In-vivo formed versus preformed metabolite kinetics of trans-resveratrol-3-sulfate (R3S) and trans-resveratrol-3-glucuronide (R3G)

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**Abbreviation List**

APAP – acetaminophen
CS – calibration standards
ia – intra-arterial administration
IS – internal standard
LC-MS/MS – liquid chromatography with tandem mass spectrometry
LOQ – limit of quantitation

MRM – multiple reaction monitoring

PK – pharmacokinetics

QC – quality control

RES - *Trans*-3,5,4'-trihydroxystilbene; *trans*-resveratrol

R3G – *trans*-resveratrol-3-O-glucuronide

R4’G – *trans*-resveratrol-4’-O-glucuronide

R3S – *trans*-resveratrol-3-sulfate

R4’S – *trans*-resveratrol-4’-sulfate
Abstract

Metabolites in safety testing (MIST) have gained a lot of attention recently. Regulatory bodies have made recommendations where it is assumed that the kinetics of preformed and in vivo formed metabolites are similar. This has been a topic of debate. We have compared the kinetics of in vivo formed and preformed metabolites. *Trans*-3,5,4′-trihydroxystilbene (*trans*-resveratrol, RES) and its two major metabolites, resveratrol-3-sulfate (R3S) and resveratrol-3-glucuronide (R3G) were used as model substrates. The pharmacokinetics of R3S and R3G were characterized under two situations. First, the pharmacokinetics of R3S and R3G were characterized (in vivo formed metabolite) after administration of RES. Then synthetic R3S and R3G were administered (preformed metabolite) and their pharmacokinetics were characterized. Pharmacokinetic (PK) models were developed to describe the data. A three compartment model for RES, two compartment model for R3S (preformed) and an enterohepatic cycling model for R3G (preformed) was found to describe the data well. These three models were further combined to build a comprehensive PK model, which was used to perform simulations to predict in vivo formed metabolite kinetics. Comparisons were made between in vivo formed and preformed metabolite kinetics. Marked differences were observed in the kinetics of preformed and in vivo formed metabolites.
Introduction

Attention has increasingly focused on the issue of drug metabolites in safety testing (MIST) by both pharmaceutical companies and regulatory agencies (Baillie et al., 2002; Hastings et al., 2003; Smith and Obach, 2005; Prueksaritanont et al., 2006; Frederick and Obach, 2010). Two regulatory guidelines were recently published on this topic (Center for Drug Evaluation and Research (U.S.), 2008; International Conference on Harmonisation, 2009). These guidances recommend metabolite safety evaluation studies to be performed as early as possible during the clinical development program. The recommendation is to synthesize the metabolite and to evaluate it in preclinical toxicity studies.

One major assumption underlying metabolite toxicity evaluation studies is that the kinetic behavior of a preformed metabolite is the same as that of the metabolite formed in vivo following administration of parent compound. This has been a topic of debate (Prueksaritanont et al., 2006; Pang et al., 2008; Pang, 2009). It is understood that the pharmacokinetics of a preformed metabolite depends on the ADME properties of the metabolite whereas the kinetics of a metabolite generated in vivo depends on the parent as well as the metabolite (Prueksaritanont et al., 2006). Therefore, differences between the kinetic behavior of a preformed metabolite and the same metabolite generated in vivo could arise due to intrinsic differences between the disposition of the parent and its metabolite, e.g., their physicochemical properties or their interactions with transporters. Metabolites are generally more polar than their precursors. A polar preformed metabolite may experience diffusional barriers to its penetration into an eliminating organ, and hence its elimination clearance may be less than that of in vivo generated metabolite,
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whose entry into the eliminating organ is in the form of a more lipophilic parent (Pang, 1985). Additionally it is important to note that the diffusional barrier to penetration in eliminating organ pertains to the biliary excretion of substance and does not refer to renal (filtration) clearance.

As observed by Pang and co-workers (Pang et al., 2008; Pang, 2009), although preformed metabolite administration may not directly reflect the time-course of the in vivo formed metabolite, the kinetic data on the preformed metabolite can be extremely useful to develop a robust model for predictions and simulations. The data generated from preformed metabolite administration can be wisely incorporated into a comprehensive pharmacokinetic (PK) model of the parent-metabolite to improve predictions of the behavior of formed metabolite through modeling and simulation.

In the present study, metabolite kinetics of preformed and in vivo generated metabolites were compared with two approaches: i) assuming similar PK of preformed versus in vivo formed metabolite, ii) assuming dissimilar PK of preformed versus in vivo formed metabolite. The first approach assumes that the systemic or elimination clearance of in vivo formed and preformed metabolites are similar (Pearson and Wienkers, 2009), whereas the second approach does not make this assumption. The goal of the present study was to build a comprehensive PK model. This comprehensive PK model was used to predict in vivo formation of R3S and R3G after RES administration. Simulation assuming different elimination clearances of preformed and in vivo formed metabolites was compared to simulation with the assumption that PK of preformed versus in vivo formed metabolites are similar.
The polyphenol resveratrol (RES, Fig 1) was used as a model substrate in this study. RES is almost completely metabolized into its sulfated and glucuronidated metabolites in humans as well as rodents (Meng et al., 2004; Hoshino et al., 2010). RES is useful as a model substrate for polyphenols that are heavily conjugated into phase II metabolites. Conjugated metabolites are generally more polar than phase I metabolites. We have previously reported the synthesis of pure RES metabolites resveratrol-3-sulfate (R3S) and resveratrol-3-glucuronide (R3G, Fig 1) (Iwuchukwu et al., 2012). The PK of RES and synthesized and purified R3S and R3G were characterized in mice. These data were utilized to develop PK models. Our models corroborate differences in the PK of preformed versus in vivo formed metabolites (Prueksaritanont et al., 2006; Pang et al., 2008; Pang, 2009).
Materials and Methods:

