Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 199 (2018) 308–314

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

A combined photophysical and computational study on the binding of mycophenolate mofetil and its major metabolite to transport proteins

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ARTICLE INFO

Article history:
Received 19 December 2017
Received in revised form 6 March 2018
Accepted 23 March 2018
Available online 27 March 2018

Keywords:
Drug-protein binding
Docking
Fluorescence
Human serum albumin
Human α_1-acid glycoprotein

ABSTRACT

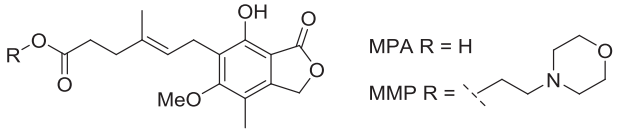
Binding of the immunosuppressive agent mycophenolate mofetil (MMP) and its pharmacologically active metabolite mycophenolic acid (MPA) to human serum albumin (HSA) and α_1-acid glycoprotein (HAAG) has been investigated by means of an integrated approach involving selective excitation of the drug fluorophore, following their UV-A triggered fluorescence and docking studies. The formation of the protein/ligand complexes was evidenced by a dramatic enhancement of the fluorescence intensity and a hypsochromic shift of the emission band. In HSA, competitive studies using oleic acid as site I probe revealed site I as the main binding site of the ligands. Binding constants revealed that the affinity of the active metabolite by HSA is four-fold higher than its proactive form. Moreover, the affinity of MMP by HSA is three-fold higher than by HAAG. Docking studies revealed significant molecular binding differences in the binding of MMP and MPA to sub-domain IIA of HSA (site 1). For MPA, the aromatic moiety would be in close contact to Trp214 with the flexible chain pointing to the other end of the sub-domain; on the contrary, for MMP, the carboxylate group of the chain would be fixed nearby Trp214 through electrostatic interactions with residues Arg218 and Arg222.

1. Introduction

The binding of drugs to plasma proteins determines their biodistribution, elimination and therapeutic or toxic effects. It is generally accepted that only the unbound form of a drug interacts with the receptor, to produce pharmacological effects. This principle is the basis for understanding the pharmacokinetic behavior and the pharmacological activity of those drugs that are fast and extensively bound to plasma proteins at therapeutic concentrations [1].

Among plasma proteins, human serum albumin (HSA) and human α_1-acid glycoprotein (HAAG) play a crucial role as drug carriers [2]. Thus, HSA is a globular protein of 585 amino acids, which accounts for about 60% of the total protein amount in blood serum. It has an exceptional binding capacity for a wide range of endogenous and exogenous ligands, being an important determinant of the pharmacokinetic behavior of many drugs. Its structure is well-known, containing three homologous domains assembled to form a heart-shaped macromolecule. Each domain contains two subdomains with common structural motifs. The principal regions of ligand binding to HSA are located in hydrophobic cavities of subdomains IIA and IIIA [3]. Concerning HAAG, it is less abundant than HSA, although its serum concentration increases in response to a systemic tissue injury, inflammation, or infection. HAAG contains 183 amino acids, two disulfide bridges, and the carbohydrate content represents 45% of the molecular weight attached in the form of five to six highly sialylated complex-type-*N*-linked glycans. This protein has the ability to bind and carry mainly basic and neutral lipophilic ligands from endogenous and exogenous origin and has one main binding site, which is large and flexible [4]. Most acidic, neutral, and basic drugs bind to HSA, and a few basic drugs bind almost exclusively to HAAG [5].

Mycophenolate mofetil (MMP) is an immunosuppressive agent, prescribed to prevent acute rejection of transplanted organs [6]. It is a prodrug that undergoes rapid hydrolysis by esterases in liver and kidney, to afford the pharmacologically active metabolite form mycophenolic acid (MPA) [7]; the latter behaves as selective and reversible inhibitor or inosine monophosphate dehydrogenase, acting on T and B lymphocytes. Other uses of MMP are as antimicrobial, anti-inflammatory, antifibrotic, proapoptotic, antiangiogenic, anticancerous or antioxidant [8].



