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BINDING OF LOPINAVIR TO HUMAN α_1-ACID GLYCOPROTEIN AND SERUM ALBUMIN

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Running Title: Lopinavir binding to human AAG and HSA

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List of non-standard abbreviations

AAG	$Alpha_1$-acid glycoprotein
HAART	Highly Active Anti-Retroviral Therapy
HSA	Human serum albumin
PBS	Phosphate buffered saline

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ABSTRACT

HIV protease inhibitors are an important component of Highly Active Antiretroviral Therapy used to treat HIV infected pregnant women. They have a low placental transfer and are highly plasma protein bound. This study was carried out to determine the unbound fraction of lopinavir in cord blood and to characterize the binding of lopinavir to α_1-acid glycoprotein (AAG) and human serum albumin (HSA), and displacement by ritonavir. Serum was obtained from cord blood from placentae obtained after cesarean section of healthy non-HIV infected women (n=4). The unbound fraction of lopinavir in serum obtained from this cord blood was 2.2±1.1%. The unbound fraction of lopinavir in separately obtained maternal serum samples (n=4) was 0.89±0.12%, which was not significantly different from that observed with cord serum samples. Varying concentrations of lopinavir, AAG, and HSA in buffer solutions were then used to characterize the lopinavir binding. The data were fit to obtain the number of binding sites (N) and equilibrium dissociation constant (K_D). Binding of lopinavir to AAG (7-23μM) was saturable with K_D of 5.0±1.1μM and N of 1.2±0.2. At low HSA concentrations (15-152μM), lopinavir binding K_D was 24.3±8.7μM and N was 1.1±0.4; however at 758μM, lopinavir binding was essentially unsaturable. Lopinavir binding to AAG and HSA was not sensitive to ritonavir and thus, efforts to enhance fetal exposure to lopinavir should be focused on other issues such as efflux transporters.

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INTRODUCTION

Highly Active Anti-Retroviral Therapy is used to treat HIV-infected patients, involving the administration of multiple antiretroviral drugs acting at different steps of the HIV life cycle. In treating HIV-infected pregnant patients, the aim of therapy is not only the treatment of the mother but also to prevent the transmission of the virus to the fetus. Among the antiretroviral drugs used, there are differences in the extent of transfer of these drugs across the placenta; HIV protease inhibitors are particularly poorly transferred.

The placenta separates maternal and fetal blood circulations, wherein, the concentrations of drug binding proteins are high in both circulations. However, they are unequal and dynamic over time. This protein concentration gradient favors partitioning of total drug on the maternal side for highly plasma protein bound drugs like HIV protease inhibitors (most > 98 %). It has been shown that concentrations of plasma proteins such as α_1-acid glycoprotein and albumin change during the gestation period. Between 12 and 41 weeks gestation, maternal serum albumin concentration ranges from 311 to 583 μM, while fetal serum albumin ranges from 114 to 603 μM (Krauer et al., 1984), and as high as high as 659 μM (Nation, 1981). Maternal serum AAG concentration, on the other hand, ranges from 7 to 24 μM (Krauer et al., 1984) and as high as 46 μM in the presence of acute or chronic inflammation (Chu et al., 1981). Fetal serum AAG concentrations range from ≤ 0.2 μM to 9 μM (Krauer et al., 1984). Similar plasma/serum protein concentration ranges have been observed in other studies as well (Laurell, 1968; Ganrot, 1972; Chu et al., 1981; Nation, 1981; Wood and Wood, 1981; Raynes, 1982; Denson et al., 1984; Krauer et al., 1984). Thus, the binding of highly plasma protein bound drugs such as HIV protease inhibitors, changes during gestation.

Additionally, because of concentration differences of proteins in the maternal and fetal circulations, it is important to determine the unbound fraction of the protease inhibitors, not only in the maternal blood but also the fetal/cord blood. Sudhakaran et al observed a higher unbound fraction of indinavir and saquinavir in cord plasma compared to maternal plasma and suggested this binding differential to be due to the transplacental AAG concentration gradient (Sudhakaran et al., 2007). Therefore, the protein concentration gradient that exists in the maternal and fetal circulations during pregnancy may unfavorably affect partitioning of the drug into the fetal compartment.