Chemicals: RES was purchased from Cayman Chemical (Ann Arbor, MI, USA). R3S, R3G and R4’G for calibration were purchased from Toronto Research Chemicals (North York, Canada), and were additionally synthesized for animal dosing studies in the laboratory of Dr. Daniel J. Canney (Temple University, Philadelphia, PA, USA) (Iwuchukwu et al., 2012). R4’S was synthesized in the laboratory of Dr. Daniel J. Canney. Other reagents were purchased from standard sources. All reagents for analytical procedures were of analytical grade.

Animals: Male C57BL/6 mice weighing between 20 and 25 g were supplied by Jackson lab and maintained in the American Association for the Accreditation of Laboratory Animal Care-accredited University Laboratory Animal Resources of Temple University. Animals were fed a normal diet and water was continuously available, and were housed in a standard 12 hr dark/light cycle. Animals were acclimatized for four days before procedure. During the procedures animals were not provided food for the initial 10 hours of sampling to rule out any variability of gall bladder emptying in response to food. Animals had free access to water during the procedure. All animal studies were approved by the Institutional Animal Care and Use Committee.

Catheterization: Right carotid artery cannulation was performed under anesthesia using the EZ-ANESTHESIA apparatus with 1.5 % isoflurane and 2 L/min oxygen. An incision was made right of midline in the neck and right carotid artery was isolated. The right carotid artery was ligated, a small cut was made and a medical grade vinyl catheter tubing
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(0.28-mm i.d. × 0.64 mm o.d., SCI, Lake Havasu City, Arizona) filled with heparin-
saline (50 IU/ml, APP Pharmaceuticals, LLC, Schaumburg, IL) was inserted into the
right carotid artery. The cannula was tied into place, exteriorized at the back of the neck
and the incision sutured. Animals were allowed to recover from the surgery.

**Drug administration and blood sampling:** RES was solubilized in 20% 2-
hydroxypropyl-β-cyclodextrin in saline (Juan et al., 2010). Synthesized and purified R3G
and R3S (Iwuchukwu et al., 2012) were solubilized in saline. The carotid artery cannula
was used for systemic drug administration and blood sampling. Heparin-saline (20 uL, 50
IU/ml) was used to flush the cannula after systemic administration or blood sampling.
RES was administered intra-arterially (i.a.) at a dose of 15 mg/kg (65.79 umol/kg). R3S
was administered at a dose of 5 mg/kg (16.23 umol/kg, i.a.) and R3G was dosed at 3.5
mg/kg (8.67 umol/kg, i.a.). These doses were selected by performing pilot studies which
gave R3S and R3G exposures (AUCs) in the range comparable to R3S and R3G
observed upon 15 mg/kg RES (i.a.) administration. Blood (20 uL) was serially sampled at
2.5, 5, 10, 15, 45, 90, 180, 300, 420 and 600 min. A 24-hr collection was additionally
made for animals with functional cannulas. A total of 3-5 mice per dose and timepoint
was utilized. For 15 mg/kg RES, 5 mice were available for all the time points except 4
mice at 600 min and 2 mice at 24 hrs. For 5 mg/kg R3S, 4 mice were available for all the
time points until 600 min and for 3.5 mg/kg R3G, 4 mice were available for all the time
points except 3 mice at 24 hrs. Blood samples were centrifuged at 14,000 rpm for 2 min,
and harvested plasma was collected and stored at -80 °C until LC-MS/MS analysis.
**LC-MS/MS Analysis:** RES, R3S, R4’S, R3G and R4’G concentrations in plasma were measured with an electrospray ionization liquid chromatography-tandem mass spectrometry system (ABSciex API 4000) set in negative ion scan mode as described previously (Iwuchukwu et al., 2012). In brief, ascorbic acid (2.5 uL of a 15% solution) was added to 10 uL plasma samples and vortexed for 1 min. Then 30 uL of methanol containing 78 ng/ml APAP (internal standard) was added and vortexed for 1 min and centrifuged at 15,000 rpm for 15 min at room temperature. The supernatant (10 uL) was injected into the liquid chromatography tandem mass spectrometry system. The chromatographic separation system consisted of a guard column (Zorbax SB-C18, 5 um, 4.6 x 12.5 mm; Agilent Technologies), an analytical column (Zorbax SB-C18, 5 um, 4.6 x 150 mm; Agilent Technologies) and a gradient mobile phase of 5mM ammonium acetate (phase A) and methanol (phase B). The elution started with 10 % B at 0 min and linearly increased to 20 % B over 2 min, then to 35 % B from 2-10 min. The gradient was further increased to 60 % from 10-12 min and remained constant at 60% B until 17 min. Subsequently methanol was decreased to 10 % over 17-19 min. Flow rate of the mobile phase was 1 mL/min and the flow from the column was split 1:3 into a ABSciex API4000 triple quadrupole mass spectrometer equipped with a Turbo Ionspray source operating at 450°C. The column temperature was maintained at 35°C. The column effluent was monitored at the following precursor-product ion transitions: m/z 227→185 for RES, m/z 150→107 for the IS (APAP), 403→113 for R4’G and R3G and 307→227 for R4’S and R3S with a dwell time of 400 ms for each ion transition. The retention time was ~ 5 min for the IS, ~ 5.9 min for R4’G, ~ 7.3 min for R3G, ~ 9.2 min for R4’S, ~ 10.2 min for R3S and ~ 14.2 min for RES. The lower limit of quantification was 3.5 ng/ml (0.012 uM) for R4’S, 2.4 ng/ml (0.008 uM) for R3S, 10 ng/ml (0.025 uM) for R4’G, R3G and 10 ng/ml
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(0.044 uM) for RES. Both the accuracy and precision of the assay were greater than 85%, expressed by < 15% intraday and interday error.
Pharmacokinetic Analysis:

