Characterization of Binding of Raltegravir to Plasma Proteins

Caroline Barau,^a Valérie Furlan,^a Yazdan Yazdanpanah,^b Catherine Fagard,^c Jean-Michel Molina,^d Anne-Marie Taburet,^a Aurélie Barrail-Tran^{a,e}

Assistance Publique, Hôpitaux de Paris, Hôpital Bicêtre, Hôpitaux Universitaires Paris-Sud, Service de Pharmacie Clinique, Paris, France^a; Assistance Publique, Hôpitaux de Paris, Hôpital Bichat-Claude Bernard, Service de Maladies Infectieuses et Tropicales, Paris, France^b; Université de Bordeaux, ISPED, Centre INSERM U897-Epidémiologie Statistique, Bordeaux, France^c; Assistance Publique, Hôpitaux de Paris, Hôpital Saint-Louis, Service de Maladies Infectieuses et Tropicales, Paris, France^d; Université Paris-Sud, Faculté de Pharmacie, Département de Pharmacie Clinique et Pharmacocinétique, Chatenay-Malabry, France^e

The objective of this study was to characterize raltegravir (RAL) binding to albumin and alpha-1-acid glycoprotein (AAG). Unbound and bound RAL were separated by ultrafiltration. The association constant (K_a) was estimated by a graphical method. In HIV-infected patients, the average plasma protein binding is 76%. RAL did not bind to AAG but bound to nonsaturable, low-affinity albumin sites with an n (number of sites) $\cdot K_a$ product of 9.8×10^2 liters/mol. A pH increase of 0.2 U led to a 2% increase in the bound fraction.

Raltegravir (RAL), the first approved HIV-1 integrase inhibitor, is a member of a promising new class of drugs for the treatment of HIV-1-infected patients (1, 2). The physicochemical properties of RAL are heavily influenced by pH, which has the potential to alter its pharmacokinetics. Indeed, the lipophilicity of RAL is pH dependent and is reduced as the pH is increased from 5 to 9. The pK_a of RAL was recently determined to be 6.7 (3). RAL is approximately 83% bound to plasma proteins (4). It is now recognized that the unbound concentration of a drug is considered the active moiety, which is available to cross cell membranes. Thus, any alteration of protein binding may have consequences for the total drug concentration. However, little is known about the determinants of RAL protein binding. Albumin and alpha-1-acid glycoprotein (AAG) are the two main plasma proteins known to bind drugs (5). Determination of the characteristics of RAL protein binding would allow a better understanding of its plasma and intracellular disposition.

This study aimed to characterize RAL protein binding to serum albumin and AAG and to examine the displacement effects of ritonavir (RTV) and darunavir (DRV), which are extensively bound to plasma proteins.

To measure RAL protein binding, human plasma (obtained from a blood bank) from healthy subjects, fatty-acid free and globulin-free albumin and AAG solutions (obtained from Sigma-Aldrich), and plasma from nine HIV-infected patients included in the ANRS139-TRIO study were used (6). The ANRS139-TRIO clinical study was designed to study the virological efficacy of combination therapy including RAL, DRV-RTV, and efavirine in HIV-infected patients with resistant viruses and few remaining treatment options (6). This study enrolled 103 patients, and 9 volunteered for an extensive pharmacokinetic substudy whose objective was to describe the pharmacokinetic parameters of RAL (7). The ANRS139-TRIO study was approved by the local ethics committee, and patients gave their written informed consent to be involved in the study. Plasma samples were collected at different time points, during a 12-h dosing interval while patients were receiving 400 mg of RAL twice a day (b.i.d.) and 600 and 100 mg of DRV and RTV, respectively, b.i.d. before the introduction of efavirine (6, 7). Human AAG and albumin solutions were prepared in pH 7.4 phosphate-buffered saline, and the concentrations used were those found in normal patients, 0.7 and 40 g/liter, respec-