Lopinavir was chosen as the drug to study as its combination with ritonavir is currently the drug combination of choice to treat HIV infected pregnant women. Thus, the purpose of this study was to determine the unbound fraction of lopinavir in cord blood and to characterize the binding of lopinavir to human AAG and HSA. So, in this study we determined the unbound fraction of lopinavir in serum obtained from cord blood. We also studied the binding of lopinavir to AAG and HSA. The AAG and HSA concentrations we used covered the concentration ranges occurring during gestation as discussed above. Since ritonavir is used clinically with lopinavir to enhance lopinavir exposure, we also determined the effect of ritonavir on lopinavir binding to these plasma proteins in a physiologic buffer. The role of ritonavir as a displacer of lopinavir protein binding for potential utility in increasing the maternal-to-fetal placental transfer was also investigated.

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METHODS

AAG was obtained from Fisher Scientific and from Sigma Aldrich. HSA was obtained from Fisher Scientific. Lopinavir and ^{3}H-lopinavir (specific activity of 100-139 mCi/mmol) were obtained from AK Scientific, CA and Moravek Biochemicals, CA, respectively. Ritonavir was obtained from Sigma Aldrich and Bosche Scientific, NJ.

Placentae were obtained from 4 women (between ages of 18-45 years, gestational length ≥36 weeks) from cesarean section deliveries following normal pregnancies at the VCU Medical Center Hospital. The study was approved by the VCU Institutional Review Board (protocol #4212) and informed consent was obtained from patients prior to delivery. Patients with hypertension, diabetes, preeclampsia, HIV infection or febrile illness; history of smoking, alcohol or drug abuse were excluded. Maternal blood samples were not available in this study. Cord blood was obtained from placentae within 30 minutes of birth, allowed to clot on ice, and centrifuged to obtain serum. Maternal serum samples were purchased from BioChemed Services (Winchester, VA). These samples were obtained from non-smoking pregnant patients aged 19-34 years between 17 and 27 weeks gestation.

Rapid equilibrium dialysis (Pierce Inc.), with a molecular weight cut-off of 8,000 Daltons, was used to determine the protein binding of lopinavir in maternal serum and in serum obtained from cord blood. These protein binding experiments with serum samples were followed by experiments with varying concentrations of lopinavir, AAG and HSA. The protein solutions were prepared in phosphate buffered saline, pH 7.4 (PBS). The final concentrations of lopinavir were 0.1, 0.32, 1, 3, 10, 30, and 100 μM. Protein binding of lopinavir was determined in the presence of AAG (7 μM and 23 μM) and HSA (15 μM, 152 μM and 758 μM).

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The protein solution (500µl) containing the drug was added to the sample (inner) chamber and 750µl of PBS was added in the buffer (outer) chamber. Non-specific binding was determined by adding PBS in both the compartments with the solution in the sample chamber containing ^{3}H-lopinavir alone. The Teflon base plate containing the inserts was covered and incubated at 37ºC on an orbital shaker at 100 rpm. Samples (50µl for serum experiments and 100µl for the other experiments) were removed from each side at various times up to 16 hours, and ^{3}H-lopinavir in sample and buffer compartments was detected by liquid scintillation counting on a Packard TR2800 scintillation analyzer. Fraction unbound was determined by taking the ratio of mass of radioactivity observed in the buffer chamber to that in the sample chamber. All determinations were made in triplicates.

The equation for drug binding to a protein is given by:

$$\frac{B}{P_T} = \frac{N[Lopinavir]_f}{K_D + [Lopinavir]_f} \qquad \text{Equation (1)}$$

where, B is the bound drug concentration, P_T is the total protein concentration, N is the number of binding sites per protein molecule, K_D is the equilibrium dissociation constant and $[Lopinavir]_f$ is the free drug concentration. Binding parameters N and K_D were determined by non-linear least-squares regression using equation 1 (GraphPad Prism version 5).

