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Elucidating the Effect of Final Day Dosing of Rifampin in Induction Studies on Hepatic Drug Disposition and Metabolism

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Running title: Differentiating Rifampin's Induction and Inhibition Effects

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Abbreviations:

OATP/Oatp, organic anion transporting polypeptide; P-gp, P-glycoprotein; CYP, cytochrome P450; DEX, dexamethasone; RIF, rifampin; Dg3, digoxin; Dg2 digoxigenin bis-digitoxoside; Dg1, digoxigenin mono-digitoxoside; Dg0 aglycone digoxigenin; LC-MS, liquid chromatography-mass spectrometry; PK, pharmacokinetics; AUC, area under the concentration-time curve; ACN, acetonitrile; MTBE, *tert*-butyl-methyl-ether; FBS, fetal bovine serum; CL, clearance; Vss, volume of distribution.

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Abstract

Because rifampin (RIF) induces hepatic enzymes and inhibits uptake transporters, dosing a drug that is a dual substrate of enzymes and uptake transporters on the final day of an inducing regimen should exhibit less inductive effect than dosing on the following day in the absence of RIF since RIF decreases drug uptake into liver. *In vitro* and *in vivo* rat studies were conducted using digoxin as a model substrate. Digoxin was administered to an uninduced control group to obtain baseline values. The second group (induced with dexamethasone) received digoxin alone mimicking administration of a test drug one day following completion of an induction regimen, whereas the third group (induced) received digoxin with RIF mimicking the concomitant dosing on the final day of an induction regimen. Results from hepatocyte concentration-time course studies showed that compared to uninduced control (26.9±1.3 μM•min/mg), digoxin AUC in induced cells when no RIF is present decreased significantly (13.7±0.9 μM•min/mg, $p<0.01$) suggesting induction of cyp3a. However, digoxin AUC for induced cells in the presence of RIF (27.3±0.9 μM•min/mg) matched the control. Rat pharmacokinetic studies showed that compared to digoxin clearance in uninduced controls (7.08±1.57 ml/min/kg), digoxin clearance in induced rats increased two-fold (15.6±3.7 ml/min/kg, $p<0.001$), but when RIF was co-administered in the induced rats, digoxin clearance (7.14±1.24 ml/min/kg) overlapped with control. That is, concomitant dosing of RIF and digoxin masked the inductive effect. To observe full inductive effects, test drugs should be administered one day after final dosing of RIF to minimize potential OATP inhibition effects.

Introduction

Rifampin (RIF) is commonly used to treat tuberculosis and other mycobacterial infections. In clinical studies, it is frequently used as an inducer of several clinically significant cytochrome P450 isoforms as well as membrane transporters (Schuetz, 2001; Kullak-Ublick and Becker, 2003). The mechanism of activation is primarily through the binding of nuclear receptor pregnane X receptor (PXR) amongst other nuclear receptors (Kliewer et al., 1998; Lehmann et al., 1998).

Recently, RIF has been shown to be an excellent substrate and inhibitor of hepatic uptake transporters that are localized on the sinusoidal membrane of hepatocytes (Tirona et al., 2003). These transporters are members of the solute carrier superfamily of transport proteins. The isoforms that are most affected by RIF are organic anion transporting polypeptides (OATP in human/Oatp in rats). In the over-expressed oocyte system, RIF has been shown to inhibit the uptake of estradiol-17β-glucuronide by Oatp1a4 (formerly Oatp2) (K_i = 1.4 μM) (Fattinger et al., 2000) and the uptake of BSP by OATP1B1 (formerly OATP-C) (K_i = 7 μM) and OATP1B3 (formerly OATP8) (K_i = 5 μM) (Vavricka et al., 2002). In a transiently transfected cell-line, RIF potently inhibited: Oatp1a4 mediated uptake of digoxin (Dg3) (K_i = 1.46±0.58 μM) (Shitara et al., 2002), OATP1B1 mediated uptake of estradiol-17β-glucuronide (EC_{50} = 0.94 μM) (Tirona et al., 2003) and atorvastatin (K_i = 2.88±1.33 μM) (Lau et al., 2006b), as well as OATP-8 mediated uptake of BSP (K_i = 1.5 μM) and amanitin (IC_{50} = 0.8 μM) (Letschert et al., 2006). In freshly isolated rat hepatocyte studies, RIF exhibited a strong inhibition effect on Oatp mediated uptake of Dg3 and erythromycin (Lam and Benet, 2004; Lam et al., 2006). In the isolated perfused rat liver system, without affecting enzymatic activities,

RIF reduced hepatic clearance of Dg3 and atorvastatin by 1.3-fold and 2-fold, respectively (Lau et al., 2004; Lau et al., 2006b). In vivo, total clearance of erythromycin decreased 1.6-fold relative to the control when RIF was co-administered i.v. in rats (Lam et al., 2006). Concomitant i.v. dosing of RIF with oral dosing of atorvastatin in rats and in humans reduced CL/F of atorvastatin by 3.5-fold and 6-fold, respectively (Lau et al., 2006a; Lau et al., 2006c).

Results from a clinical study conducted by Stone et al. (2004) suggested that RIF both induced and inhibited the disposition of caspofungin, which has been shown to be a substrate of OATP1B1 (Sandhu et al., 2005). In another clinical study, when RIF and repaglinide were co-administered, RIF acted as an inducer and inhibitor of repaglinide metabolism (Bidstrup et al., 2004). When repaglinide was administered on the same day as the final (7th) inducing dose of RIF, repaglinide AUC decreased by 50% compared to the non-induced control; whereas, a sharper AUC decrease of 80% was observed when repaglinide was dosed one day after the last day of RIF dosing. Niemi et al. (2005) showed that OATP1B1 is important in the disposition of repaglinide by examining effects of transporter polymorphism on pharmacokinetics. Compared to measures for the 521TT reference genotype, the 521CC subjects exhibited a 152% increase in AUC and a 188% increase in C_{max}. Since it has been well demonstrated that RIF is an excellent inhibitor of OATP1B1, it is reasonable to speculate that the conflicting results from the Bidstrup study could be due in part to inhibition of OATP1B1 by RIF when administered on the same day with repaglinide. All of these findings lead to our hypothesis that because RIF induces hepatic enzymes and inhibits uptake transporters, dosing of a drug that is a dual substrate of enzymes and uptake transporters on the final day of an inducing regimen

should exhibit less inductive effect than on the following day in the absence of RIF since the presence of RIF decreases drug uptake into the liver.

To test our hypothesis, in vitro and in vivo studies were conducted in rats using Dg3 as a model compound. While in human, Dg3 is primarily excreted unchanged; in rats, it is an excellent substrate of Oatp1a4, cyp3a and p-gp (Tanigawara et al., 1992; Salphati and Benet, 1999; Shitara et al., 2002). Biotransformation of Dg3 involves a stepwise hydrolysis of digitoxosides by cyp3a to form digoxigenin bis- and mono-digitoxoside and the aglycone digoxigenin before conjugation and elimination (Harrison and Gibaldi, 1976; Salphati and Benet, 1999). Since the RIF inducing effect is species specific to human, dexamethasone (DEX) was used instead to induce cyp3a, p-gp and Oatp1a4 through PXR (Salphati and Benet, 1998; Guo et al., 2002). In this study, we examined the effects of RIF on Dg3 disposition and subsequent metabolism in vitro using freshly isolated rat hepatocytes and in vivo comparing pharmacokinetic profiles.