Noncompartmental Analysis

Pharmacokinetic parameters of RES, R3G and R3S were obtained by noncompartmental analysis with Phoenix, WinNonlin (version 6.1, Pharsight Corporation, Palo Alto, CA). All concentrations and masses were expressed as molar quantities. The area under the plasma concentration-time curve (AUC) was calculated by the linear trapezoidal method; clearance (Cl) was calculated as Cl = Dose/AUC\(_{0-\infty}\); volume of distribution at steady state (Vss) was calculated as Vss = Cl\times MRT\(_{0-\infty}\); the terminal half-life (t\(_{1/2}\)) was calculated as 0.693/k, and k was the slope of the terminal regression line, where AUC\(_{0-\infty}\) is the area under the curve from time zero to infinity and AUC\(_{0-t}\) is the area under the curve from time zero to last sampling point.

Characterization of metabolite kinetics:

i) Non-compartmental estimation: The apparent fraction of RES converted to R3S and R3G (fm), can be calculated assuming that R3S and R3G are formed directly and only from RES, entry of the metabolite into the eliminating organ is not diffusion limited, and elimination is not perfusion limited, with the following equations:

\[
fm_{R3S} = \left( \frac{AUC_{RES}^{R3S}}{AUC_{R3S}^{R3S}} \right) \times \left( \frac{Dose_{R3S}}{Dose_{RES}} \right) \times \left( \frac{Cl_{RES}^{R3S}}{Cl_{R3S}^{R3S}} \right) \quad [\text{Eq 1}]
\]

\[
fm_{R3G} = \left( \frac{AUC_{RES}^{R3G}}{AUC_{R3G}^{R3G}} \right) \times \left( \frac{Dose_{R3G}}{Dose_{RES}} \right) \times \left( \frac{Cl_{RES}^{R3G}}{Cl_{R3G}^{R3G}} \right) \quad [\text{Eq 2}]
\]
If metabolite elimination clearance is assumed to be the same whether preformed or in vivo formed, equations 1 and 2 simplify to equations 3 and 4 respectively (Pang and Kwan, 1983):

\[
fm_{R3S} = \left[ \frac{(AUC_{R3S})_{RES}}{(AUC_{R3S})_{R3S}} \right] \times \left[ \frac{Dose_{R3S}}{Dose_{RES}} \right] \quad \text{[Eq 3]}
\]

\[
fm_{R3G} = \left[ \frac{(AUC_{R3G})_{RES}}{(AUC_{R3G})_{R3G}} \right] \times \left[ \frac{Dose_{R3G}}{Dose_{RES}} \right] \quad \text{[Eq 4]}
\]

where \( AUC_{R3S}^{RES} \) is the AUC of R3S when RES is administered i.a., \( AUC_{R3G}^{RES} \) is the AUC of R3G when RES is administered i.a., \( AUC_{R3S}^{R3S} \) is the AUC of R3S when R3S is administered i.a. and \( AUC_{R3G}^{R3G} \) is the AUC of R3G when R3G was administered i.a.

ii) Compartmental analysis: Since there was wide variability in the individual animal data, most likely due to enterohepatic recycling, a naïve averaged data approach was used for modeling (Gabrielsson and Weiner, 2006). This approach has been commonly used, and for example reported by other researchers (Ogiso et al., 1998). The average concentration of each administration group at each sampling time point was used to perform the PK data analysis using SAAM II software system (version 1.2, SAAM Institute, Seattle, WA, 1997). For each dataset, the simplest compartment model was tested, and complexity was built into the model in subsequent steps. Model selection was based on goodness of fit and comparison of objective functions. Different weighting schemes were tested in SAAM II. A fractional standard deviation of 0.1 was used, and data- and model- relative as well as absolute variance models were evaluated. Model-absolute variance was selected and used, as it gave parameter estimates with the lowest
CV. Criteria for goodness of fit of each proposed model to the observed data was based on Akaike’s Information Criterion (AIC) as the objective function (Akaike, 1974). The standard error (SE) of the parameter estimation was expressed as % CV (SE/estimate x 100). The PK parameters for RES (model 1), R3G (model 2) and R3S (model 3) were first characterized as independent parent compounds. Linear PK were assumed at the dose levels used, and elimination solely from the central compartment was assumed.