These studies were followed by selecting a physiologic maternal concentration of AAG (9 µM) or HSA (530 µM), and carrying out the determination of lopinavir protein binding at lopinavir concentrations ranging from 0.1 to 30 µM in the presence of different concentrations (0, 1, 10, 25, and 50 µM) of ritonavir. Lopinavir protein binding to HSA (530 µM) was also determined in the presence of a higher ritonavir concentration of 100 µM. Data from HSA and AAG experiments were analyzed by ANOVA with Dunnett's post-test; significance was

assessed at $p<0.05$. Binding of lopinavir in cord and maternal serum was compared using an unpaired t-test with Welch's correction (GraphPad Prism version 5).

RESULTS AND DISCUSSION

Protein binding equilibrium was achieved within 16 hours, and non-specific binding was negligible. Fraction unbound for lopinavir in serum obtained from cord serum from term placentae of healthy patients was 2.2 ±1.1 % (mean ± SD, n=4). Our results are similar to unpublished data of Else et al. (1.73 ±1.02%) who reported the protein binding of lopinavir in plasma from cord blood of HIV positive pregnant women at term and on lopinavir/ ritonavir therapy (Else et al., 2007). Fraction unbound for lopinavir in maternal serum samples was 0.89±0.12% (mean ± SD, n=4), which was not significantly different from that observed with cord serum obtained from term placentae ($p=0.056$). Notably, the variances were significantly different ($p<0.01$), which may imply more variability in unbound fraction of lopinavir in fetal blood.

Lopinavir binding to 7 and 23 µM AAG at varying drug concentrations (0.1-30µM) is shown in figure 1A. The binding of lopinavir to AAG was dependent on protein and drug concentrations. Fraction bound varied from 0.59±0.03 to 0.27±0.01 at 7 µM AAG and from 0.87±0.01 to 0.62±0.03 at 23 µM AAG over a lopinavir concentration range of 0.1-30µM. It was not possible to determine the binding of lopinavir at 100 µM due to the low solubility of lopinavir. The data obtained for binding of lopinavir at both AAG concentrations were simultaneously fit to Equation (1) with weighting ($1/y^2$). Figure 1B shows the saturation binding curve of lopinavir at 7 and 23 µM AAG concentrations in triplicate determinations. K_D was 5.0±1.1µM and N was 1.2±0.2. Binding of lopinavir to AAG thus appeared saturable.

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Similar triplicate experiments were carried out at several HSA concentrations (15, 152, or 758 µM). Lopinavir binding to 15, 152 and 758 µM HSA at varying drug concentrations (0.1-100µM) is shown in figure 2A. In contrast to binding of lopinavir to AAG, binding to HSA was less dependent on lopinavir concentration; however, binding was dependent upon HSA concentration. With HSA, fraction bound ranged from 0.41±0.06 to 0.29±0.06 over a lopinavir concentration range of 0.1-30µM at 15 µM HSA and from 0.90±0.01 to 0.81±0.02 over a lopinavir concentration range of 0.1-100µM at 152 µM HSA. It was not possible to determine the binding of lopinavir at 100 µM in the presence of a low HSA concentration (15 µM) due to the low solubility of lopinavir. At 758 µM HSA, fraction bound of lopinavir (0.96±0.02) was independent of lopinavir concentration (0.1-100µM). The data obtained for binding of lopinavir to varying HSA concentrations were also simultaneously fit to Equation (1) with weighting ($1/y^2$). Figure 2B shows the saturation binding curve of lopinavir at 15, 152, and 758 µM HSA concentrations. K_D was estimated to be 24.3±8.7µM and N was 1.1±0.4. However, at a physiologic concentration of 758 µM HSA, lopinavir binding was essentially non-saturable.

The K_D values obtained in the case of experiments with AAG and HSA can be compared to the therapeutic concentrations of lopinavir to determine if the binding is saturable or non-saturable. Lopinavir C_{max} following administration of 400mg lopinavir with 100mg ritonavir to healthy male volunteers is 13.5µM (http://www.fda.gov/cder/foi/nda/2000/21-226_Kaletra_biopharmr_P1.pdf). In our study, K_D for lopinavir binding to AAG of 5.0±1.1 µM was approximately one-third of the lopinavir C_{max}, suggesting saturable binding of lopinavir to AAG at therapeutic concentrations. In contrast, the K_D for lopinavir binding to HSA of 24.3±8.7 µM is approximately twice the reported lopinavir C_{max}, consistent with non-saturable binding of lopinavir to HSA.

