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Short communication

In vitro influence of fatty acids and bilirubin on binding of mycophenolic acid to human serum albumin

Yoann Vial[a], Michel Tod[b,c], Marilyne Hornecker[a], Saik Urien[d,e], Filomena Conti[f], Alain Dauphin[a], Yvon Calmus[f], Benoit Blanchet[a,*]

[a] *Laboratoire de pharmacologie-toxicologie, service de Pharmacie, GH Cochin Saint Vincent de Paul, 75014 Paris, APHP, France*
[b] *Pharmacie, Hôpital de la Croix-Rousse, Hospices civils de Lyon, Lyon, France*
[c] *Université de Lyon, F69008, Lyon, France*
[d] *Unité de Recherche Clinique, Hopital Tarnier, 89 rue d'Assas, 75006 Paris, APHP, France*
[e] *CIC-0901 Inserm Necker-Cochin and EA-3620, Paris, France*
[f] *Unité de Transplantation hépatique, Hôpital Saint-Antoine, 75011 Paris, APHP, France*

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ABSTRACT

Mycophenolic acid (MPA) is 98–99% bound to albumin. Because MPA is restrictively cleared and has a low extraction coefficient, increase in its free fraction related to decreased albumin binding results in lower total concentrations but unchanged unbound concentrations. Multiple factors, including hypoalbuminemia, impaired renal function, and accumulated mycophenolic acid glucuronide are known to reduce MPA protein binding. Little is known about the influence of fatty acids and bilirubin on this issue. By using quenching fluorescence method, the aims of this study were to investigate *in vitro* the binding properties of MPA, then the influence of myristic acid and bilirubin on MPA binding to albumin. The estimate of dissociation constant (Kd) of MPA was 13.2 [CI 95 12.7–13.8] μM. In the presence of myristic acid (concentration range 4–100 μM), apparent Kd (Kd_{app}) of MPA was approximately 1.5–10-fold greater. For myristic acid/albumin molar ratio reachable in clinical settings (2:1 and 5:1), Kd_{app} of MPA rose about a factor 1.5 and 2.2, respectively. In the presence of bilirubin (concentration range 0.5–5 μM), Kd_{app} of MPA was approximately 1.5–5-fold greater than MPA Kd. For bilirubin/albumin molar ratio reachable in clinical settings (1:4 and 1:2), Kd_{app} of MPA rose about a factor 1.5 and 1.9, respectively. These data suggest that hypertriglyceridemia or cholestasis may significantly increase MPA free fraction in clinical settings, thereby lowering MPA total concentration in plasma while the free concentration remains unchanged. These results may help to optimize the therapeutic drug monitoring of MPA.

1. Introduction

Mycophenolate mofetil (MMF), a prodrug of mycophenolic acid (MPA), is widely used to prevent acute graft rejection after solid organ transplantation. MPA is 98–99% bound to albumin and primarily metabolized by uridine diphosphate glucuronyl transferases (UGT) to the pharmacologically inactive phenolic MPA glucuronide metabolite (MPAG) [1]. Because MPA is restrictively cleared and has a low extraction coefficient, increase in its free fraction (MPA_{fu}) related to decreased albumin binding results in lower total concentrations but unchanged unbound concentrations, so long as the intrinsic clearance of the drug is unaffected [2]. A high variability in MPA_{fu} has been largely described in transplant recipients [1]; and multiple factors, including hypoalbuminemia, impaired renal function and accumulated MPAG are known to influence MPA protein binding [1,3].

The primary physiological role of albumin in plasma is to transport bilirubin and fatty acids [4] that can accumulate to relatively high concentrations in plasma. The influence of bilirubin on MPA binding to albumin has been commonly suggested but the quantitative relationship has not been established so far [1,5,6]. Besides, hypertriglyceridemia is a common problem in transplant recipients, occurring in up to 60% in renal recipients [7]. However, to our knowledge, the influence of fatty acids on MPA binding has still not been investigated. By using the quenching fluorescence method, the aims of this study were to investigate *in vitro* the binding properties of MPA, then the influence of bilirubin and fatty acids on MPA binding to albumin.

2. Materials and methods

2.1. Reagents and apparatus

Human serum albumin (fraction V fatty acid free), MPA, myristic acid (MYR) and Tris–HCl were purchased from Sigma (St. Louis, MO,

* Corresponding author. Tel.: +33 1 58 41 23 13; fax: +33 1 58 41 23 15.
E-mail address: benoit.blanchet@cch.aphp.fr (B. Blanchet).

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USA). Human serum albumin (HSA) solution (2 μM) was prepared in pH 7.4 Tris–HCl buffer solution. Stock solution of MPA (1 g/l) was prepared in methanol, then stored at −20 °C in the dark. Stock solution of MYR (5000 μM) was prepared in methanol, then stored at 4 °C in the dark up to one week. Stock solution of bilirubin (250 μM) was daily prepared in 0.5 M NaOH. A Shimadzu RF-1501 fluorescence spectrophotometer (Champs sur Marne, France) equipped with a 10 mm quartz cell was used to measure the fluorescence intensity. The maximum excitation wavelength (λex) and maximum emission wavelength (λmax) for HSA were 280 and 354 nm, respectively.