Next, a comprehensive PK model (model 4) for the formation of R3S and R3G was built combining models 1, 2 and 3. This model was used to predict the concentration of its two major in vivo formed metabolites (R3G and R3S) when RES was administered by an i.a. bolus dose. The volume of distribution of central compartment was calculated as $V_c = \frac{\text{Dose}}{C_0}$, where $C_0$ is the initial concentration of drug in plasma. CI calculated as product of elimination rate constant and volume of central compartment was calculated and reported (Table 2). Formation clearances of R3S and R3G were calculated by multiplying the volume of central compartment of RES to corresponding formation rate constants i.e. $k_f,_{R3S}$ and $k_f,_{R3G}$. Fraction metabolized was calculated assuming that RES was completely metabolized into R3S and R3G.
Results:

Non-compartmental analysis

The concentration-time profiles of RES and its metabolites after administration of RES are shown in Fig 2A. R3S and R3G were the major metabolites and R4’G and R4’S were minor metabolites based on the relative plasma exposure upon i.a. administration. Four metabolites R3S, R3G, R4’G and R4’S exhibited early peak plasma concentration after i.a. RES administration. RES exhibited high clearance and high volume of distribution. Its clearance was interestingly higher compared to the normal blood flow to liver (90 ml/min/kg) in mouse (Davies and Morris, 1993). The results of the noncompartmental pharmacokinetic analysis are summarized in Table 1.

The concentration-time profiles of R3S and its metabolites after R3S administration at 5 mg/kg i.a. dose are shown in Fig 2B. R3G was observed in the plasma after R3S administration, and decreased rapidly to levels below the LOQ after 15 min. Subsequently R3G levels were detectable at some points after 180 min in some animals. Due to paucity of data points the R3G plasma profile could not be characterized after 15 min. The results of noncompartmental pharmacokinetic analysis are summarized in Table 1. RES was not detected upon the 5 mg/kg R3S dose. The results also indicated that R3S exhibits a high clearance compared to mouse liver blood flow (Davies and Morris, 1993) and a high volume of distribution.

The concentration-time profiles of R3G and its metabolites after R3G administration at 3.5 mg/kg are shown in Fig 2C. R4’G was observed in plasma after R3G administration. The AUC ratio of R4’G metabolite to total AUC0-inf (R3G + R4’G) was less than 1 %.
The results of the noncompartmental pharmacokinetic analysis are summarized in Table 1. R3G exhibits a low clearance and a high volume of distribution in mice. Reentry peaks of R3G in the plasma, likely due to enterohepatic recirculation, were clearly observed at 5 hours after i.a. administration of 3.5 mg/kg R3G.

Estimation of metabolite kinetics assuming similar characteristics of preformed versus in vivo formed metabolites:

Fraction of RES being metabolized into R3S and R3G using equations 3 and 4 ($f_{mR3S}$ and $f_{mR3G}$) was found to be 0.16 and 0.17 respectively. The sum of apparent fm values from R3S and R3G was 0.34.

Estimation of metabolite kinetics assuming dissimilar characteristics of preformed versus in vivo formed metabolites:

PK modeling of RES, preformed R3S and preformed R3G: (Models 1, 2 and 3). R3S and R3G are the major metabolites based on exposure when RES (15 mg/kg i.a.) was administered (Fig 2A). R4’G and R4’S were minor metabolites and together account for only 0.2% of the total exposure of RES and metabolites combined (Table 1). We have therefore ignored these minor metabolites in the model for the sake of simplicity. One, two and three compartment linear models were evaluated to explain the concentration data obtained after 15 mg/kg of RES, 5 mg/kg of R3S and 3.5 mg/kg of R3G i.a. administration. An open three compartment model with elimination from the central compartment (model 1, Fig 3A) was found to characterize well the concentration-time profiles for RES following its i.a. bolus administration. The predicted and observed RES plasma concentrations from Model 1 are shown in Fig 3B. An open two compartment
model (model 2, Fig 3C) best described the observed R3S concentration following R3S 5 mg/kg i.a. bolus administration (Fig 3D). An open three compartment model with a delay compartment (model 3, Fig 3E) and elimination from central compartment was used to characterize the concentration-time profile of R3G following R3G 3.5 mg/kg i.a. bolus administration (Fig 3F). A delay compartment was included to better describe the data (Davis et al., 2000), as a secondary peak was observed in R3G concentration which might be due to enterohepatic cycling. The delay site is characterized by two parameters that are estimated from the data: the delay time and the number of delay compartments. Mass entering the delay site passes through each of the delay compartments. The delay time was fixed as 180 min based on visual examination of data and 10 delay compartments were used. Table 2 shows the compartmental pharmacokinetic parameter estimates of RES, R3S and R3G. Run tests were performed for Models 1, 2 and 3, and resulted in large p-values (p = 0.26, 0.25, and 0.26 respectively), indicative of a lack of run of signs. The mean clearance estimate of RES and preformed R3G and R3S from models 1, 2 and 3 were comparable to the non-compartmental clearance estimates respectively (Tables 1 and 2).