Materials and Methods

Materials. Dg3, digoxigenin (Dg0), RIF, corticosterone, DEX, corn oil, HPLC grade methanol and *tert*-butyl-methyl-ether (MTBE) were purchased from Sigma-Aldrich (St Louis, MO). [^{3}H] Dg3 (37 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). Digoxigenin bis-digitoxoside (Dg2) and digoxigenin mono-digitoxoside (Dg1) were kind gifts from Professor Emil Lin, UCSF. Lanoxin (GlaxoSmithKline, Kirkland, Canada) and RIF (Bedford laboratories, Bedford, OH) for i.v. infusion were purchased from the UCSF pharmacy for research use only. Male Wistar rats (200-350 g) from Charles-River Laboratories (Wilmington, MA) were housed in the UCSF animal care facility with a 12 hour light/dark cycle and allowed free access to water and food. The studies described here were approved by the Committee on Animal Research, UCSF.

Study Design. For in vitro studies, control rats received 4 daily intraperitoneal (i.p.) injections of vehicle (corn oil) and induced rats received 4 daily i.p. injections of 80 mg/kg DEX (Salphati and Benet, 1998). On day 5, hepatocytes were isolated from the control rats and the induced rats. In vitro uptake studies and time course studies were carried out.

For in vivo studies, 18 rats were equally divided into 3 groups, the uninduced control group, the induced Dg3 alone group and the induced Dg3 with RIF group. For the uninduced control group, rats received i.p. corn oil injections for 4 days followed by pharmacokinetic (PK) studies of 1 mg/kg Dg3 on day 5 to obtain baseline PK values. For the two induced groups, rats received 80mg/kg of DEX for 4 days. On day 5, the induced Dg3 alone group received 1 mg/kg Dg3, and PK studies were carried out to mimic the clinical situation where a test drug was administered one day after final dosing of RIF.

The induced Dg3 with RIF group received 1 mg/kg Dg3 and 45 mg/kg RIF together on day 5 to simulate the situation in which a test drug was administered concomitantly with RIF on the last day of an induction regimen.

Hepatocyte Isolation. Cells from 6 rats per set were pooled for counting and subsequent studies. The cell isolation procedure is as follows: anesthesia was induced by intraperitoneal injection with a 1 ml/kg dose of ketamine/xylazine (80 mg/ml, 12 mg/ml) prior to surgery. The portal vein was cannulated with an IV catheter (catalog number 2007-04, Becton Dickinson, Sandy, UT) and perfused with oxygenated liver perfusion buffer (Invitrogen, Carlsbad, CA) for 5 minutes at 30 ml/min followed by perfusion with an oxygenated hepatocyte washing buffer (Invitrogen, Carlsbad, CA) modified with 2 mM L-glutamine, 10 mM HEPES, and 1.2 U/ml collagenase (Sigma-Aldrich, St. Louis, MO) for 5 minutes at 20 ml/min. The digested livers were excised and broken down. Hepatocytes were then washed twice with an ice cold hepatocyte wash buffer containing 2 mM L-glutamine and 10 mM HEPES and centrifuged at 50 x g for 2-3 minutes. Cell viability was determined using the trypan blue exclusion method. Cells with viability of greater than 90% were used for further studies.

Hepatocyte Uptake Studies. Prior to each incubation, 2 million hepatocytes were pre-warmed in Krebs-Henseleit buffer (pH7.4) containing 0.21 g/L of sodium bicarbonate and supplemented with 1% BSA and 10 mM glucose for 5 minutes. For the Dg3 uptake studies, [^{3}H] Dg3 (500nM) with and without 100 μM RIF were added to the cells. After 2 minutes, each reactions was terminated by transferring 1 million hepatocytes into a centrifuge tube containing 150 μl of 2 N NaOH under a layer of 500 μl of a mixture of silicone oil and mineral oil (Shitara et al., 2003) and centrifuged at 13,000

× g for 10 seconds. The cell pellets were left at 65°C overnight to ensure complete lysis. After removing the oil layer, 150 µl of 2 N HCl and 5ml of scintillation cocktail were added to each sample and radioactivity was measured using a scintillation counter (LS6000TA, Beckman Coulter, Fullerton, CA).

To measure Dg3 metabolite uptake, Dg2 (500nM), Dg1 (500nM) and Dg0 (500nM) with and without 100 µM RIF were added to the cells. After 2 minutes, each reaction was stopped by transferring 1 million hepatocytes into a centrifuge tube containing 700 µl of a mixture of silicone oil and mineral oil and centrifuged at 13,000 x g for 10 seconds. After removing the oil layer, the cell pellet was resuspended in 100 µl of water to lyse the cells followed by addition of 200 µl of ACN to precipitate the protein. After a quick vortex, samples were spun down at 13,000 × g for 10 minutes. The supernatants were transferred into HPLC vials ready for LC-MS analysis. Protein concentrations of uninduced and induced cells were measured using a BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's directions. All further hepatocyte calculations were normalized to protein concentration.

Hepatocyte Time Course Studies. Incubations were carried out in 50 ml round-bottom flasks undergoing continuous rotation while gassed with 95% O_2/5% CO_2 at 37°C (Li et al., 2002). At the end of the study, an aliquot of hepatocytes in Krebs-Henseleit buffer (pH7.4) containing 2.73 g/L of sodium bicarbonate and supplemented with 1% BSA and 10 mM glucose was removed from each flask to determine drug effects on cell viability using the trypan blue exclusion method. At 5, 10, 15, 20, 30, 45 and 60 minute time points, two aliquots of two million hepatocytes were transferred to a glass cell culture tube containing 2 ml of MTBE and internal standard corticosterone, followed by a

quick vortex to stop the reaction. All samples were spun down at 2000×g for 10 minutes. After quick-freezing the aqueous layer in a methanol/dry ice bath, the organic layer was poured into a new tube and evaporated under nitrogen gas. One group of aliquots was used to measure Dg3 and metabolites by LC-MS analysis as described below. To each glass tube of the second group of aliquots, 150 µl of 4mM phosphate buffer (pH 6.8) containing 50 units of β-glucuronidase (Sigma-Aldrich, St. Louis, MO) was added to cleave possible glucuronide adducts. After a brief vortex, these samples were left in the 37ºC incubator over night. ACN (150 µl) was added to each sample to stop the reaction followed by centrifugation at 13,000 × g for 10 minutes to precipitate the protein. The supernatants were transferred into HPLC vials ready for LC-MS analysis. All further hepatocyte calculations were normalized to protein concentrations.

Surgery and Pharmacokinetic Studies in Rats. Male Wistar rats (250g-300g) were under anesthesia induced by intraperitoneal injection with a 1 ml/kg dose of ketamine:xylazine (80 mg/ml:12 mg/ml, Sigma-Aldrich, St. Louis, MO) prior to surgery and several times during the 4 hour study to ensure complete anesthetization. The femoral vein and the femoral artery were cannulated using a 10 cm PE-10 tube (I.D. 0.28 mm, O.D. 0.61 mm, BD Intramedic, Sparks, MD) and a 10 cm SP-35 tube (I.D. 0.5 mm, O.D. 0.9 mm, Natume Co., Tokyo, Japan), respectively. The lines were washed immediately with saline containing 10 Unit/ml heparin to prevent clotting. Dg3 (1mg/kg) with either vehicle control (saline) or together with rifampin (45 mg/kg) was co-administered through the femoral vein. Blood (250 µl) samples were collected at time points 0, 3, 5, 15, 30, 60, 90, 120, 150, 180, and 240 minutes via femoral artery and transferred to heparin-pretreated microtainer tubes (Becton-Dickinson, Franklin Lakes,

NJ). Plasma was obtained by centrifugation at 10,000×g for 5 minutes. Rat livers were excised at 240 min and weighed. To each 100 µl of plasma and liver homogenate sample, 200 µl of ACN containing IS was added, and samples were centrifuged for 15 minute at 10,000×g. The supernatants were transferred into HPLC vials and assayed using LC-MS.

