Evidence for Time-Dependent Interactions between Ritonavir and Lopinavir/Ritonavir Plasma Levels Following P-Glycoprotein Inhibition in Sprague-Dawley Rats

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Received May 25, 2010; accepted October 4, 2010; published online October 29, 2010

The interaction between verapamil, a P-glycoprotein (P-gp) inhibitor, with ritonavir and lopinavir/ritonavir (LPV/r) after acute and chronic treatment was investigated in rats. Rats were divided into 4 groups, *viz.* Group 1: ritonavir, 20 mg/kg/d ($n=18$), group 2: ritonavir, 20 mg/kg/d plus verapamil 5 mg/kg/d ($n=18$), group 3: LPV/r, 80 and 20 mg/kg/d ($n=17$) and group 4: LPV/r, 80 and 20 mg/kg/d plus verapamil 5 mg/kg/d ($n=18$). Blood samples were collected after decapitation on days 1, 7 and 21. Lopinavir and ritonavir plasma levels were simultaneous determined by a validated LC/MS/MS method. The lower limit of quantification for both ritonavir and lopinavir was 0.078 μg/ml. Verapamil significantly increased ritonavir plasma levels, administered as monotherapy, following acute ($p<0.005$) and chronic treatment (day 21) ($p<0.005$). During acute (but not chronic) LPV/r treatment, verapamil also increased the lopinavir levels ($p<0.05$). A time or exposure dependent pharmacokinetic interaction was thus observed between verapamil and ritonavir whether administered alone or after the lopinavir-ritonavir combination (LPV/r). This interaction occurred most prominently after acute treatment, and became less pronounced over time. This study indicates the importance of a longer time frame to investigate enzyme based drug interactions in rat models.

Key words animal model; P-glycoprotein inhibition; lopinavir; ritonavir; pharmacokinetic interaction

Protease inhibitors (PI's), such as ritonavir and lopinavir, form an integral part of combination antiretroviral therapy. The metabolism of the PI's is complex and is influenced by both the cytochrome P450 (CYP P450) enzymes in the liver as well as the efflux transporter, P-glycoprotein (P-gp).[1)] However, there is conflicting reports in the literature on the influence of these two systems on the plasma levels of lopinavir and ritonavir after acute and chronic treatment.[1,2)]

The PI's are substrates for CYP P450 oxidative metabolism, predominantly CYP3A4.[3)] However, most of the PI's inhibit CYP3A4 specifically,[3)] with ritonavir being one of the most potent. Earlier studies have found that ritonavir markedly increases the plasma concentration and prolongs the elimination of many concomitant drugs.[4)] Indeed, the combination of ritonavir and lopinavir (LPV/r) has been designed specifically on the ability of ritonavir to enhance the bioavailability of lopinavir.[4)] Studies in rats have noted that the bioavailability of lopinavir can be lower by as much as 25% when administered alone as compared to its levels following the administration of LPV/r. Moreover, the combination is associated with a significant increase in maximum plasma concentration (C_{max}) and area under the curve (*AUC*) for both ritonavir and lopinavir.[5)] However, contradictory reports have found that plasma concentrations of ritonavir, or other co-administered drugs, are 'reduced' after chronic ritonavir treatment.[2,6,7)]

The multidrug efflux transporter, P-gp, is responsible for extracting substrate drugs from the site of absorption, and as such limits their intracellular drug accumulation and can thus contribute to treatment failure.[8)] P-gp is an ATP-dependant membrane transporter with broad substrate specificity, capable of mediating efflux of a variety of structurally diverse drugs.[9)] There is a strong suggestion that different binding sites exist on the P-gp transporter,[10,11)] while there is also evidence of competitive and non-competitive inhibition of these sites.[11)] P-gp is expressed in a multitude of organs such as the liver, intestinal epithelial cells, kidney and blood–brain barrier in both humans and rodents.[12—14)] The expression of this glycoprotein and its action to limit drug absorption and penetration into a desired target organ has important therapeutic implications, including the potential for sub-therapeutic drug levels, viral resistance and contributing to treatment failure.[15)] Controversy relating to the effect of PI's on the P-gp transport system is widely recognised, including whether these drugs are responsible for P-gp induction,[16)] whether ritonavir and lopinavir are P-gp inhibitors [17)] or whether or not PI's affect P-gp expression.[18)]

The calcium channel blocker, verapamil, is often used as an inhibitor of P-gp to investigate interactions between antiretroviral (ARV) drugs.[19)] Despite the aforementioned controversy, the effect of P-gp on ARV therapy is nevertheless significant. A number of studies in the literature have investigated the possibility that variation in P-gp expression may have an impact on treatment outcomes of highly active antiretroviral therapy (HAART).[20,21)] To further illustrate the important contribution of P-gp to treatment response, an earlier study in rats by Usansky *et al.*[22)] found that the absorption of saquinavir was increased 20-fold when the expression of P-gp was inhibited. Furthermore, the authors also found that the inter-individual variability of saquinavir decreased following the inhibition of P-gp.