Simulation of in vivo formed metabolites using model 4:

A great deal of time was invested in trying to fit comprehensive models to resveratrol and its in vivo formed metabolites simultaneously, but the models did not converge (possibly due to the large number of parameters (n=17) in relation to the data collected). Hence simulations were performed instead of model-fitting. The parameters obtained from model 1, model 2 and model 3 were fixed in model 4 and then the in vivo formed R3S and R3G after RES administration (Fig 4) predicted under two conditions. In the first
condition the elimination clearance of the preformed metabolites was assumed to be equal to that of the \textit{in vivo} formed metabolites. For this all the parameters in model 4 were fixed using parameters from model 1, 2 and 3. Next this was used to predict formation rate constants $k_{f,R3S}$ and $k_{f,R3G}$ (see Fig 4). Rate constants $k_{R3S}$ and $k_{f,R3G}$ were converted to clearance parameters by multiplying with central volume of distribution ($V_c$). Simulations were performed, but resulted in poor fit of the predicted versus observed plasma concentrations of RES (Fig 5A), R3S (5B) and R3G (5C).

In the second condition the elimination clearance of preformed metabolites was assumed to be dissimilar to \textit{in vivo} formed metabolites. For this all the parameters in model 4 were fixed using parameters from models 1, 2 and 3 except for the elimination rate constants of R3S ($k_{1,0,R3S}$) and R3G ($k_{1,0,R3G}$). The parameters $k_{f,R3S}$ (0.032 min$^{-1}$), $k_{f,R3G}$ (0.030 min$^{-1}$), $k_{1,0,R3S}$ (0.453 min$^{-1}$) and $k_{1,0,R3G}$ (0.295 min$^{-1}$) were predicted from the model (Fig 4). Next the simulation was performed by fixing $k_{f,R3S}$, $k_{f,R3G}$, $k_{1,0,R3S}$ and $k_{1,0,R3G}$ providing a good fit of observed versus model predicted RES, R3S and R3G plasma concentrations after RES 15 mg/kg i.a. administration as shown in Fig 6A, 6B and 6C respectively. The second condition also provided a more realistic estimate of the fraction of RES metabolized to R3S and R3G to be 52% and 48% respectively. Parameters were estimated under the assumption that there was no elimination of RES other than R3S and R3G. Elimination clearances of \textit{in vivo} formed R3S (313.08 ml/min/kg) and R3G (67.86 ml/min/kg) (Table 3) predicted under the second condition were found to be higher than the elimination clearances of preformed R3S (76.29 ml/min/kg) and R3G (13.78 ml/min/kg; Table 1).
Discussion:

RES pharmacokinetics has been reported previously (Marier et al., 2002; Boocock et al., 2007; Brown et al., 2010; Patel et al., 2010). RES is extensively metabolized into its sulfated and glucuronidated metabolites in mammals (Goldberg et al., 2003; Walle et al., 2004). This study characterized the kinetics of the major metabolites of RES, R3S and R3G, by administering the preformed metabolites. This is the first report of the metabolite kinetics of R3S and R3G after the administration of these preformed metabolites and their quantification against synthetic standards. The utility of the present work lies in development of models to explain the complex kinetics of highly conjugated substrates such as polyphenols. Further, models such as those presented here will be further developed in future studies to evaluate a) kinetics of conjugated metabolites that might be active, and b) potential interactions between polyphenols and co-administered drugs e.g enzyme induction or inhibition.

Similar to rats (Juan et al., 2010), RES was extensively metabolized to R3G in mice in the present study. Both RES and R3G underwent enterohepatic recirculation. The systemic clearance of RES observed in the present study (118.77 ml/min/kg, Table 1) was comparable to that reported in rats (195 ml/min/kg 183 ml/min/kg by (Marier et al., 2002; Kapetanovic et al., 2011). The high clearance is likely due to the rapid metabolism of RES. Interestingly, the value for total body clearance (118.77 ml/min/kg, Table 1) greatly exceeds hepatic blood flow rate (90 ml/min/kg) in mice (Davies and Morris, 1993). With the hepatic extraction ratio of RES assumed to be 100 percent, the maximum possible hepatic clearance is calculated to be 90 ml/min/kg. Extrahepatic clearance (28.77 ml/min/kg) was calculated as the difference between total body clearance (118.77
ml/min/kg for 15mg/kg i.a. dose) and hepatic clearance (90ml/min/kg), which represents 24.22% of total body clearance. This indicates the possibility of extrahepatic metabolism playing a role in the clearance of RES. A clear secondary peak was observed when preformed R3G was administered (Figure 2C) which can be attributed to the enterohepatic circulation of R3G. Enterohepatic circulation of R3G has also been shown earlier (Marier et al., 2002). Interestingly no RES was observed in plasma after administration of preformed R3S or preformed R3G.

Preformed major metabolites of RES - R3S and R3G - were administered to delineate the metabolite kinetics and to determine the fraction of RES converted to respective metabolites. The present data (Table 1) and literature reports clearly indicate much greater conversion of RES to R3S and R3G (Yu et al., 2002; Wenzel and Somoza, 2005) than predicted by equations 1 and 2, which assume similar kinetics of preformed versus in vivo formed metabolite (Table 1). Since these formation clearances are calculated based on preformed metabolite data, the difference in preformed metabolite kinetics as compared to in vivo formed metabolite kinetics might be responsible for underprediction of metabolite formation clearances. This prompted the use of a modeling approach to delineate the kinetics of in vivo formed metabolites.