Our data indicate that binding to HSA and AAG can account for the total protein binding of lopinavir by multiplying the expected unbound fractions of lopinavir at those protein concentrations. For the AAG and HSA concentration ranges previously discussed, the predicted lopinavir protein binding would vary between 96-99% in the maternal serum and 86-98% in the fetal serum during the course of pregnancy, as protein concentrations in maternal and fetal serum change between 12 and 41 weeks gestation. The range of these predictions includes our observed unbound fractions in both fetal and maternal serum. The trend in the present results toward a higher unbound fraction for lopinavir in fetal serum is consistent with the higher unbound fraction in cord blood reported for indinavir and saquinavir (Sudhakaran et al., 2007).

This study shows that protein binding of lopinavir is characterized by saturable binding to AAG and non-saturable binding to albumin at physiologic protein concentrations. Although HAS binds lopinavir with a somewhat lower affinity and both proteins appear to bind lopinavir at a single site, the physiologic concentration of HSA is much greater resulting in a much higher binding capacity. As a result, HSA would contribute more to the high degree of total plasma protein binding of lopinavir.

The concentration of serum proteins, the number of binding sites, and the apparent K_D determine the extent to which drugs such as lopinavir are bound to proteins. It is anticipated that in the absence of active transport processes, equilibrium would occur for unbound concentrations in fetal and maternal blood for drugs with a long half-life. Thus changes in fraction unbound (due to alterations in binding kinetics) in either circulation would change the fetal to maternal serum total drug concentration ratios, while the unbound concentration ratios would presumably remain constant.

Effect of ritonavir on lopinavir binding to AAG and HSA

Figure 3A shows the lopinavir binding to 9 μM AAG at various lopinavir concentrations (0.1-30μM) in the presence of ritonavir (0, 1, 10, 25, or 50μM). Lopinavir binding to AAG was significantly displaced by ritonavir only at 50μM but not lower concentrations; it was not possible to obtain a reliable estimate of K_i. Figure 3B shows lopinavir binding to 530 μM HSA at various lopinavir concentrations (0.1-30μM) in the presence of ritonavir (0, 1, 10, or 100μM). Ritonavir (1, 10μM) had no apparent effect on unbound fraction of lopinavir in the presence of 530 μM HSA. In the presence of 100μM ritonavir (but not lower concentrations), lopinavir binding to HSA was significantly altered.

Additionally, ritonavir which is co-administered with lopinavir to HIV infected pregnant women may not act to displace lopinavir from binding to AAG or HSA at its therapeutically relevant ritonavir concentrations (C_{max} =0.83μM, C_{trough} =0.30μM) (Murphy et al., 2001). Therefore, efforts to enhance fetal exposure to lopinavir should likely focus on other mechanisms such as ATP-binding cassette transporters present on the apical (maternal-facing) syncytiotrophoblast. Additionally, mechanistic studies of transplacental transfer of highly protein bound drugs should consider unbound fetal to maternal concentration ratios, especially for highly protein bound drugs.

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LEGENDS TO FIGURES:

Figure 1. **Lopinavir binding to AAG:** (A) Lopinavir binding at varying drug concentrations (0.1-30μM) to 7μM AAG (filled circles) and at 0.1-100μM to 23μM AAG (open squares). (B) Bound lopinavir concentrations normalized for AAG concentration as a function of unbound lopinavir concentrations. AAG: 7μM (filled circles); 23μM (open squares).

Figure 2. **Lopinavir binding to HSA:** (A) Lopinavir binding at varying drug concentrations (0.1-30μM) to 15 μM HSA (filled circles), and at 0.1-100μM lopinavir to 152 μM (open squares) and 758 μM HSA (filled triangles). (B) Bound lopinavir concentrations normalized for HSA concentration as a function of unbound lopinavir concentrations. HSA: 15 μM (filled circles); 152μM (open squares); 758μM (filled triangles).