Measurement of Drug Concentrations. Samples were analyzed on a liquid chromatography-mass selective detector system (Hewlett Packard) consisting of the 1100 HPLC components HPLCI and HPLCII as described previously (Christians et al., 2000). The two HPLC systems were connected via a 7240 Rheodyne six-port switching valve mounted on a step motor (Rheodyne, Cotati, CA). The system was controlled and data were processed using ChemStation Software revision A.06.01 (Hewlett Packard). Samples (100 µl) were injected onto a 10 x 6-mm extraction column (Keystone Scientific, Bellefonte, PA) filled with Hypersil ODS-1 of 5-µm particle size (Shandon, Chadwick, UK). Samples were washed with a mobile phase of 0.1% formic acid supplemented with 1 mM sodium acetate. The flow was 1.5 ml/min, and the temperature for the extraction column was set to 65°C. After 0.9 minutes, the switching valve was activated, and the analytes were eluted with 100% ACN in the backflush mode from the extraction column onto a 100 x 4.6-mm C_8, 3.5-µm analytical column (Zorbax XDB, C_8; Hewlett Packard). The mobile phase consisted of methanol and 0.1% formic acid supplemented with 1 mM sodium acetate:ACN (80:20, v:v). The following gradient was run: time 0 min, 50% methanol; 6 min, 90% methanol. The flow rate was 0.5 ml/min. The analytical column was also maintained at 65°C. Two minutes after sample injection, the mass-selective

detector was activated. The detection limits were 50 ng/ml, 5 ng/ml, 10 ng/ml, 10 ng/ml and 100 ng/ml for Dg3 (Dg3), Dg2, Dg1, Dg0 and rifampin, respectively.

Transient Transfection and Uptake Transport Assays. The Oatp1a4 cDNA in pCI-neo mammalian expression plasmid (Promega, Madison, WI) was sequenced and its expression was verified using the Western blotting method. HEK293 cells (University of California Cell Culture Facility, San Francisco, CA) were cultured in Dulbecco's modified Eagle's medium H21 (4.5 g/liter glucose) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 100 µg/mL streptomycin and 100 U/mL penicillin. Cells were seeded into poly-D-lysine-coated 12-well plates (BD Biosciences, Bedford, MA) at a density of 0.5×10^6 cells/well 1 day prior to transfection. Transfection of the Oatp1a4 plasmid and the pCI-neo vector control using ExGen500 transfection reagents (Fermentas, Hanover, MD) were according to the manufacturer's direction. Cell culture media was replaced 24 hours prior to uptake studies. The cells were washed with 37°C transport medium (Hank's balanced salt solution containing 1% FBS and 25 mM HEPES, pH 7.4) for 5 minutes immediately before the uptake experiment. Uptake studies were initiated by adding 0.7 ml of 37°C transport medium containing substrates. The cells were incubated for 2 minutes at 37°C. Drug uptake was stopped by removing the incubation medium followed by washing three times with ice-cold phosphate buffered saline (pH 7.4). Air-dried cells were scraped off the plates and resuspended in 200 µL of double-distilled water. After adding ACN to precipitate the protein, the samples were spun down at 13,000 × g for 10 minutes at 4˚C. The supernatants were transferred into HPLC vials ready for LC-MS analysis. Protein concentrations of cells were measured using a BCA protein assay kit (Pierce, Rockford,

IL) according to the manufacturer's directions. All further calculations were normalized to protein concentrations.

Data Analysis. For the pharmacokinetic studies, terminal half-life ($T_{1/2}$) was determined from log linear regression. Area under the time-concentration curve (AUC) was calculated by the linear trapezoid rule and extrapolated to infinite time from the last measured concentration (C_{last}) by adding [$C_{last} \times T_{1/2}/0.693$]. Clearance (CL) was calculated as dose divided by extrapolated AUC. Volume of distribution at steady-state (Vss) was calculated using moments (Benet and Galeazzi, 1979). Analysis of variance was used to analyze differences among the three groups. The significance between two means in these groups was evaluated using Tukey's multiple comparison test. The *p*-value for statistical significance was set at < 0.05.

Results

Uptake of [^{3}H]-Dg3 by Freshly Isolated Rat Hepatocytes. Effects of RIF on Dg3 uptake were examined in the freshly isolated rat hepatocytes as depicted in Figure 1. Compared to that of the control in the uninduced cells (2.15±0.17 nM/mg protein), there was a more than 5-fold increase in intracellular concentration of Dg3 in the induced cells (12.6±0.8 nM/mg protein, $p<0.001$), over 2 minutes. This finding is consistent with up-regulation of Oatp1a4 through PXR observed using RT-PCR (Guo et al., 2002). However, when 500 nM of [^{3}H]-Dg3 was co-incubated with 100 μM RIF in the induced hepatocytes, the uptake was reduced more than 3-fold (3.55±0.34 nM/mg protein, $p<0.001$) versus that without RIF (12.6±0.8 nM/mg protein). Results from the uptake study indicate the importance of Oatp1a4 in the disposition of Dg3.

Hepatocyte Time Course Studies. Three sets of time course studies were carried out. In the first set, Dg3 with vehicle was incubated in hepatocytes that were not induced as control. In the second set, Dg3 with vehicle was incubated using induced cells to mimic clinical situations in which a test drug was administered one day after final dosing of RIF in induction studies. In the third set, Dg3 was co-incubated with RIF in the induced cells to mimic conditions in which a test drug was administered concomitantly with RIF on the final day of an induction study. Total drug concentrations (cell and media) were measured over 60 minutes (Fig. 2). Only Dg3 and Dg2 were found in the cell suspension, whereas Dg1 and Dg0 levels were below the detection limits.

Table 1 summarizes the areas under the concentration-time curve over 60 minutes and related calculations for the 3 sets of studies for samples not undergoing glucuronidase cleavage. Compared to the Dg3 AUC of the uninduced control, AUC of

Dg3 decreased 2-fold when incubated alone in the induced cells. However, when RIF was co-incubated with Dg3 in the induced cells, AUC increased 2-fold versus the Dg3 alone to a level that overlapped with the control in the uninduced cells as shown in Figure 2A. Conversely, AUC of Dg2 increased considerably (13-fold) for the induced group compared to the uninduced control, which indicates induction of cyp3a. Dg2 AUC decreased (3-fold) significantly with RIF compared to when no RIF was added to induced cells, as shown in Figure 2B. Ratios of Dg2 AUC to Dg3 AUC and mass balance were also calculated.

For the samples measured following glucuronidase cleavage, none of the values in Table 1 changed significantly except for the mass balance percentage in the induced Dg3 control set that increased from 43.3±0.6% to 52.4±1.2% ($p<0.05$).

In Vivo Pharmacokinetic Study of Dg3. To determine the effects of final day dosing of RIF on the pharmacokinetics of a representative drug, we tested our hypothesis in vivo in rats. Effects of RIF on Dg3 metabolism were investigated in the induced rats. All three groups of rats received an i.v. bolus dose of Dg3 (1mg/kg) with one induced group concomitantly receiving 45 mg/kg of RIF. Plasma concentrations of drugs were measured (Fig. 3), and pharmacokinetic parameters were estimated (Table 2). Compared to that of the uninduced control rats, $AUC_{(0\text{-inf})}$ of Dg3 decreased and CL increased 2- fold when Dg3 alone was given to induced rats. Vss also increased significantly. However, when RIF was co-administered with Dg3 in the induced rats, $AUC_{(0\text{-inf})}$ increased and CL decreased compared to that without RIF to levels close to that of uninduced controls. Vss also decreased significantly. Plasma levels of RIF were also measured (Fig. 3A). Plasma levels of Dg2 were only slightly and non-significantly lower in the induced rats than in

the uninduced control rats. However, when RIF was co-administered with Dg3 in the induced rats, Dg2 levels increased substantially (Fig. 3B, Table 2). As opposed to the hepatocyte studies, Dg0 concentrations were measurable in the plasma in all three groups (Fig. 3C, Table 2). Compared to the control Dg0 level in the uninduced rats, Dg0 concentrations in both induced groups were higher; however, levels of Dg0 between Dg3 with and without RIF in the induced rats were not significantly different. AUCs of Dg2 and Dg0 were summed and ratios of metabolite AUC to parent drug AUC were calculated (Table 2). The AUC ratio increased more than 5-fold in the induced Dg3 alone group compared to the uninduced control rats. In the induced rats, when RIF was present, AUC ratio decreased significantly compared to induced without RIF. The trend of AUC ratio changes correlated well with that of hepatocyte studies.