The average plasma concentration-time profile of RES after 15mg/kg of RES administration was explained by a 3 compartment model (Figure 3A). Similar models have been used to explain disposition of drugs undergoing enterohepatic cycling (Hasselström and Säwe, 1993). RES was modeled to be distributed from a central compartment into two peripheral compartments, with elimination from the central
compartment. It has been shown earlier (Colburn, 1982) that enterohepatic circulation increases the apparent volume of distribution. Therefore, a very high volume of distribution of RES can be partially attributed to tissue binding and partially to enterohepatic circulation of RES.

R3S plasma profile was explained by a 2 compartment model with elimination from the central compartment (Figure 3C). Although enterohepatic cycling of R3S is also a possibility, a two compartment model was found to explain well the plasma profile of R3S after R3S (preformed) administration. An enterohepatic circulation model described the disposition of R3G after R3G (preformed) administration (Figure 3E). Similar models have been developed for morphine 3-glucuronide (Ouellet and Pollack, 1995), morphine (Dahlström and Paalzow, 1978), phenolphthalein (Colburn et al., 1979) and isoflavones (Moon et al., 2006). These models used either a series of cycling compartments linked by first order rate constants or a single compartment with a lag time to account for the delay observed in the appearance of a secondary peak in plasma. A similar approach was used in the present model by using a delay compartment which was comprised of 10 compartments with a single rate constant \( k_{1,3, \text{R3G}} \) and a fixed delay time of 3hr. Thus in Figure 3E, Compartment 1 depicts the blood as well as quickly equilibrating tissues. Compartment 2 depicts more slowly equilibrating tissues and compartment 3 can depict the intestinal compartment. The rate constant \( k_{1,3, \text{R3G}} \) represents several different processes including biliary transport of R3G, transit of R3G through the gastrointestinal lumen, and possible hydrolysis of glucuronides into RES. The rate constant \( k_{3,1, \text{R3G}} \) may denote absorption of re-formed RES and its subsequent glucuronidation into R3G or absorption of R3G from the lower intestine. A lag time of 3 hr is included between biliary
transport and absorption to account for transit from the liver to the site of de-glucuronidation, and subsequent metabolism and reabsorption. The clearance estimates obtained after compartmental analysis of pooled data were found to be comparable to those estimated with individual data by non-compartmental methods (Tables 1 and 2).

Models 1, 2 and 3 were combined to form a comprehensive model 4 to predict the \textit{in vivo} formed R3S and R3G after RES administration (Figure 4). Simulation using the assumption that elimination clearances of preformed and \textit{in vivo} formed metabolites are the same, led to a poor overlap of the observed and predicted \textit{in vivo} formed R3S and R3G (Fig 5A, B & C). Simulation using the assumption that elimination clearances of preformed and \textit{in vivo} formed metabolites are different, led to a much improved prediction (Fig 6A, B & C). The second approach also gave a more realistic formation ratio of R3S and R3G as 52% and 48% respectively. This value was comparable to the formation ratio of \textit{in vivo} formed R3S (46%) and R3G (54%) predicted by Colom and co-workers (Colom et al., 2011). With the second approach the elimination clearance of R3S and R3G used for the simulation was higher than the preformed metabolites’ elimination clearances. It has been suggested that phase II metabolites like glucuronides and sulfates are more hydrophilic and preformed metabolites may experience difficulty penetrating into an eliminating organ, and hence the extent of its elimination may be less than that of \textit{in vivo} generated metabolite, whose entry into the eliminating organ is in the form of a more lipophilic precursor (Pang et al., 1984; Pang, 1985). The formation and elimination clearances of the formed and preformed metabolites differ markedly. Differences in metabolite kinetics of preformed and \textit{in vivo} formed metabolites were clearly visible in the present study.
It has been observed by Pang and coworkers (Pang et al., 2008; Pang, 2009) that although preformed metabolite administration might not be able to provide a complete correlation of the formed metabolite time-course, the accompanying information can be incorporated to build a comprehensive PK model. This can improve the predictions of in vivo formed metabolites. In the present work, model 4 was useful for purposes of predicting the disposition of metabolites as well as RES exposure. This and similar models can be further developed and improved to predict events such as interactions with xenobiotics that lead to enzyme induction or inhibition.

As a first study of RES metabolite PK, the present study did not include data collection such as urine, feces, or bile. This is an obvious limitation of the study, as additional data would provide a more detailed picture of RES disposition. Also, single dose plasma data collected here did not aid in discerning elimination from the central versus peripheral compartment (Berezhkovskiy, 2004; Yates and Arundel, 2008). Thus, a criticism of the models presented is the assumption of elimination solely from the central compartment. If peripheral elimination were to play a role in the elimination of RES or its metabolites (e.g. metabolism in tissues kinetically different from the central compartment), the steady-state volume of distribution estimates might be predicted inaccurately with the present models.

In summary, the kinetics of R3S and R3G were studied for the first time by administering the preformed metabolites. PK models were developed to adequately explain the kinetics of RES and its two major metabolites, R3S and R3G. Preformed and in vivo formed R3S
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and R3G kinetics were compared and a marked difference was observed between the preformed and *in vivo* formed metabolite kinetics. Due to observed kinetic differences between *in vivo* formed metabolites and preformed metabolites, safety and toxicity studies conducted with preformed metabolites are useful only when there is a similarity in the kinetics of preformed and *in vivo* formed metabolites, or when sufficient tissue exposure of preformed metabolites is ensured. However, achieving high tissue exposure can be especially difficult for very hydrophilic metabolites.
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Authorship Contributions

Participated in research design: Sharan, Nagar.