Figure 3. **Effect of ritonavir on lopinavir binding to AAG and HSA:** (A) Lopinavir binding to 9 μM AAG at various concentrations of lopinavir (0.1-30μM) in the presence of ritonavir (0, 1, 10, 25, or 50μM; ●, ○, ■, □, ▲, respectively). (B) Lopinavir binding to 530 μM HSA at various concentrations of lopinavir (0.1-30μM) in the presence of ritonavir (0, 1, 10, or 100μM; ●, ○, ■, □, respectively).

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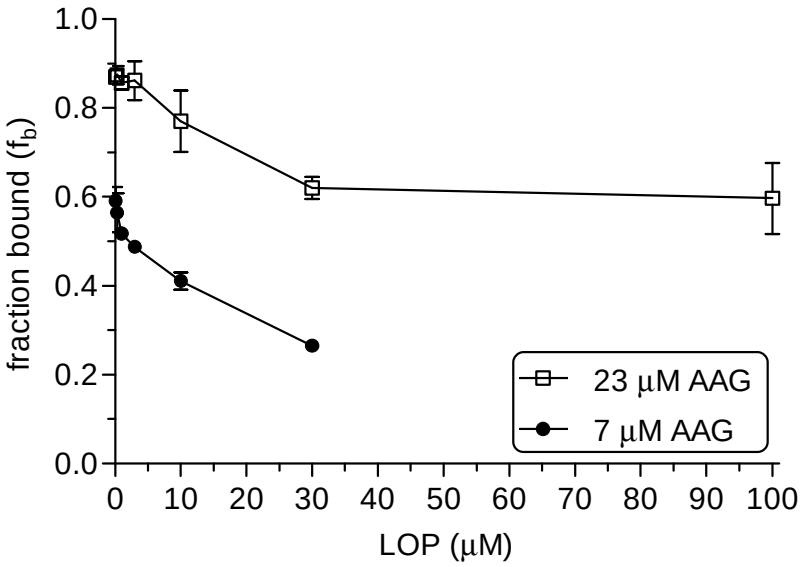
Figure 1:
(A)



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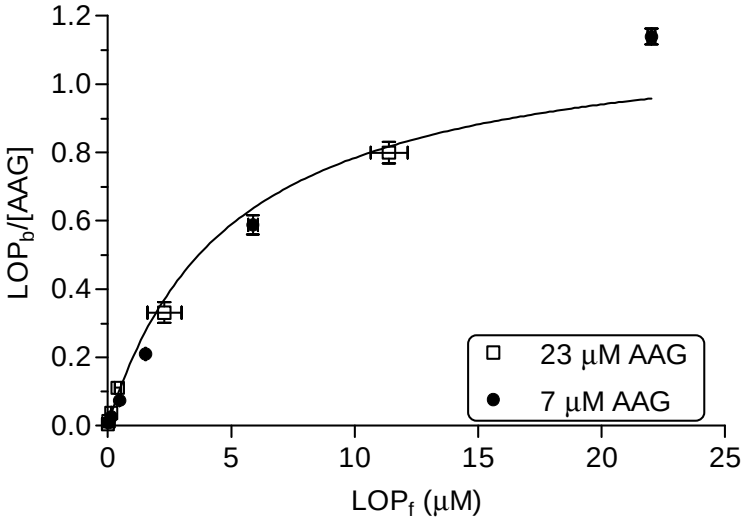