Rat livers were excised and weighed immediately following the final blood draw at 240 minutes. Liver homogenates were made and concentrations of drugs were measured. Amounts of Dg3 and its metabolites were expressed as percents of the original Dg3 dose (Fig. 4). The amount of Dg3 did not differ significantly among the the 3 groups. However, Dg2 levels were significantly higher in the induced Dg3 alone group and the induced with RIF group compared to the uninduced control group. Furthermore, the amount of Dg2 in the induced with RIF group was substantially higher than the without RIF group. RIF concentration was 254±69 μg/ml/g liver (data not shown). The amount of Dg0 in the induced with RIF group is significantly higher than that in the uninduced control group and the induced group.

Uptake of Dg3 Metabolites in Freshly Isolated Hepatocytes and in Oatp1a4 Transfected HEK293 Cells. To test whether Dg3 metabolites are substrates of hepatic

uptake transporters, 2 min uptake studies were carried out in the presence and the absence of RIF using freshly isolated hepatocytes. Results showed that in the presence of RIF, Dg0 uptake was 55.1±1.4 % of its control, Dg1 uptake was 32.3±5.1 % of its control, and Dg2 uptake was 31.7±0.8 % of its control (Fig 5A). To further investigate which uptake transporters are responsible for the uptake of the metabolites, Oatp1a4, Oatp1b2, and vector control plasmids were transiently transfected into HEK293 cells. Compared to each respective empty vector control, Dg0 uptake was 188±23 %, Dg1 uptake was 380±18 %, and Dg2 uptake was 465±39 % in Oatp1a4 transfected cells (Fig 5B). However, there were no significant changes in uptake found in Oatp1b2 transfected cells compared to vector controls for all three metabolites (data not shown). These data collectively indicate that Dg3 metabolites are substrates of hepatic uptake transporters, specifically Oatp1a4 in rats.

Discussion

The present study examined cellular and animal models that may predict the inhibitory effect of rifampin on metabolism of a test drug when dosed together on the final day of an induction regimen in humans. Three treatment groups were used to mimic clinical induction studies. First, the uninduced control group gives baseline values of metabolism for the test drug. Second, the induced group is representative of dosing the test drug alone one day following the final RIF dose of an induction regimen. Third, the induced with RIF group mimics concomitant dosing of the inducing agent RIF with the test drug on the last day of the induction regimen.

Dg3 was used as a model compound to test our hypothesis because in rats it is a substrate of Oatp1a4, cyp3a, and P-gp. DEX was used to induce cyp3a and the transporters instead of RIF due to species-specific differences between human and rats. In contrast to human OATPs, rat Oatp1a4 is under the regulation of PXR. As a result, 4 day treatment of DEX also induced Oatp1a4 as shown in Figure 1. There is a 5-fold increase in intracellular concentration of Dg3 in induced cells versus the uninduced baseline control. However, it is important to note that we are making no assumption that the induction by dexamethasone in rats is the same as the induction of rifampicin in humans, nor that the interplay between rifampin induction and inhibition is similar to dexamethasone induction and rifampin inhibition. The purpose of our model is to show that the presence of rifampin under conditions of induction can mask the induction effect by inhibiting hepatic uptake of an Oatp substrate.

In vitro time course studies in hepatocytes showed that without affecting the enzymatic activity, concomitant dosing of rifampin and Dg3 in induced cells diminished

the induction effect on Dg3 metabolism (Fig. 2). When Dg3 was given alone in the induced cells, a 2-fold decrease in Dg3 AUC and a 14-fold increase in Dg2 AUC were noted compared to that of the uninduced control cells. This profile is consistent with marked up-regulation of cyp3a. However, when RIF was concomitantly dosed with Dg3 in the induced cells, Dg3 AUC increased 2-fold to a level no different from that of the uninduced control set. This significant change in AUC is most likely due to inhibition of Oatp1a4, because the RIF concentration used was 100 μM, a concentration previously shown to have minimum inhibitory effect on cyp3a and P-gp (Lam and Benet, 2004). AUC of Dg2 decreased significantly in the induced cells in the presence of RIF compared to induced cells without RIF, but was still significantly greater than that seen with the uninduced control cells. As expected, fewer drug molecules entered the cells and fewer metabolites were formed when the uptake transporters were inhibited. Only very small amounts of Dg2 were formed, which resulted in greater changes in Dg2 AUCs (13-fold) compared to Dg3 AUCs (2-fold). Mass balance for the induced Dg3 alone group was only 43% (Table 1). This is likely due to sequential metabolism of Dg2; however, the levels of Dg1 and Dg0 were below the LC-MS detection limit. Glucuronidase assays were carried out to free Dg3 and its metabolites from the glucuronide adduct for LC-MS measurement, however the treatment only improved the mass balance from 43% before digestion to 52% after digestion. Thus, it appears that DEX may induce other unknown pathways that eliminate Dg3 and its metabolites, but this change is not observed when RIF is present to decrease drug transport into hepatocytes, as no significant difference in mass balance is noted between control and induced Dg3 with RIF (Table 1).

The in vivo rat studies showed that concomitant dosing of RIF and Dg3 significantly reduced the apparent induction effects compared to that without RIF in the induced group by inhibiting Oatp1a4 (Fig. 3). Total clearance (CL) for the induced Dg3 alone group was 2-fold higher than that of the uninduced Dg3 control group suggesting induction of cyp3a (Table 2). When RIF was co-administered with Dg3 in the induced rats, CL was no different than for the Dg3 control group. Since rifampin concentrations in the blood are at low levels that should not inhibit cyp3a, this decrease in CL is most likely due to inhibition of Oatp1a4. Half life for the induced Dg3 alone group was expected to be significantly lower than that of the uninduced group, however $T_{1/2}$ for all 3 groups were not significantly different from each other. This results from the finding that volume of distribution increased (2.8-fold) significantly in the Dg3 alone induced group versus that of the uninduced group, but in the presence of RIF, Vss returned to the control value. In rats, Dg3 has a low hepatic extraction ratio, thus intrinsic clearance plays an important role in Dg3 overall clearance. We previously showed that inhibition of Oatp1a4 can alter intrinsic clearance of Dg3 by preventing drug entrance into the hepatocytes (Lam and Benet, 2004). However, the increase in Vss with DEX induction suggests that upregulation of transporters throughout the animal leads to an overall increase in the space available in which Dg3 may distribute. This is reversed when RIF is present to inhibit transporters.