The use of animal models to investigate ARV drug metabolism is well documented in the literature, while *in vitro* (cell culture) models for P-gp interactions have also been developed.[23)] Importantly, the induction of CYP3A in Sprague-Dawley rats following ritonavir treatment and the measurement of ritonavir activity in the blood–brain barrier and intestinal mucosa has been described.[24)] Although studies in

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rats to investigate drug interactions between ritonavir and lopinavir have primarily focussed on treatments of shorter duration,[2] studies over a longer period of time are limited and require clarification, especially because of the obligatory role of long-term drug treatment in patients receiving these drugs. To our knowledge, the present study is the first to investigate the time-dependent effects of P-gp inhibition over 1, 7 and 21 d on the plasma levels of the ritonavir and lopinavir (LPV/r) combination, compared to that of ritonavir given alone. Since lopinavir is not available as a stand-alone preparation in the clinic, always being administered in combination with ritonavir, we have not included a study on lopinavir with and without P-gp inhibition.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats ($n=72$), weighing 250—350 g (6—10 weeks old) sourced from the Animal Research Centre of North-West University, were grouped six rats per cage and housed in identical cages. The rats were raised on a commercial pellet diet with access to water provided *ad libitum*. All animals were handled according to the code of ethics in research, training and testing of drugs as laid down by the Animal Ethics Committee of the North-West University (Ethics approval number: NWU-00029-07-S9).

Drugs and Dosages Ritonavir (Norvir® 80 mg/ml); LPV/r (Kaletra® 4 : 1 80/20 mg/ml) and verapamil (Vasomil® 80 mg) were purchased from a local retail pharmacy after obtaining a valid prescription for research purposes. Ritonavir and LPV/r were administered as a solution. Verapamil was mixed with distilled water and homogenized to attain a fluid texture. The drugs were administered orally once daily by oral gavage in order to maximise the first-pass effect.[25] Blood samples were drawn 1 h after dosing to ensure experimental consistency and uniformity. The drugs were administered at the following dosages: Ritonavir, (20 mg/kg/d)[26] LPV/r (lopinavir 80 mg/kg/d and rotinavir 20 mg/kg/d)[26] and verapamil, (5 mg/kg/d).[27] The doses were calculated to attain the therapeutic effect needed and based upon data from the literature.[26—28]

Study Design The study design consisted of 4 different treatment groups (72 rats in total), destined to receive either ritonavir ($n=18$), ritonavir plus verapamil ($n=18$), LPV/r ($n=17$), and LPV/r plus verapamil ($n=18$). The rats were allocated into groups according to treatment duration (1, 7, 21 d) and the day of sacrifice. Blood samples were collected after 1 d of treatment (single dose, $n=6$ per treatment group), after 7 d of treatment (day 7, $n=6/5$) and after 21 d of treatment (day 21, $n=6$). The blood samples were collected 1 h after administration to ensure experimental consistency and scientific certainty. The duration of treatment was selected to investigate both the acute and chronic effects of the P-gp inhibition by verapamil on the pharmacokinetics of ritonavir and lopinavir.

Method of Sacrifice Rats were sacrificed by decapitation. Decapitation is less stressful to the animal and excludes the metabolic effects of drugs used during euthanasia and is therefore the ideal method of sacrifice.[26]

Sample Collection and Storage Blood collection occurred directly after decapitation. The blood was collected in ethylenediamine tetraacetic acid (EDTA) vacutainers with the blood volume varying between individual rats (3.5 ml±500 μl). After collection, the vacutainer was capped and slightly tilted for a few seconds, before being centrifuged at 5000 rpm for 10 min. The plasma layer was subsequently extracted in duplicate into labelled micro centrifuge tubes and frozen at −80 °C.