Conducted experiments: Sharan

Contributed new reagents or analytic tools: Sharan, Canney, Iwuchukwu

Performed data analysis: Sharan, Nagar, Zimmerman

Wrote or contributed to the writing of the manuscript: Sharan, Nagar, Zimmerman
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resveratrol and quercetin in humans, mice, and rats after ingestion of pure
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diacid metabolite, enalaprilat, in a perfused rat liver preparation. Presence of a
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Footnotes

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Figure Legends

Figure 1: Structure of RES and its four monoconjugated metabolites i.e. R3S, R4’S, R3G and R4’G.

Figure 2: Mean plasma concentration-time profiles after administration of, A: RES i.a. 15 mg/kg; B, R3S i.a. 5 mg/kg and C, R3G i.a. 3.5 mg/kg. Data are presented as mean ± SD, n = 4 – 5.

Figure 3: Compartmental modeling of RES, R3G and R3S disposition. A: Three compartment PK model 1 describing the disposition of RES after administration of RES (15 mg/kg, i.a.). Vc: volume of the central compartment, k: first order rate constants for RES disposition. B: Observed average RES concentration (data points) and PK model 1 predicted (solid line) RES concentration time profiles after RES administration, plot of weighted residuals versus predicted RES concentration (inset). C: Two compartment PK model 2 describing the disposition of R3S after administration of R3S (5 mg/kg, i.a.). Vc,R3S: volume of the central compartment, k: first order rate constants for R3S disposition. D: Observed average R3S concentration (data points) and PK model 2 predicted (solid line) R3S concentration time profiles after R3S administration, plot of weighted residuals versus predicted R3S concentration (inset). E: Enterohepatic cycling PK model 3 describing the disposition of R3G after administration of R3G (3.5 mg/kg, i.a.). Vc,R3G: volume of the central compartment, k: first order rate constants for R3G disposition. F: Observed average R3G concentration (data points) and PK model 3 predicted (solid line) R3G concentration time profiles after R3G administration, plot of weighted residuals versus predicted R3G concentration (inset).

Note: The notation used throughout is k(from, to). In SAAM II software, the rate constants have a different notation k(to, from).
Figure 4: PK model 4 describing the disposition of \textit{in vivo} formed metabolite R3S and R3G after RES (15 mg/kg, i.a.) administration. Individual models for RES, R3S, and R3G are as described in Figure 3. Vc: volumes of central compartments, k: first order disposition rate constants, kf: first order formation rate constants for RES metabolites. Note: The notation used throughout is k(from, to). In SAAM II software, the rate constants have a different notation k(to, from).

Figure 5: Observed and PK model 4 simulated concentration time profiles of RES (A), R3S (B) and R3G (C) after RES administration, assuming elimination clearance of preformed metabolites to be equal to \textit{in vivo} formed metabolites.

Figure 6: Observed and PK model 4 simulated concentration time profiles of RES (A), R3S (B) and R3G (C) after RES administration, assuming elimination clearance of preformed metabolites to be not equal to \textit{in vivo} formed metabolites.
Table 1. Noncompartmental pharmacokinetic analysis upon a single 15 mg/kg (i.a.) RES, 5 mg/kg (i.a.) R3S or 3.5 mg/kg (i.a.) R3G dose. Data are presented as Mean ± SD

<table>
<thead>
<tr>
<th>ESTIMATE</th>
<th>RES 15 mg/kg</th>
<th>R3S 5 mg/kg</th>
<th>R3G 3.5 mg/kg</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt;</td>
<td>510.01 ± 105.54</td>
<td>243.29 ± 113.10</td>
<td>650.66 ± 216.50</td>
<td>min*uM</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-inf&lt;/sub&gt;</td>
<td>591.08 ± 167.29</td>
<td>255.84 ± 124.98</td>
<td>710.10 ± 273.30</td>
<td>min*uM</td>
</tr>
<tr>
<td>Cl</td>
<td>118.77 ± 33.36</td>
<td>76.29 ± 37.07</td>
<td>13.78 ± 5.75</td>
<td>ml/min/kg</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>37.59 ± 23.70</td>
<td>6.37 ± 2.36</td>
<td>4.55 ± 1.07</td>
<td>L/kg</td>
</tr>
<tr>
<td>Terminal t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>190.58 ± 69.65</td>
<td>128.08 ± 26.21</td>
<td>272.48 ± 17.07</td>
<td>min</td>
</tr>
<tr>
<td>Cmax</td>
<td>15.27 ± 9.07</td>
<td>14.79 ± 3.15</td>
<td>27.84 ± 4.70</td>
<td>uM</td>
</tr>
<tr>
<td>Tmax</td>
<td>2.5 ± 0</td>
<td>2.5 ± 0</td>
<td>2.5 ± 0</td>
<td>min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R3S</th>
<th>RES</th>
<th>R3S</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt;</td>
<td>163.87 ± 42.38</td>
<td>NA</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-inf&lt;/sub&gt;</td>
<td>174.94 ± 45.75</td>
<td>NA</td>
</tr>
<tr>
<td>Terminal t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>201.12 ± 158.12</td>
<td>NA</td>
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</table>