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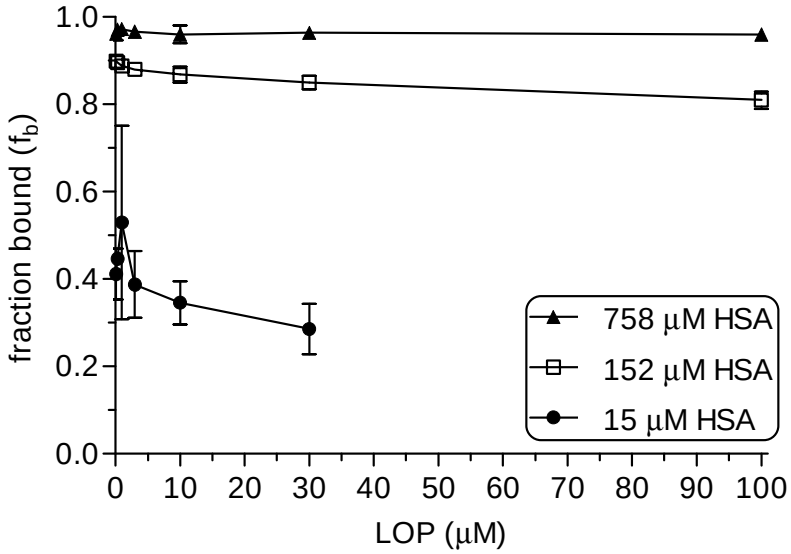
Figure 2:
(A)



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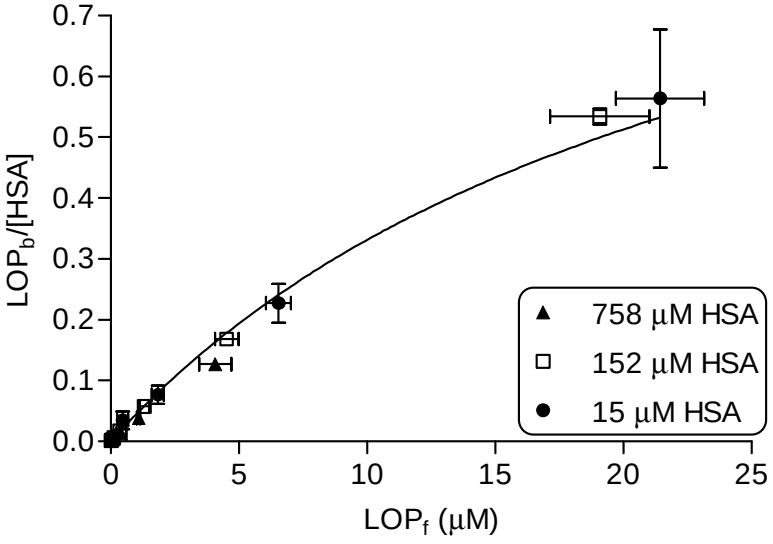

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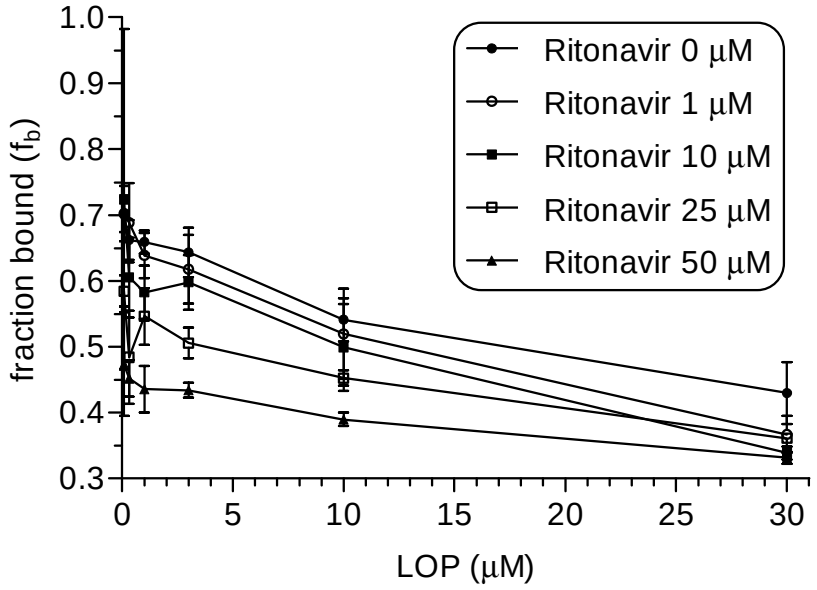
Figure 3:
(A)



(B)