Analytical Methods. Chemicals Solvents used as elements were high purity water, methanol and acetonitrile (HPLC grade Burdick and Jackson Laboratory Co. (Anateck, Sloane Park, SA), Muskegon, Michegan, U.S.A.), acetic acid (SAARCHEM, Muldersdrift, SA), ammonium acetate (Merck, Midrand, SA Dormstadt, GMBH). Reserpine (Fluka (Sigma-Aldrich, Aston Monor, SA), St. Louis, Missouri, U.S.A.) was used as internal standard (IS). Reagents utilized for plasma protein precipitation included methanol and zinc sulphate. Ritonavir and lopinavir reference standards were donated by Abbott S.P.A, Italy.

Liquid Chromatographic (LC) Tandem Mass Spectrometer (MS) Instrumentation An Agilent 1200 series liquid chromatographic system, interfaced to a 6410 triple quadruple mass spectrometer and coupled with ESI (electrospray ionization), was used for plasma sample determinations. The analytes ritonavir, lopinavir and reserpine (IS) were separated on a cation-exchange column (Column Zorbax SB-C3 100 mm×3 mm×3.5 μm, Agilent Technologies, Waldbronn, Germany). The method optimization and development was based on a previously published method by Koal *et al.*[29]

The novelty and benefits of this modified and validated LC/MS/MS method included the MS operating in positive multiple reaction modes only, allowing high efficiency, selective and sensitive detection. Moreover, small quantities of plasma (100 μl) are suitable for quantification, requiring only a single injection to quantify ritonavir and lopinavir within 8 min. Isocratic separation was performed at a flow rate of 0.5 ml/min. The mean intra- and inter-day variation for two quality control samples injected repeatedly was found to be 9.1%. The limit of quantification for both the compounds was 0.078 mg/ml.

Statistical Analysis Data are expressed as mean±S.E.M. in the figures, with $p<0.05$ regarded as statistically significant. Assessment for statistical significance was processed by means of two-way analysis of variance (ANOVA) with the treatment type and the treatment day as factors. Non-parametric binary sample comparisons within each of the treatment days were analysed using the Mann–Whitney U test and the Kruskal–Wallis test for multiple sample comparisons within each treatment type over time. Analyses were performed using Graph Pad Prism® (version 5.01 for Microsoft Windows, Graph Pad Software, San Diego, CA, U.S.A.).

RESULTS

The results are represented in Figs. 1 and 2. In Fig. 1 the influence of verapamil on ritonavir, administered as monotherapy (Fig. 1A), ritonavir in the LPV/r combination (Fig. 1B) and lopinavir in the LPV/r combination (Fig. 1C) at days 1, 7 and 21 can be seen. Figure 2 reflects the change in concentration over the time period for ritonavir, administered as monotherapy (Fig. 2A), ritonavir in the LPV/r combination (Fig. 2B) and lopinavir in the LPV/r combination (Fig. 2C).

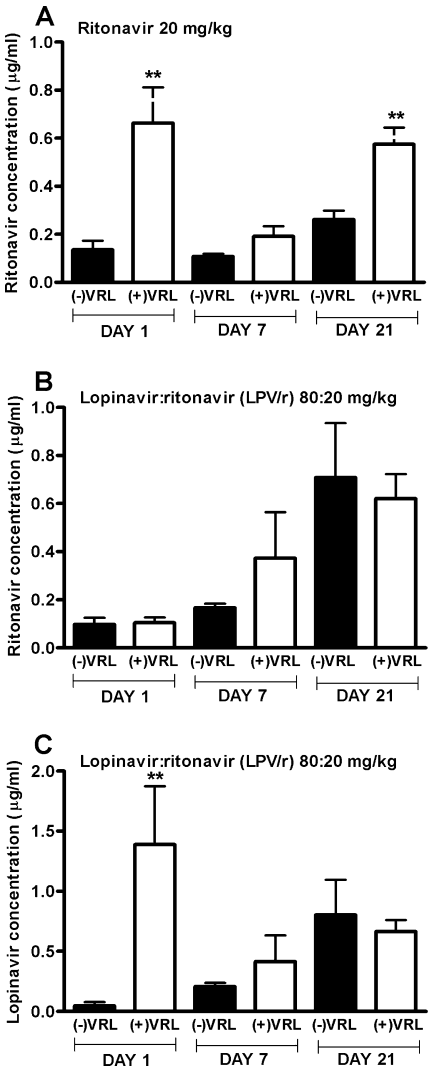

Fig. 1. The Effect of Verapamil on the Plasma Concentrations of (a) Ritonavir, (b) Ritonavir (Lopinavir : Ritonavir) and (c) Lopinavir (Lopinavir : Ritonavir) on Days 1, 7 and 21 of Treatment

Data are expressed as mean±S.E.M. $^{**}p<0.005$; Mann–Whitney U test.