It is known that MPA is mostly bound to HSA (98–99%), and that its pharmacological activity is linked to the concentration of unbound drug.

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https://doi.org/10.1016/j.saa.2018.03.064
1386-1425/

Factors such as hypoalbuminemia influence the protein binding of MPA, altering the free concentration *in vivo*. In addition, MPA-protein binding is relevant in renal transplantation and its insufficiency [9].

In this context, the binding of MPA to HSA has been explored in the past by following the fluorescence quenching of the Trp residue of HSA in the presence of increasing amounts of MPA. However, it is not possible to excite Trp selectively, due to spectral overlap with MPA, so fluorescence quenching can be masked by filtering effects, resulting in misleading interpretations [8a,10]. The problems are circumvented in the present work by interrogating the binding of MMP and its major metabolite MPA to transport proteins (HSA and HAAG), which allows selective excitation of the drug fluorophore, following their UV-A triggered fluorescence. The results are consistent with those obtained by means of computational studies.

2. Results and Discussion

In this section, the photophysical properties in homogenous drug solutions, corresponding to the free forms, are presented first. This is followed by the results obtained with the bound forms, using HSA and HAAG as host proteins. Variations observed in the steady-state and time-resolved emission experiments, specifically the fluorescence quantum yields and lifetimes, can be associated with the binding process. This, in combination with the results obtained using displacement probes, is exploited to provide key information on the nature of the binding site and the magnitude of the binding constant. Finally, computational studies are presented that explain the experimental results.

2.1. Photophysical Properties of MMP and MPA in PBS

The absorption spectrum of MMP in PBS (phosphate buffered saline solution, pH = 7.4) is shown in Fig. 1A (black trace). Two bands can be observed above 280 nm, in the UV-A and UV-B regions, with maxima at λ = 340 nm and 310 nm, respectively; they are ascribed to phenolate and phenol MMP species, according to previous reports on the absorption spectra at different pH values [11]. As expected, the absorption spectrum of MPA is very similar (Fig. 1B), since both MMP and MPA contain the same active chromophore.

The fluorescence emission and excitation spectra of MMP and MPA were also recorded in PBS (Fig. 2A and B, respectively). For both compounds, the emission band exhibited a maximum at λ = 432 nm, which did not depend on the excitation wavelength (310 or 340 nm); such emission is attributed to deprotonated phenolate singlet excited state, according to the excitation spectrum with maximum at λ = 340 nm. From the intersection between normalized excitation and emission spectra (values between 380 and 382 nm in case of MMF and 377 and 381 nm in case of MPA), the singlet energy of the phenolate species is *ca.* 75 kcal/mol.

The fluorescence quantum yields (ϕ_F) of MMP and MPA were determined using carbazole as standard [12], the corresponding values are presented in Table 1, entries 1 and 4; both were lower than 0.1, although ϕ_F (MMP) was somewhat higher than ϕ_F (MPA). The fluorescence lifetimes (τ_F) were under the detection limit of our apparatus (lower than 1 ns).

2.2. Photophysical Properties of MMP and MPA Within HSA

The fluorescence spectra of MMP and MPA in the presence of increasing amounts of HSA are shown in Fig. 3A and B, respectively. For both MMP and MPA, addition of protein led to a hypsochromic shift of the band, with the new maximum located at 422 nm; besides, the fluorescence intensity increased dramatically after HSA addition. These trends agree with an efficient ligand/protein interaction, as revealed further by the enhanced ϕ_F and τ_F values, determined at 1:1 ligand/protein molar ratio (Table 1, entries 2 and 5).