Dg3 metabolites observed in the plasma were Dg2 and Dg0. Compared to the plasma concentrations of Dg3, Dg2 concentrations were substantially lower, ranged from 1-12% of Dg3 in the three groups, while Dg0 concentrations ranged from 25- 145% of Dg3 concentrations (Fig. 3). The lack of measurable Dg1 reflects the fast conversion of

Dg3 to Dg2 (V_{max}/K_m = 2.92±0.42 µl/min/mg protein), Dg1 to Dg0 (V_{max}/K_m = 10.95 µl/min/mg protein), and slow formation of Dg1 from Dg2 (V_{max}/K_m = 0.11±0.03 µl/min/mg protein) by cyp3a in rats (Salphati and Benet, 1999). Dg2 AUC for the induced Dg3 alone group did not differ from that of uninduced control, but the corresponding Dg0 AUC was more than twice that of the uninduced control group indicating induction of cyp3a. In the presence of rifampin, AUC of Dg2 increased significantly versus that of the induced Dg3 alone group (Table 2). This finding does not follow the results from the hepatocyte time course study (Table 1); however, we have repeatedly observed results similar to those in Table 2 for our isolated perfused rat liver (Lau et al., 2006b) and in vivo studies (Lam et al., 2006; Lau et al., 2006c). Since Dg2 is also a substrate for Oatp1a4 (Fig. 5), it is likely that in the presence of RIF, re-uptake of Dg2 back into the liver after exiting into the blood is inhibited. AUC of Dg0 for the induced Dg3 with RIF group did not differ significantly from that of AUC for the induced Dg3 alone group (Table 2). Metabolites to Dg3 AUC ratios followed the same pattern as the ratios from the hepatocyte time course study. Compared to that of uninduced Dg3 control group, AUC ratios increased significantly in the induced Dg3 alone group suggesting increased metabolism due to induction of cyp3a; whereas without affecting cyp3a activity, inhibition of Oatp1a4 by RIF resulted in a decreased AUC ratio relative to that of the induced Dg3 alone group indicating less metabolism occurred. Assays following glucuronidase cleavage were not conducted for in vivo samples due to sample size limitations.

Amounts of Dg3 and its metabolites in the liver (at 240 minutes) were measured and expressed as percentages of the original dose. In all treatment groups, the amount of

Dg3 stayed relatively constant (Fig. 4). The amount of Dg2 in the induced Dg3 alone group was markedly higher than that of the uninduced Dg3 control group indicating induction of cyp3a; whereas little difference was observed for Dg0. Compared to that of the induced Dg3 alone group, amounts of Dg2 and Dg0 in the induced Dg3 with RIF group were significantly higher. In vitro, Oatp1a4 has been shown to be a bi-directional transporter (Li et al., 2000) depending on substrate gradient and intracellular level of glutathione. At 240 minutes, plasma concentrations of RIF were 2.13±0.55 μg/ml, and liver concentrations were 254±69 μg/ml/g liver. It is likely that the liver concentration of RIF is high enough to inhibit basolateral efflux of the metabolites into the blood. The basolateral inhibition should be more prominent for the metabolites since they are more polar than Dg3.

During early-phase drug development, the hepatic uptake transporters should be taken into consideration for their potential effects on drug disposition and metabolism of substrates. Currently, induction study protocols are designed to administer a test drug either on the last day of the 7-day inducing regiment with RIF or a day later. Here we showed that concomitant dosing of RIF and Dg3 completely diminished the induction effect. In order to observe the full induction effect, a test drug (an OATP substrate) should be administered on day 8, one day following the completion of the inducing regimen.

References

Benet LZ and Galeazzi RL (1979) Noncompartmental determination of the steady-state volume of distribution. *J Pharm Sci* **68**:1071-1074.

Bidstrup TB, Stilling N, Damkier P, Scharling B, Thomsen MS and Brosen K (2004) Rifampicin seems to act as both an inducer and an inhibitor of the metabolism of repaglinide. *Eur J Clin Pharmacol* **60**:109-114.

Fattinger K, Cattori V, Hagenbuch B, Meier PJ and Stieger B (2000) Rifamycin SV and rifampicin exhibit differential inhibition of the hepatic rat organic anion transporting polypeptides, Oatp1 and Oatp2. *Hepatology* **32**:82-86.

Guo GL, Choudhuri S and Klaassen CD (2002) Induction profile of rat organic anion transporting polypeptide 2 (oatp2) by prototypical drug-metabolizing enzyme inducers that activate gene expression through ligand-activated transcription factor pathways. *J Pharmacol Exp Ther* **300**:206-212.

Harrison LI and Gibaldi M (1976) Pharmacokinetics of Dg3 in the rat. *Drug Metab Dispos* **4:**88-93.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**:73-82.

Kullak-Ublick GA and Becker MB (2003) Regulation of drug and bile salt transporters in liver and intestine. *Drug Metab Rev* **35**:305-317.

Lam JL and Benet LZ (2004) Hepatic microsome studies are insufficient to characterize in vivo hepatic metabolic clearance and metabolic drug-drug interactions: studies

of Dg3 metabolism in primary rat hepatocytes versus microsomes. *Drug Metab Dispos* **32**:1311-1316.

Lam JL, Okochi H, Huang Y and Benet LZ (2006) In vitro and in vivo correlation of hepatic transporter effects on erythromycin metabolism: characterizing the importance of transporter-enzyme interplay. *Drug Metab Dispos* **34**: 1336-1344.

Lau YY, Wu CY, Okochi H and Benet LZ (2004) Ex situ inhibition of hepatic uptake and efflux significantly changes metabolism: hepatic enzyme-transporter interplay. *J Pharmacol Exp Ther* **308**:1040-1045.

Lau YY, Huang Y, Frassetto L and Benet LZ (2006a) Effects of OATP1B1 inhibition of the disposition and metabolism of atorvastatin in healthy volunteers. *Clin Pharmacol Ther* **Submitted**.

Lau YY, Okochi H, Huang Y and Benet LZ (2006b) Multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of in vitro and ex situ systems. *J Pharmacol Exp Ther* **316**:762-771.

Lau YY, Okochi H, Huang Y and Benet LZ (2006c) Pharmacokinetics of atorvastatin and its hydroxy metabolites in rats and the effects of concomitant rifampicin single doses: Relevance of first-pass effect from hepatic uptake transporters, intestinal and hepatic metabolism. *Drug Metab Dispos* **34**: 1175-1181

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016-1023.

Letschert K, Faulstich H, Keller D and Keppler D (2006) Molecular Characterization and Inhibition of Amanitin Uptake into Human Hepatocytes. *Toxicol Sci*.

Li L, Meier PJ and Ballatori N (2000) Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Mol Pharmacol* **58**:335-340.

Li C, Benet LZ and Grillo MP (2002) Enantioselective covalent binding of 2-phenylpropionic Acid to protein in vitro in rat hepatocytes. *Chem Res Toxicol* **15**:1480-1487.

Niemi M, Backman JT, Kajosaari LI, Leathart JB, Neuvonen M, Daly AK, Eichelbaum M, Kivisto KT and Neuvonen PJ (2005) Polymorphic organic anion transporting polypeptide 1B1 is a major determinant of repaglinide pharmacokinetics. *Clin Pharmacol Ther* **77**:468-478.

Salphati L and Benet LZ (1998) Modulation of P-glycoprotein expression by cytochrome P450 3A inducers in male and female rat livers. *Biochem Pharmacol* **55**:387-395.

Salphati L and Benet LZ (1999) Metabolism of Dg3 and digoxigenin digitoxosides in rat liver microsomes: involvement of cytochrome P4503A. *Xenobiotica* **29**:171-185.

Sandhu P, Lee W, Xu X, Leake BF, Yamazaki M, Stone JA, Lin JH, Pearson PG and Kim RB (2005) Hepatic uptake of the novel antifungal agent caspofungin. *Drug Metab Dispos* **33**:676-682.

Schuetz EG (2001) Induction of cytochromes P450. *Curr Drug Metab* **2**:139-147.