2.2. MPA albumin binding

Appropriate amounts of MPA were transferred in glass tube and evaporated to dryness under a gentle stream of nitrogen gas. Then, the dry residues were reconstituted in 1 ml of 2 μM HSA solution. The final concentrations of MPA in HSA solution were 1, 2, 10, 20, 50, 100, 500 and 1000 μM. The resultant mixture was incubated 1 h at 37 °C. A blank system containing only 1 ml of 2 μM HSA was similarly prepared. After 1-h incubation, fluorescence intensity was measured. Each experiment was performed in triplicate.

2.3. Bilirubin–albumin binding

Appropriate amounts of bilirubin (20 μl) were added to 980 μl of 2 μM HSA solution. The final concentrations of bilirubin in HSA solution were 0.5, 1, 5, 10, 20, 40, 60, 80, 100, 150 and 250 μM. The resultant mixture was incubated only 15 min at 37 °C to avoid the photodegradation of bilirubin. A blank system containing only 1 ml of 2 μM HSA was similarly prepared. After 15-min incubation, fluorescence intensity was measured. All procedures were carried out under minimal light. Each experiment was performed in triplicate.

2.4. Influence of MYR and bilirubin on MPA albumin binding

Appropriate amounts of MPA were transferred in 5 ml glass tube and evaporated to dryness under a gentle stream of nitrogen gas. Dry residues were reconstituted in 1 ml of 2 μM HSA solution including either MYR or bilirubin solution 2% (v/v). MYR or bilirubin was added in HSA solution during MPA dry residues reconstitution. The incubation times for MPA in the presence of MYR or bilirubin were 60 and 15 min, respectively. The final concentrations of MPA in HSA solution were 1, 2, 10, 20, 50, 100, 250 and 500 μM. Regarding competitors, the final concentrations of MYR were 4, 10, 20, 40, 80 and 100 μM and those of bilirubin were 0.1, 0.5, 1 and 5 μM.

2.5. Data analysis

The fluorescence intensity versus MPA concentration data were analyzed by non-linear regression. The fluorescence intensity was calculated as the mean of the three experiments. The exact mathematical expression for describing competitive binding of two different ligands to a protein molecule [8] was fitted to the data by weighted least squares. The results are expressed as point estimate [95% confidence interval].

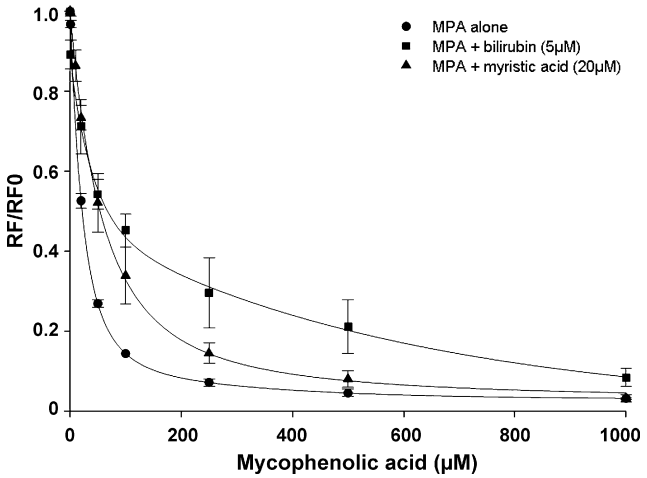

Fig. 1. Example of quenching curves illustrating competition between mycophenolic acid (MPA) and myristic acid (MYR, 20 μM) or bilirubin (5 μM). RF0 is the fluorescence intensity of albumin in the presence of MYR or bilirubin. RF is the fluorescence intensity in the presence of MPA and endogenous ligand (MYR, bilirubin).

3. Results

3.1. Binding properties of MPA

MPA quenched HSA fluorescence in a concentration-dependant manner (Fig. 1). The estimates of dissociation constant of MPA (Kd_{MPA}) and binding capacity (B_{max}) were 13.2 [12.7–13.8] and 3.44 [0.97–5.91] μM. As a 2 μM HSA solution was used for this analysis, we calculated that 1 mol of HSA could bind 1.72 mol of MPA. At physiological albumin concentration of 680 μM (45 g/l) and 453 μM (30 g/l), MPA_{fu} would be 1.1% and 1.7% respectively.

3.2. Influence of MYR on MPA albumin binding

Whatever the MYR/HSA molar ratio tested (2–50), no significant change in HSA relative fluorescence was observed. In the presence of MYR, the profiles of MPA quenching curve were significantly changed compared to that obtained without MYR (Fig. 1). For MYR concentrations ranging from 4 to 100 μM, the apparent Kd (Kd_{app}) of MPA was approximately 1.5–10-fold greater than Kd of MPA (Table 1).

