anthracycline concentration.[16] All these quantities were determined from the fluorescence spectra for each step of the titration.

As used here, K'_f is the first stepwise binding constant of albumin as defined by Spector et al.[17]

$$K'_f=\frac{[D_t]-[D_f]}{[D_f]([HSA]-[D_t]+[D_f])}$$

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