tively. Protein solutions were spiked with known amounts of RAL to yield final concentrations of 0, 0.1, 0.2, 1.1, 2.3, 4.5, 11.2, 22.5, 33.8, 67.6, and 112.6 μ M (0 to 50,000 ng/ml). RAL concentrations were converted into micromolar units by using a RAL molecular mass of 444 g/mol. For the interaction study, albumin solutions were spiked with DRV at 2,000, 5,000, and 10,000 ng/ml and RTV at 500 and 1,000 ng/ml in addition to RAL at 100, 1,000, and 10,000 ng/ml. The influence of pH on RAL binding was studied in an albumin solution spiked with RAL at 200 and 2,000 ng/ml at pHs ranging from 7.0 to 7.6, including the normal pH range of arterial blood of 7.38 to 7.44 (5). These solutions were adjusted with 1 M hydrochloric acid or 1 M sodium hydroxide to create the required pH. Total concentrations of RAL were measured by liquid chromatography-tandem mass spectrometry by protein precipitation with acetonitrile. Chromatographic separations were performed by using a gradient program with 2 mM ammonium acetate containing 0.1% formic acid and acetonitrile with 0.1% formic acid (8). The limit of quantification was 2.5 ng/ml. The laboratory participates in an external quality control program. Bound and unbound RAL were separated by ultrafiltration of 400- μ l samples performed on Centrifree devices (cutoff, 30 kDa; Millipore, Molsheim, France) at $2,000 \times g$ for 30 min at 25°C (9). Approximately 50 μ l of filtrate was collected, which represents 12.5% of the total sample volume (10). All of the samples were ultrafiltered three times. RAL was then measured in the filtrate as previously described with a limit of quantification of 1 ng/ml. As drug adsorption to the filter can affect the measurement of unbound drug concentrations, we performed six ultrafiltrations of three aqueous solutions spiked with known amounts of RAL to yield 25, 125, and 600 ng/ml to ensure that binding was minimal. The coefficients of variation of intra- and interassay precision and accuracy were evaluated with three concentrations and were 9, 10,

Received 17 April 2013 Returned for modification 20 May 2013

Accepted 9 July 2013

Published ahead of print 15 July 2013

Address correspondence to Caroline Barau, caroline.barau@yahoo.fr.

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doi:10.1128/AAC.00625-13

FIG 1 Mean concentrations ($\pm$ standard deviations) of total and unbound RAL in nine HIV-infected patients in the ANRS139-TRIO study in semilogarithmic coordinates.

and 10% for total RAL and 8, 10, and 4% for unbound RAL, respectively.

The bound fraction was the ratio of bound to total RAL concentrations. The graph of the bound drug concentration (C_b) as a function of the total drug concentration (C_{tot}) depicts the protein binding system (10). The slope of the line $C_b = f \cdot C_{tot}$ (where f is a function) is the product of the association constant (K_a), the number of binding sites (n), and the protein concentration (P). We used a graphical Scatchard plot method to estimate the protein binding parameters. K_a and n were estimated by linear regression analysis with the following equation: $(C_b/P)/C_u = n \cdot K_a - (C_b/P) \times K_a$. Results are expressed as medians and ranges. All statistical tests were performed with Statgraphics 5 plus (Manugistics, Inc.), and pharmacokinetic parameters were calculated with WinNonlin Professional 5.1 (Pharsight, Mountain View, CA).

In nine HIV-infected patients (eight males; median age, 46 [range, 18 to 61] years), the total RAL minimum and maximum concentrations in plasma (C_{min} and C_{max} , respectively) and area under the concentration-time curve from 0 to 9 h (AUC_{0-9}) were 135 (range, 24 to 706), 1,176 (range, 219 to 5,086) ng/ml and 4,485 (range, 901 to 8,245) ng $\cdot$ h/ml, respectively. The unbound RAL C_{min} , C_{max} , and AUC_{0-9} were 23 (range, 4 to 320) ng/ml, 101 (range, 43 to 1,246) ng/ml, and 957 (range, 210 to 1,926) ng $\cdot$ h/ml, respectively. The median bound fraction of RAL was 76.2% (range, 73.3 to 83.9%) and was negatively correlated with albumin concentrations ($r^2 = 0.8013$; $P = 0.04$). Figure 1 depicts the mean concentrations (and standard deviations) of total and unbound RAL in the nine HIV-infected patients included in the extensive pharmacokinetic analysis of the ANRS139-TRIO clinical trial.

In vitro, RAL did not bind to AAG, but in a 40-g/liter albumin solution and in pooled plasma of healthy subjects, the median RAL protein binding was 60.9% (range, 53.0 to 68.6%) and 73.8% (range, 70.9 to 76.5%), respectively. Protein binding remained unchanged, whatever the total RAL concentration. RAL was found to bind to one class of nonsaturable, low-affinity albumin sites. The Scatchard plot of the data showed a nearly horizontal line whose equation was $y = -0.00087 + 9.8 \times 10^2$ ($r^2 = 0.02280$) for a wide range of concentrations, indicating nonsaturable binding. The parameters n and K_a could not be differentiated, and the $n \cdot K_a$ product was 9.8×10^2 liters/mol. No displacement of RAL from its albumin binding sites was found in the presence of DRV

FIG 2 *In vitro* mean bound RAL fractions ($\pm$ standard deviations) in a 40-g/liter human serum albumin solution at various pHs spiked with two concentrations of RAL. fp, bound fraction.

nor RTV, regardless of the concentration. Interestingly, in the pH range of 7.0 to 7.6, a 0.2-U decrease in pH led to a 2% decrease in RAL protein binding ($P = 0.03$) (Fig. 2).