Shitara Y, Sugiyama D, Kusuhara H, Kato Y, Abe T, Meier PJ, Itoh T and Sugiyama Y (2002) Comparative inhibitory effects of different compounds on rat oatpl (slc21a1)- and Oatp2 (Slc21a5)-mediated transport. *Pharm Res* **19**:147-153.

Shitara Y, Itoh T, Sato H, Li AP and Sugiyama Y (2003) Inhibition of transporter-mediated hepatic uptake as a mechanism for drug-drug interaction between cerivastatin and cyclosporin A. *J Pharmacol Exp Ther* **304**:610-616.

Stone JA, Migoya EM, Hickey L, Winchell GA, Deutsch PJ, Ghosh K, Freeman A, Bi S, Desai R, Dilzer SC, Lasseter KC, Kraft WK, Greenberg H and Waldman SA (2004) Potential for interactions between caspofungin and nelfinavir or rifampin. *Antimicrob Agents Chemother* **48**:4306-4314.

Tanigawara Y, Okamura N, Hirai M, Yasuhara M, Ueda K, Kioka N, Komano T and Hori R (1992) Transport of Dg3 by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *J Pharmacol Exp Ther* **263**:840-845.

Tirona RG, Leake BF, Wolkoff AW and Kim RB (2003) Human organic anion transporting polypeptide-C (SLC21A6) is a major determinant of rifampin-mediated pregnane X receptor activation. *J Pharmacol Exp Ther* **304**:223-228.

Vavricka SR, Van Montfoort J, Ha HR, Meier PJ and Fattinger K (2002) Interactions of rifamycin SV and rifampicin with organic anion uptake systems of human liver. *Hepatology* **36**:164-172.

Footnotes

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Legends for Figures

Fig. 1. Two-minute uptake of [^{3}H] Dg3 into freshly isolated hepatocytes (right axis) for the three treatment groups is expressed as percentage of uninduced control (left axis). *, $p<0.05$; **, $p<0.001$, significantly different from the uninduced control group. ††, $p<0.001$, significantly different from the induced Dg3 alone group. Data are depicted as the mean ± S.D., n=6.

Fig. 2. Hepatocyte time course studies of the three treatment sets. Measurements of (A) Dg3 (Dg3) and (B) Dg2 in the cell suspensions are shown. Data are depicted as the mean ± S.D., n=6.

Fig. 3. Rat in vivo studies of the three treatment groups. Plasma concentrations of (A) Dg3 (Dg3) and RIF, (B) Dg2, and (C) Dg0 are shown. Data are depicted as the mean ± S.D., n=6.

Fig. 4. Amounts of Dg3 (Dg3), Dg2 and Dg0 retained in the liver at 240 minutes expressed as percentages of the original Dg3 dose. *, $p<0.05$; **, $p<0.001$, significantly different from the uninduced control group. †, $p<0.05$, significantly different from the induced Dg3 alone group. Data are depicted as the mean ± S.D., n=6.

Fig. 5. Uptake of Dg3 metabolites in (A) freshly isolated hepatocytes with and without the addition of RIF and (B) in Oatp1a4 transfected HEK293 cells and vector controls. *, $p<0.005$; **, $p<0.0001$, significantly different from the respective control. Data are depicted as the mean ± S.D., n=3.

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Table 1. Area under the curve (AUC) of Dg3 (Dg3) and Dg2 in rat hepatocyte incubations were determined for the three treatment sets. Metabolite to Dg3 ratio and mass balance were calculated. Each value represents mean ± S.D., n = 6.

	Uninduced Dg3 control	Induced Dg3 alone	Induced Dg3 with RIF
$AUC_{(0-60)}$ of Dg3 (μM • min/mg)	26.9 ± 1.3	13.7 ± 0.9 *	27.3 ± 0.9 †
$AUC_{(0-60)}$ of Dg2 (μM • min/mg)	0.463 ± 0.009	6.06 ± 0.21 *	2.18 ± 0.87 * †
$AUC_{(0-60)}$ ratio (Dg2/Dg3)	0.0172 ± 0.0072	0.442 ± 0.294*	0.0799 ± 0.0950 †
Mass Balance (%)	89.3 ± 5.7	43.3 ± 0.6 *	93.3 ± 9.7†

*, $p<0.01$, significantly different from the uninduced Dg3 control set

†, $p<0.01$, significantly different from the induced Dg3 alone group set.

Table 2. Pharmacokinetic parameters were calculated for the three treatment groups.

Each value represents mean ± S.D., n = 6.

	Uninduced Dg3 control	Induced Dg3 alone	Induced Dg3 with RIF
$T_{1/2}$ (hr)	3.93 ± 1.07	4.97 ± 0.83	4.68 ± 1.18
$AUC_{0\text{-inf}}$ (µg/ml•min)	146 ± 25	66.9 ± 14.9 **	144 ± 30 ††
CL (ml/min/kg)	7.08 ± 1.57	15.6 ± 3.7 **	7.14 ± 1.24 ††
Vss (l/kg)	1.89 ± 0.77	7.64 ± 1.47 **	2.23 ± 0.45 ††
Dg2 $AUC_{(0\text{-}240)}$ (µg/ml•min)	1.14 ± 0.15	1.01 ± 0.23	9.59 ± 1.17 ††**
Dg0 $AUC_{(0\text{-}240)}$ (µg/ml•min)	23.7 ± 8.7	48.1 ± 21.7	57.2 ± 27.0 *
$AUC_{(0\text{-}240)}$ ratio (met[a]/Dg3)	0.266 ± 0.101	1.449 ± 0.621 *	0.803 ± 0.335 †*

*, $p<0.05$; **, $p<0.001$, significantly different from the uninduced Dg3 control group.

†, $p<0.05$; ††, $p<0.001$, significantly different from the induced Dg3 alone group

[a] met: sum of Dg0 $AUC_{(0\text{-}240)}$ and Dg2 $AUC_{(0\text{-}240)}$.