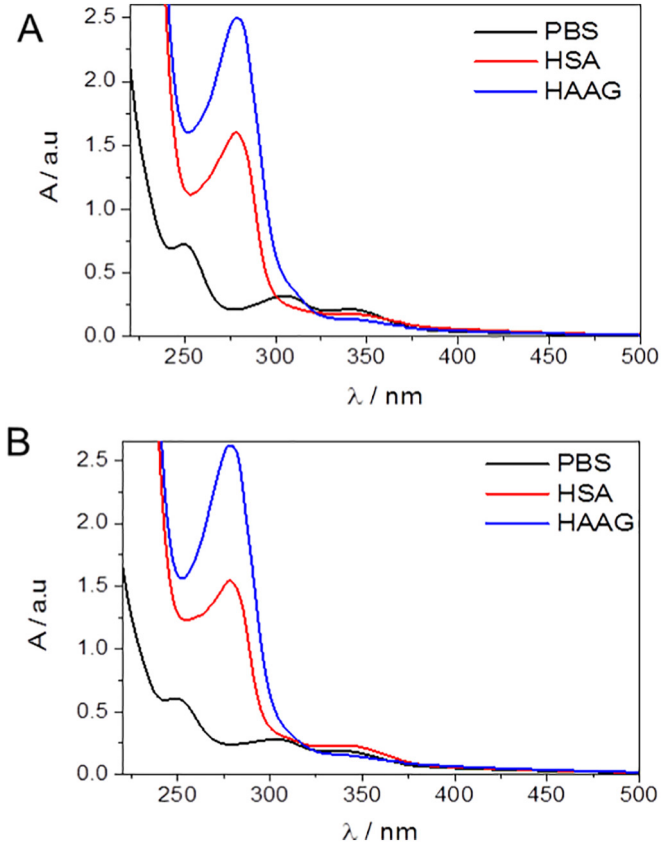

Fig. 1. UV absorption spectra of A: MMP and B: MPA in different media: PBS (black), HSA at 1:1 ligand@protein molar ratio (red) and HAAG at 1:2 ligand@protein molar ratio (blue). Concentration of ligand was 4.0×10^{-5} M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fluorescence quantum yields were obtained using carbazole as standard ($\phi_{F(std)}$ (EtOH) = 0.42) [12], using Eq. (1)

$$\phi_{F(i)} = \frac{A_{(i)}}{A_{std}} \cdot \frac{Abs_{(std)}}{Abs_{(i)}} \cdot \frac{n}{n_{std}} \cdot \phi_{F(std)} \quad (1)$$

where, $A_{(i)}$ is the fluorescence area of the drugs, A_{std} is the fluorescence area of standard, *Abs* and $Abs_{(std)}$ corresponds to the absorbance intensity at 340 nm of drug and standard, respectively, and *n* is the refraction index of solvent employed.

The binding constants (K_B) for MMP@HSA and MPA@HSA complexes were determined using the modified Benesi-Hildebrand equation (Eq. (2)) [13], where the stoichiometry of the complex was taken as 1, according to the Job-plot curves (Fig. S1 in Supporting Information) [14]

$$\frac{1}{F - F_0} = \frac{1}{(F_{max} - F_{min})} + \frac{1}{K_B (F_{max} - F_0)} \cdot \frac{1}{C^n} \quad (2)$$

where, *F* corresponds to the fluorescence intensity of the drugs at each protein concentration (0 to 6.0×10^{-5} M), F_0 is the fluorescence of the MMP or MPA in the absence of proteins, *C* is the molar protein concentration and *n* is the stoichiometry of the complex, determined by the Job-plot graphs (1 in all cases).

Thus, from the plot of $1/(I - I_o)$ *vs* 1/[HSA] (Fig. 4), K_B values of $3.2 \times 10^4\ M^{-1}$ (MMP) and $1.3 \times 10^5\ M^{-1}$ (MPA) were obtained, indicating a moderate-strong drug/protein interaction, somewhat smaller than that of warfarin ($K_B = 3.3 \times 10^5\ M^{-1}$) but higher than those of the antihistaminic epinastine hydrochloride ($K_B = 2.72 \times 10^4\ M^{-1}$) or the antiallergic cetirizine hydrochloride ($K_B = 2.4 \times 10^4\ M^{-1}$) [15].