To our knowledge, no previous study has aimed to describe the characteristics of RAL plasma protein binding. It is, however, an important parameter of the distribution and disposition of antiretroviral drugs since they have an intracellular mechanism of action (11). Our important finding is that RAL binds extensively to albumin at one class of nonsaturable, low-affinity sites with a pH effect. No binding of RAL to AAG could be evidenced. These binding characteristics differ slightly from those of other available integrase inhibitors, such as elvitegravir, which was shown to be 99.4% bound to plasma protein, with preferential binding to albumin (12). The median unbound RAL C_{min} on a b.i.d. regimen is 23 ng/ml, which is in the range of the 95% inhibitory concentration for wild-type viruses (14.6 ng/ml) (4). Such data warrant optimal adherence to avoid virological failure.

Albumin and AAG are known to be the main drug-binding proteins in plasma (5). Albumin plays an important role in the distribution, metabolism, elimination, and therapeutic effectiveness of drugs. Acidic (anionic) drugs reportedly bind predominantly to albumin in plasma, but interactions with AAG have also been reported (13). Unfortunately, estimates of the fractions bound to the individual proteins in plasma are generally not available in the literature for acidic drugs. Our *in vitro* study established that RAL did not bind to AAG but is approximately 61% bound to albumin, which is consistent with its physicochemical properties. Indeed, RAL is an acidic drug (3) and acidic drugs reportedly bind predominantly to albumin in plasma. It is now well documented that numerous factors can significantly alter plasma albumin concentrations and induce important variations in the plasma protein binding of drugs. For instance, it has been reported that as liver dysfunction progresses, the albumin concentration decreases (14). A study is currently being conducted to evaluate how plasma protein concentration variation in end-stage liver disease influences RAL protein binding (C. Barau, J. Braun, C. Vincent, S. Haïm-Boukoba, J. M. Molina, P. Mialhes, J. P. Aboulker, J. C. Duclos-Vallée, A. M. Taburet, and E. Teicher, unpublished data).

As the median bound RAL fraction is 73.8% in healthy patients and 76.2% in HIV-infected patients, RAL likely binds to another plasma protein. Indeed, other blood components, such as erythrocytes, lipoproteins, and alpha, beta, and gamma globulins, are also protein binding sites (10). Further studies are warranted to

identify other RAL binding proteins. As previously described for acidic drugs, we found that RAL binds to one class of nonsaturable, low-affinity albumin sites with an undifferentiated $n \cdot K_a$ product of 9.8×10^2 liters/mol. In HIV-infected patients, the course of the unbound concentration-versus-time curve appears to be similar to that of the total concentration-versus-time curve, indicating that protein binding remained unchanged during a dosing interval. Drug-drug interaction by displacement from protein binding sites is generally not clinically relevant but can explain the decreased total concentration while the unbound concentration remained unchanged. Indeed, changes in protein binding caused by a drug interaction are assumed to change unbound drug concentrations instantaneously, but this is transitory while the body re-equilibrates (15). Interestingly, DRV and RTV were not found to displace RAL from its albumin binding sites despite their high protein binding, probably because they bind mainly to AAG, like most weak organic bases (16). These data, with the lack of potent UDP-glucuronosyltransferase induction by coadministered drugs, are in keeping with the reported lack of clinically significant drug-drug interaction between RAL and RTV-boosted protease inhibitors (17).

The observed pH sensitivity of RAL binding could be explained by pH-induced conformational changes in albumin. Albumin is negatively charged at physiological pH (5) and undergoes conformational changes in the pH range of 6.0 to 9.0, which is termed the neutral-base transition (18). Moreover, principal binding sites I and II on albumin were postulated to be pH sensitive (19). As the pK_a of RAL is 6.7 (3), the ionized fraction of RAL will increase considerably as the pH is increased in the pH range studied. Similar results were previously shown by Wilting et al., who demonstrated that the neutral-base transition causes enhanced binding of warfarin (20). Some of these conformational changes could be intrinsic, but most of them are probably related to the binding of acids, which can induce local conformational changes at binding sites on albumin (5). This pH sensitivity of RAL protein binding may favor its intracellular and sanctuary site penetration, as demonstrated in the male genital tract. Prostatic fluid has a pH of 6.6 and a low protein content (21); therefore, unbound RAL concentrations are assumed to be higher in prostatic fluid than in plasma. This can explain, in part, the finding of good RAL diffusion in semen previously published (22). In addition, quiescent human peripheral blood lymphocytes are pH homeostatic, maintaining an intracellular pH of 7.1 over an extracellular physiological pH of 7.4 (23). Such pH differences could favor RAL penetration within the cell, assuming little effect of uptake and efflux transporters (11, 24). Whether such findings could be directly related to the potent virological efficacy of RAL at some sanctuary sites and the exact clinical significance of pH variations for RAL binding to proteins in HIV-infected patients remain to be further explored.

ACKNOWLEDGMENT

We thank Merck Sharp & Dohme-Chibret Laboratories for graciously providing RAL for this trial.

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