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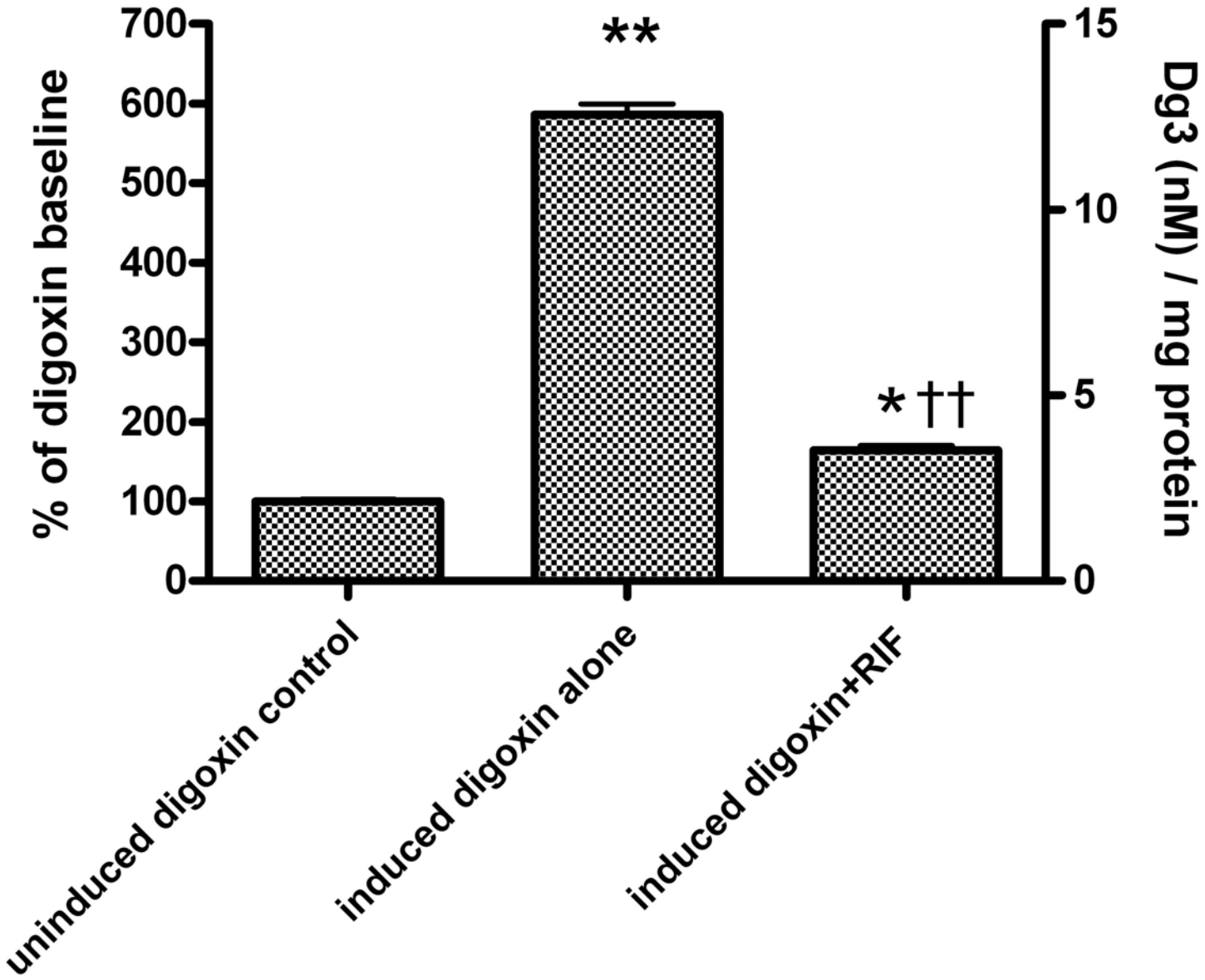
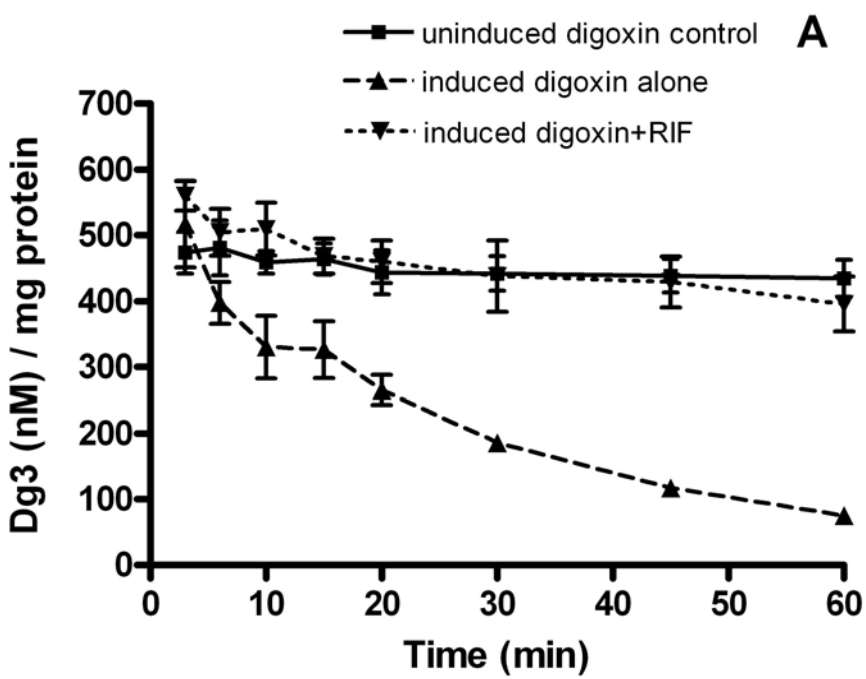


Figure 1



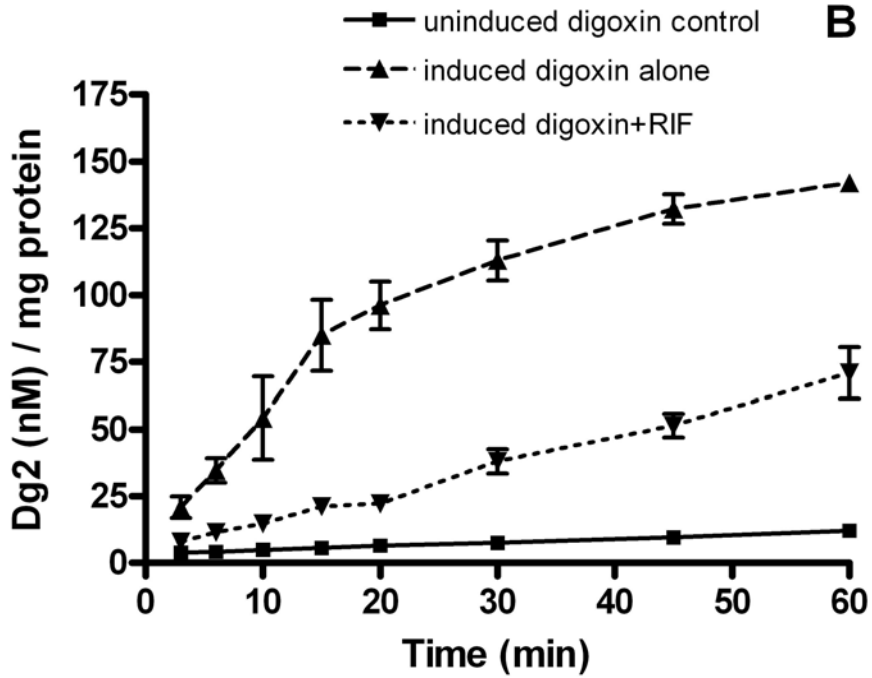


Figure 2

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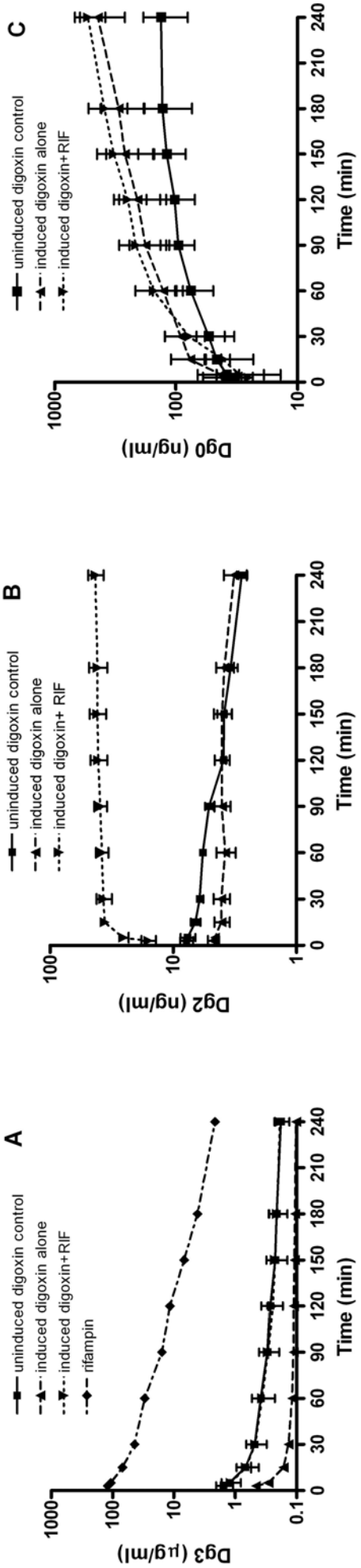