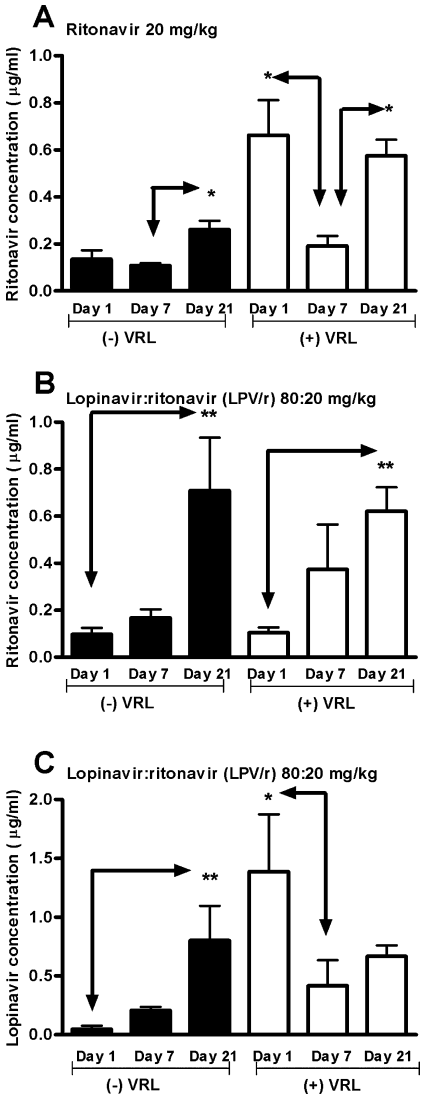

Fig. 2. The Change of Plasma Concentrations 'Over Time' (with and without Verapamil) for (a) Ritonavir, (b) Ritonavir (Lopinavir : Ritonavir) and (c) Lopinavir (Lopinavir : Ritonavir) on Days 1, 7 and 21 of Treatment

Data are expressed as mean±S.E.M. $^{**}p<0.005$; Krustal–Wallis test and $^{*}p<0.05$; Krustal–Wallis test.

Ritonavir Levels during Ritonavir Treatment Alone Ritonavir plasma concentrations on days 1, 7 and 21, when administered as monotherapy, were found to be $0.1358\pm0.0909\,\mu$g/ml ($n=6$), $0.1077\pm0.0260\,\mu$g/ml ($n=6$) and $0.2615\pm0.0915\,\mu$g/ml ($n=6$), respectively. When verapamil was added to the regimen, plasma ritonavir concentrations were $0.6632\pm0.3619\,\mu$g/ml ($n=6$), $0.1910\pm0.1045\,\mu$g/ml ($n=6$) and $0.5746\pm0.1695\,\mu$g/ml ($n=6$) on days 1, 7 and 21, respectively. The difference in plasma concentrations between the two regimens on days 1, 7 and 21 can be seen in Fig. 1A, while change in plasma concentrations over the 21 d period with and without verapamil, and their statistical analysis, are represented in Fig. 2A. A two-way analysis of variance (ANOVA) was performed for plasma ritonavir concentrations with time and treatment as factors. Since a significant time by treatment interaction was found ($F(2,30)=4.72$, $p<0.0001$), the two treatments were compared at each time. Plasma ritonavir concentrations were found to be significantly higher on day 1 ($p<0.005$) and day 21 ($p<0.005$) of treatment in the ritonavir plus verapamil combination compared to that of the ritonavir monotherapy group, but with no difference observed on day 7 (Fig. 1A). In the ritonavir plus verapamil combination, studied over the 21 d, a significant decrease in ritonavir levels was observed between day 1 and 7 ($p<0.005$, Fig. 2A) and a significant increase between day 7 and 21 ($p<0.05$, Fig. 2A), with no change noted between days 1 and 21 (Fig. 2A).