3.3. Influence of bilirubin on MPA albumin binding

The estimates of Kd and B_{max} for bilirubin were 1.06 [0.89–1.24] and 5.27 [4.77–5.77] μM. To investigate the influence of bilirubin on MPA binding, Ki in the competition model was set to the Kd

Table 1
Estimates of apparent dissociation constant of mycophenolic acid in the presence of increasing concentrations of myristic acid.

MYR concentration (μM)	MPA Kd_{app} (μM)	CI 95	MYR Ki (μM)	CI 95
0	13.24	12.68–13.81		
4	19.85	18.49–21.21	7.97	6.51–9.42
10	28.54	27.55–31.38	8.11	7.27–8.96
20	51.66	46.04–52.44	7.31	6.76–7.87
40	73.33	79.92–98.28	6.94	6.16 – 7.72
80	104.50	101.51–120.09	10.81	9.90–11.73
100	128.80	118.09–139.52	11.41	10.48–12.35

Values are expressed as estimate point with 95% confidence interval (CI 95) MPA, mycophenolic acid; MYR, myristic acid; Kd_{app}, apparent dissolution constant; Ki is the dissociation constant of myristic acid.

Table 2
Apparent constant of dissociation of mycophenolic acid in the presence of increasing concentrations of bilirubin.

Bilirubin concentration (μM)	MPA Kd_{app} (μM)	CI 95
0	13.24	12.68–13.81
0.1	14.43	13.81–15.05
0.5	19.18	18.34–20.02
1	25.12	23.99–26.25
5	70.79	67.39–74.19

Values are expressed as estimate point with 95% confidence interval (CI 95). MPA, mycophenolic acid; Kd_{app}, apparent dissociation constant.

value of bilirubin. For bilirubin concentrations ranging from 0.5 to 5 μM, Kd_{app} of MPA increased approximately 1.5–5-fold than Kd of MPA (Table 2).

4. Discussion

By using an ultrafiltration procedure, Nowak et al. previously found a MPA binding constant of 13.76 μM [3]. Additionally, they calculated that 1 mol of albumin could bind 1.8 mol of MPA. In the current work, Kd of MPA was estimated at 13.24 μM, and we found that 1.72 mol of MPA could be bound by 1 mol of albumin. The great concordance between these two investigations proves the reliability of the quenching fluorescence method to determine the binding characteristics of MPA. Regarding experimental conditions, the high intrinsic fluorescence of albumin related to its tryptophan and tyrosine residues requires the use of low albumin concentrations (2 μM) to avoid inner filter effect. In this context, concentrations of MYR and bilirubin far from those observed in physiological conditions were used to investigate their influence on MPA binding to albumin, but the ratio MYR/HSA or bilirubin/HSA were in the physiological range. Therefore, these experiments were expected to provide helpful information about the likelihood of competition in clinical settings.

MYR has already been used to investigate the influence of fatty acids on drug binding to albumin, and especially with the quenching fluorescence method [9]. In contrast with our observations, Bojko et al. documented changes in albumin fluorescence within MYR/HSA ratio range 2–50. The discrepancy between these investigations may derive from the experimental conditions such as the albumin supplier and the solvent used for MYR solution. The main finding of this work is that fatty acids can displace MPA from albumin binding sites. Indeed, the consistency of Ki up to 40 μM MYR suggests a competitive displacement of MPA by MYR and thereby a common binding site for both ligands. In contrast, the increase in Ki beyond 80 μM MYR may be related to a conformational change in the albumin molecule. In clinical settings, the fatty acids/albumin molar ratio can rise up to 6:1 or greater under pathological conditions [4]. In transplant recipients, there are multiple causes of hypertriglyceridemia including renal dysfunction, hypoalbuminemia secondary to nephrotic syndrome, mammalian target of rapamycin (mTor) therapy and highly active antiretroviral therapy in HIV-infected patients. In all these situations, a fatty acids/albumin molar ratio above 2 can be frequently observed. Our data show that fatty acids/albumin molar ratio of 2:1 and 5:1 cause a 1.5 and 2.2-fold increase in Kd_{app} of MPA, respectively. Therefore, hypertriglyceridemia may have a significant influence on MPA binding to albumin in clinical settings.

Bilirubin Kd is strongly dependent on assay conditions (albumin concentration, buffer composition, ionic strength). Our value of Kd determined without added salt is in accordance with those previously obtained (Kd range of 0.1–10 μM) at zero ionic strength [10]. In the current work, the increase in Kd_{app} of MPA about a factor 1.4–1.9-fold for bilirubin/albumin molar ratio of 1:4 and 1:2 supports the hypothesis that in clinical settings, severe hyperbilirubinemia can contribute to decrease MPA binding to albumin in patients experiencing cholestasis.

In conclusion, these data suggest that hypertriglyceridemia or cholestasis may significantly increase MPA free fraction in clinical settings, thereby lowering MPA total concentration in plasma while the free concentration remains unchanged. Therefore, hypertriglyceridemia or hyperbilirubinemia should be taken into account to rationalize MPA dosing regimens in transplant recipients.

Conflict of interest

None.

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