Figure 3

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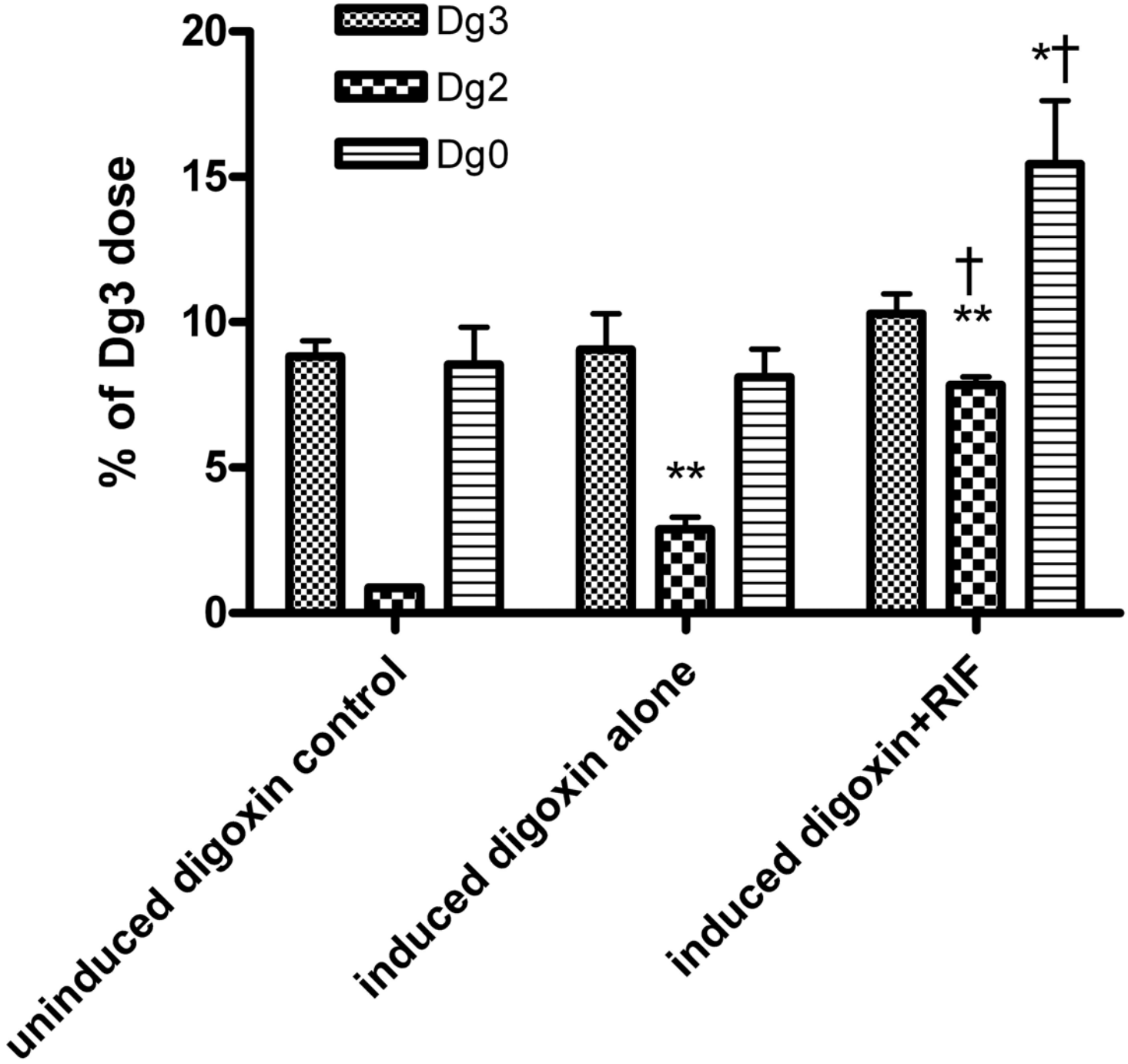
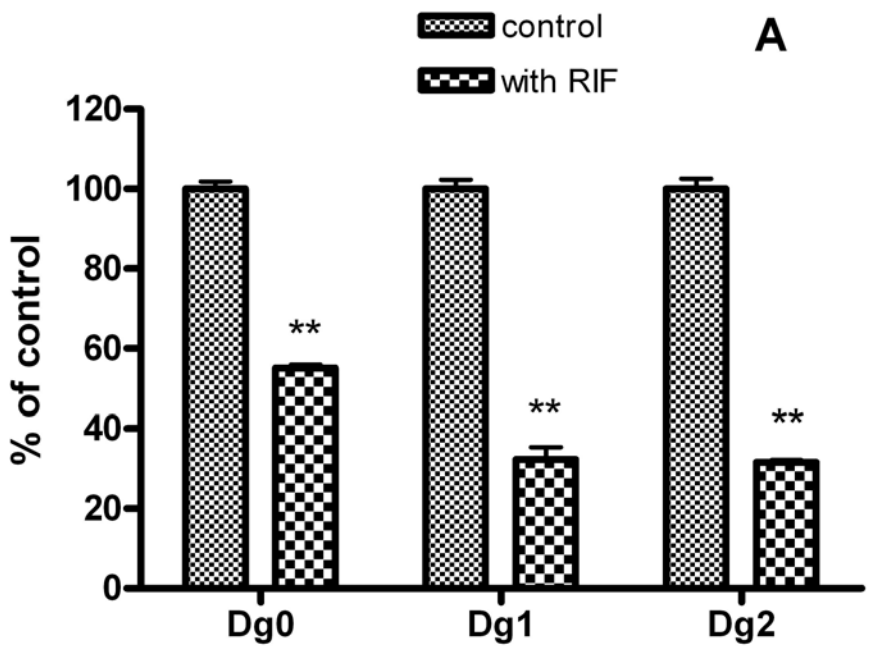


Figure 4



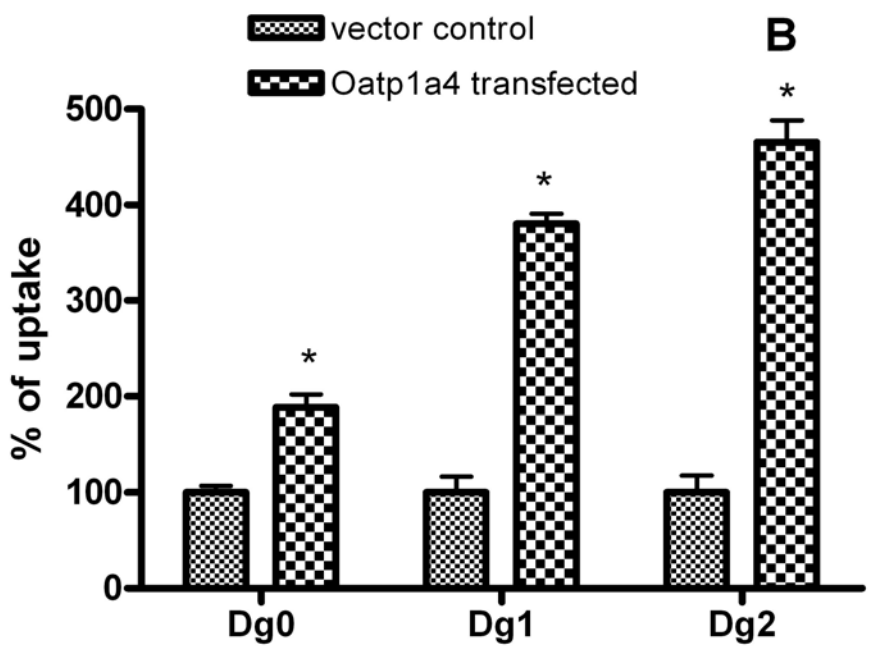


Figure 5