Ritonavir Levels during LPV/r Treatment Plasma ritonavir concentrations on days 1, 7 and 21 of treatment were $0.0980\pm0.0635\,\mu$g/ml ($n=6$), $0.1669\pm0.0369\,\mu$g/ml ($n=5$), and $0.7087\pm0.5537\,\mu$g/ml ($n=6$), respectively, when administered as the ritonavir (LPV/r) combination. When verapamil was added to the regimen, the ritonavir (LPV/r) plasma concentrations were $0.1048\pm0.0517\,\mu$g/ml ($n=6$), $0.3728\pm0.4698\,\mu$g/ml ($n=6$), and $0.6215\pm0.2477\,\mu$g/ml ($n=6$) on days 1, 7 and 21 respectively. The difference in plasma concentrations between the two regimens on days 1, 7 and 21 can be seen in Fig. 1B, while change in plasma concentrations over the 21 d period with and without verapamil, and their statistical analysis, are represented in Fig. 2B. A

two-way analysis of variance (ANOVA) was performed for plasma ritonavir (LPV/r) concentrations with time and treatment as factors. Since a significant time by treatment interaction was found ($F(2, 29)=5.34$, $p<0.05$), the two treatments were compared at each time. There was no statistically significant differences between the ritonavir (LPV/r) and the ritonavir (LPV/r) plus verapamil combinations on days 1, 7 and 21 of treatment (Fig. 1B). There was, however, a significant increase in ritonavir levels over time either in the absence ($p<0.005$) or presence ($p<0.05$) of verapamil between day 1 and 21 of treatment (Fig. 2B).

Lopinavir Levels during LPV/r Treatment Lopinavir plasma concentrations were $0.0468\pm0.0743\,\mu$g/ml ($n=6$), $0.2075\pm0.0658\,\mu$g/ml ($n=6$), and $0.8018\pm0.7217\,\mu$g/ml ($n=6$) on days 1, 7 and 21 of treatment when administered as the LPV/r combination. When verapamil was added to the regimen, lopinavir plasma concentrations were $1.3878\pm1.1899\,\mu$g/ml ($n=6$), $0.4150\pm0.5341\,\mu$g/ml ($n=6$) and $0.6657\pm0.2347\,\mu$g/ml ($n=6$), on days 1, 7 and 21 of treatment, respectively. The difference in plasma concentrations between the two regimens on days 1, 7 and 21 can be seen in Fig. 1C, while change in plasma concentrations over the 21 d period with and without verapamil, and their statistical analysis, are represented in Fig. 2C. A two-way analysis of variance (ANOVA) was performed for plasma lopinavir (LPV/r)/r) concentrations with time and treatment as factors. Since a significant time by treatment interaction was found ($F(2, 29)=4.52$, $p<0.05$), the two treatments were compared at each time. A significant difference in lopinavir levels in the lopinavir (LPV/r) versus the lopinavir (LPV/r) plus verapamil combination was only found on day 1 of treatment, where levels were significantly elevated in the presence of verapamil ($p<0.005$; Fig. 1C). There was a statistically significant increase in lopinavir (LPV/r) plasma levels between day 1 and 21 (Fig. 2C; $p<0.005$) in the absence of verapamil, and a statistically significant decrease in lopinavir levels in the lopinavir (LPV/r) plus verapamil plasma levels on day 7 *versus* day 1 ($p<0.5$; Fig. 2C).

DISCUSSION

The lopinavir/ritonavir (LPV/r) combination forms a crucial part of HAART. Drug interactions can lead to sub-therapeutic or toxic levels that can negatively affect the therapeutic outcome of ARV therapy.

In this study we have investigated the influence of the P-gp inhibitor, verapamil, on the metabolism of ritonavir, administered as monotherapy (Fig. 1A), and on the lopinavir/ritonavir (LPV/r) combination (Figs. 1B, C). After acute treatment with verapamil (day 1), a statistically significant increase in the plasma concentrations of ritonavir (monotherapy) ($p<0.005$) and lopinavir (LPV/r) ($p<0.005$) was observed, with a non-significant change in ritonavir (LPV/r) plasma concentrations. Since earlier studies have confirmed that doses of verapamil used in this study inhibit P-gp in rats,[22] our results concur with previously published data that the inhibitory effect of verapamil does indeed have a rapid onset.[29] Data from the literature, regarding the acute effect of P-gp inhibitors, is contradictory and little data on the sustained effect of P-gp inhibition after chronic treatment could be found.[16—18]

From our results it can be suggested that the P-gp inhibition effect, after acute treatment, only occur when ritonavir is administered as monotherapy and not as the LPV/r combination. Competition for the binding sites on the P-gp protein between these two structurally similar PI's (lopinavir and ritonavir) can be argued as a possible explanation. Indeed, there is evidence that different binding sites exist on the P-gp transporter protein and that drugs compete with each other for these sites.[10,11] According to the literature, all three drugs used in this study show affinity for the binding sites on the P-gp protein, with verapamil having the highest affinity.[2,30,31] However in the LPV/r combination the concentration of lopinavir is higher and lopinavir out-competes ritonavir for binding to the P-gp protein, as seen in Fig. 1C. Lopinavir is also a substrate for P-gp efflux, demonstrating inhibition after acute treatment with a P-gp inhibitor, and induction after chronic treatment.[32]