<table>
<thead>
<tr>
<th>R3G</th>
<th>R3G</th>
<th>RES</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt;</td>
<td>857.36 ± 396.17</td>
<td>1.04 ± 0.29</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-inf&lt;/sub&gt;</td>
<td>921.23 ± 457.07</td>
<td>1.59 ± 0.73</td>
</tr>
<tr>
<td>Terminal t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>264.75 ± 248.66</td>
<td>10.05 ± 3.98</td>
</tr>
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<tr>
<td><strong>AUC_{0-t}</strong></td>
<td>2.03 ± 1.78</td>
<td>NA</td>
</tr>
<tr>
<td><strong>AUC_{0-inf}</strong></td>
<td>2.61 ± 1.87</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Terminal t_{1/2}</strong></td>
<td>14.11 ± 5.98</td>
<td>NA</td>
</tr>
<tr>
<td><strong>R4'S</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUC_{0-t}</strong></td>
<td>0.83 ± 0.39</td>
<td>NA</td>
</tr>
<tr>
<td><strong>AUC_{0-inf}</strong></td>
<td>1.07 ± 0.43</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Terminal t_{1/2}</strong></td>
<td>8.85 ± 4.37</td>
<td>NA</td>
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</table>
Table 2. Compartmental pharmacokinetic parameters of RES, R3S and R3G administered as parent compound using model 1, 2 and 3, respectively.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RES, Model 1, Estimate (CV%)</th>
<th>R3S, Model 2, Estimate (CV%)</th>
<th>R3G, Model 3, Estimate (CV%)</th>
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<tbody>
<tr>
<td>Vc, RES (l/kg)</td>
<td>1.77 (27.29)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cl, RES (ml/min/kg)</td>
<td>104.54 (3.97)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>k_{1,0}, (min^{-1})</td>
<td>0.06 (25.66)</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>k_{1,2} (min^{-1})</td>
<td>0.23 (23.74)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>k_{2,1} (min^{-1})</td>
<td>0.06 (14.74)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>k_{1,3} (min^{-1})</td>
<td>0.09 (24.73)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>k_{3,1} (min^{-1})</td>
<td>0.004 (11.84)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Vc, R3S (l/kg)</td>
<td>NA</td>
<td>0.69 (11.43)</td>
<td>NA</td>
</tr>
<tr>
<td>Cl, R3S (ml/min/kg)</td>
<td>NA</td>
<td>63.11 (3.56)</td>
<td>NA</td>
</tr>
<tr>
<td>k_{1,0}, R3S (min^{-1})</td>
<td>NA</td>
<td>0.09 (9.37)</td>
<td>NA</td>
</tr>
<tr>
<td>k_{1,2} R3S (min^{-1})</td>
<td>NA</td>
<td>0.15 (7.17)</td>
<td>NA</td>
</tr>
<tr>
<td>k_{2,1} R3S (min^{-1})</td>
<td>NA</td>
<td>0.012 (7.45)</td>
<td>NA</td>
</tr>
<tr>
<td>Vc, R3G (l/kg)</td>
<td>NA</td>
<td>NA</td>
<td>0.23 (10.95)</td>
</tr>
<tr>
<td>Cl, R3G (ml/min/kg)</td>
<td>NA</td>
<td>NA</td>
<td>10.63 (6.81)</td>
</tr>
<tr>
<td>k_{1,0} R3G (min^{-1})</td>
<td>NA</td>
<td>NA</td>
<td>0.05 (11.86)</td>
</tr>
<tr>
<td>k_{1,2} R3G (min^{-1})</td>
<td>NA</td>
<td>NA</td>
<td>0.04 (15.53)</td>
</tr>
<tr>
<td>Reaction</td>
<td>Rate Constant (min⁻¹)</td>
<td>k2,1 F3G (min⁻¹)</td>
<td>k1,3 F3G (min⁻¹)</td>
</tr>
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<td>------------------</td>
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<td>NA</td>
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<tr>
<td>k₂,₁ F₃G (min⁻¹)</td>
<td></td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>k₃,₁ F₃G (min⁻¹)</td>
<td></td>
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</table>
**Table 3.** Comparison of elimination or systemic clearance of *in vivo* formed metabolites and preformed metabolites.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Preformed Metabolite</th>
<th><em>In-vivo</em> formed metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl,R3S (ml/min/kg)</td>
<td>76.29 ± 37.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>313.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cl,R3G (ml/min/kg)</td>
<td>13.78 ± 5.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.86&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>fm&lt;sub&gt;R3S&lt;/sub&gt; (%)</td>
<td>16.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>fm&lt;sub&gt;R3G&lt;/sub&gt; (%)</td>
<td>17.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.00&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> From Table 1  
<sup>b</sup> Fractions calculated using equations 1 and 2.  
<sup>c</sup> Values used for simulations in Fig 6B and 6C  
<sup>d</sup> Fractions of RES converted to R3S and R3G, calculated assuming complete metabolism of RES into R3S and R3G for Fig 6B and 6C.
Fig 1

R4'S

R4'G

RES

R3G

R3S