On day 7 a totally different scenario emerged, as seen in Figs. 2A—C. Here the inhibitory effect of verapamil in general diminished while the inhibitory effect of ritonavir became more pronounced. There was a statistically significant decrease in both ritonavir (administered as monotherapy) ($p<0.005$) and lopinavir (LPV/r) ($p<0.05$) levels on day 7 in the presence of verapamil, (Figs. 2A, C), but not in the ritonavir (LPV/r) combination (Fig. 2B).

In summary, there was a steady increase in the plasma concentrations over the 21 d for ritonavir (Fig. 2A), ritonavir (LPV/r, Fig. 2B) and lopinavir (LPV/r, Fig. 2C) administered without verapamil. In the presence of verapamil a steady increase was only observed for ritonavir (LPV/r, Fig. 2B) over the 21 d. In the ritonavir, administered as monotherapy and lopinavir (LPV/r) treatment groups a reduction of plasma levels were observed on day 7 and an increase on day 21 when verapamil was added to the regimen.

These results illustrate the complex interaction between CYP3A and P-gp, although it is difficult to differentiate whether the observed kinetic changes are mediated by P-gp or CYP enzyme involvement after chronic treatment. Similar observations have been reported by Kageyama *et al.*[2] with ritonavir administered as monotherapy, who demonstrated that ritonavir concentrations decrease over a period of time following chronic administration. However, the study by Kageyama *et al.*,[2] was over a period of 14 d while our study represents treatment for up to 21 d. The authors concluded that ritonavir inhibits the CYP3A enzymes in the early stages of chronic treatment, resulting in an increase in plasma levels.[2] After 3 to 5 d, ritonavir induces CYP3A and intestinal P-gp activities such that a reduction in ritonavir levels was observed after 14 d. The same observation was reported by van Heeswijk *et al.*[1] who argued that the function of P-gp in the gut cells or bile duct is enhanced by repeated administration of ritonavir or following up regulation of P-gp with prolonged exposure to lopinavir or ritonavir. The latter study was, however, also conducted over 14 d. Our data would therefore indicate that lopinavir and ritonavir respond differently to P-gp inhibition when administered in combination due to competition. This may have important clinical relevance.

Ritonavir, and to a lesser extend lopinavir, are eminent CYP3A inhibitors,[5] further contributing to elevated PI's plasma levels. Over time, this presents as chronic auto-inhi-

bition[1)] eventually leading to higher plasma levels. Due to the short half-lives of the PI's in rodents,[33)] if auto-inhibition was not present and if steady-state concentrations had been reached prior to day 7, we would have expected plasma levels to remain more or less constant.[33)]

Recent studies found that P-gp inhibition could possibly have a superior influence over CYP3A4 modification in altering pharmacokinetics.[34)] Over time, an equilibrium is reached and an increase in levels are observed. This is supported by the results of our study. Indeed, P-gp seems to play a time- and/or exposure-dependent role in the response to ritonavir, where verapamil evokes a drastic elevation in ritonavir levels on day 1, but which is lost on day 7, but again rises to prominence by day 21 (Fig. 2A). This would indicate that duration of exposure may explain the varied response of PI's to P-gp inhibition.

In summary, the inhibitory effect of verapamil on P-gp, and its resulting effects on ritonavir (monotherapy) and lopinavir (LPV/r) plasma levels, was only observed after acute treatment. This effect was lost after sub-chronic (7 d) treatment, with a reduction in plasma concentrations occurring by day 7 for ritonavir and lopinavir (LPV/r) and again a steady increase in all the levels by day 21. The effect of verapamil was only present on day 21 when ritonavir was administered as monotherapy suggesting that lopinavir also induces P-gp after chronic treatment.

The net effect of the interaction is an increase in plasma concentrations after steady state. This study demonstrates the importance of a longer time frame to investigate enzyme based drug interactions in rat models.

Acknowledgments The authors would like to thank Bernhard Wüst from Chemetrix (Pty) Ltd. for his specialist knowledge on mass spectrometry, and Professor Faans Steyn from the Department of Statistical Consultation for his expertise and assistance. The authors would like to acknowledge the National Research Foundation (Thuthuka) and the North-West University for financial support. There is no financial relationship, or conflict of interest, with these funding agencies.

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