Pulmonary metabolism of resveratrol: in vitro and in vivo evidence

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Abbreviation List

APAP – acetaminophen

CS – calibration standards

i.a. – intra-arterial administration
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IS – internal standard

i.v. – intravenous administration

LC-MS/MS – liquid chromatography with tandem mass spectrometry

LOQ – limit of quantitation

MRM – multiple reaction monitoring

PAPS – 3’-Phospho-adenosine-5’-phosphosulphate

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

SULT - Sulfotransferase

UDPGA – Uridine 5’-diphosphoglucuronic acid

UGT - Uridine 5’-diphosphoglucuronosyltransferase

Uppercase UGT denotes human protein and lowercase ugt denotes mouse protein

Uppercase SULT denotes human protein and lowercase sult denotes mouse protein

Uppercase MRP and BCRP denotes human protein and lowercase Mrp and Bcrp denotes mouse protein
Abstract

The role of pulmonary metabolism in trans-resveratrol (RES) pharmacokinetics was studied in a mouse model. Plasma concentrations of RES and its major metabolites trans-resveratrol-3-sulfate (R3S) and trans-resveratrol-3-glucuronide (R3G) were compared after administration of RES by intravenous (i.v.) and intra-arterial (i.a.) routes. Total AUC of RES decreased by approximately 50% when RES was administered i.v. compared to i.a. route. The AUC of R3G was also significantly higher in mice administered RES i.v. compared to i.a. route. In vitro studies performed with mouse and human lung fractions confirmed pulmonary metabolism of RES. Interestingly, mouse lung fractions gave rise to both R3S and R3G whereas human lung fractions yielded R3S. This indicates marked interspecies variation in RES conjugation, especially in the context of extrapolating rodent data to humans. Taken together, results presented here underline for the first time, the impact of pulmonary metabolism on resveratrol pharmacokinetics and interspecies differences in RES pulmonary metabolism.
The knowledge of extrahepatic metabolism in drug disposition is important. Extrahepatic drug metabolism can modify the systemic as well as tissue exposure of drug/metabolites and this becomes especially important in cases of active metabolite formation and certain disease states. For example, in severe cirrhosis of the liver, extrahepatic metabolic pathways might compensate for the impaired hepatic elimination of a drug (Patwardhan et al., 1981). Tissue levels of drug/active metabolites might change depending upon the site of metabolism. For example, in case of irinotecan, its active metabolite SN-38 is conjugated to SN-38 glucuronide in liver, which is eliminated in the bile and metabolized by gut β-glucuronidase to regenerate SN-38. Locally formed SN-38 is thought to cause diarrhea (Araki et al., 1993; Takasuna et al., 1996; Michael et al., 2004). Thus, information about sites of metabolism is important for appropriately selecting dosage regimen for various clinical conditions, as well as for selection of appropriate route of drug administration.

The route of drug administration can significantly change the disposition of parent drug and metabolites if extrahepatic eliminating organs are involved. Although a bioavailability of 100% is assumed upon intravenous administration of a drug, pulmonary metabolism can decrease the bioavailability even upon i.v. administration. Compounds administered orally must cross the gut and lungs in addition to the liver before reaching the arterial blood supply for distribution to tissues. The lung as a site of metabolism assumes significance as the entire cardiac output (approximately four times liver blood flow) perfuses the lung and can play an important role in drug disposition (Davies and...
Morris, 1993). It has been shown that phenolic compounds e.g. harmol (Mulder et al., 1984) and phenol (Cassidy and Houston, 1984) undergo pulmonary metabolism.

Trans-resveratrol (RES) is a dietary phytochemical known to have beneficial health effects via numerous mechanisms (Baur and Sinclair, 2006). RES has been shown to induce apoptosis in human lung adenocarcinoma cells (Alex et al., 2010; Zhang et al., 2011; Zhang et al., 2012b). It has been also shown to have lung cancer chemopreventive activity by altering the expression of genes involved in the phase I metabolism of polycyclic hydrocarbons (Mollerup et al., 2001). Although the role of gut and liver is well known in the metabolism of RES (Kuhnle et al., 2000; Miksits et al., 2005; Brill et al., 2006; Iwuchukwu and Nagar, 2008; van de Wetering et al., 2009), the role of lungs has not been evaluated in the metabolism of RES. RES is known to be extensively metabolized into its two major metabolites i.e. trans-resveratrol-3-sulfate (R3S) and trans-resveratrol-3-glucuronide (R3G) (Figure 1) in humans as well as rodents (Yu et al., 2002; Meng et al., 2004; Hoshino et al., 2010; Sharan et al., 2012). A monosulfated metabolite of RES, R3S has recently been shown to be biologically active in in vitro studies (Hoshino et al., 2010). Although glucuronides have generally been assumed to be pharmacologically inactive, examples do exist of active glucuronidated metabolites (Osborne et al., 1988; Kroemer and Klotz, 1992; Sperker et al., 1997). No biological activity of R3G has been reported to date.

In our previous study with intra-arterial (i.a.) administration of RES (Sharan et al., 2012), we observed that murine systemic clearance of RES was higher than hepatic blood flow,
indicating the possibility of extrahepatic conjugation in the mouse. These results led us to investigate the lung as a possible metabolizing organ for RES. We studied the contribution of pulmonary metabolism of RES in vivo by using multiple sites of administration and a single site of sampling (Cassidy and Houston, 1980), which exploits the anatomical arrangement of lungs. When administered i.v., RES enters the right atrium of the heart and crosses the lungs in a relatively undiluted form prior to reaching the general arterial system for distribution throughout the body. In contrast, when given i.a. into the right carotid artery, RES is available immediately for tissue distribution. By comparison of plasma concentration time profiles following administration of RES by both routes, we calculated the contribution of lungs in the first pass metabolism of RES across the lungs. In vivo findings in mouse were further confirmed by in vitro experiments using lung fractions of mouse. Finally, in vitro studies using human lung fractions were also performed to compare mouse versus human RES pulmonary conjugation.

Materials and Methods:
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**Chemicals:** RES was purchased from Cayman Chemicals (Ann Arbor, MI, USA). R3S and R3G for calibration were purchased from Toronto Research Chemicals (North York, Canada). The cofactors UDPGA and PAPS were purchased from Sigma-Aldrich (St. Louis, MO). Mouse and human lung fractions were purchased from XenoTech, LLC (Lenexa, Kansas). 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. Animals were housed in a standard 12 hr dark/light cycle and were acclimatized for four days before procedure. Animals had free access to food and water during the procedure. All animal studies were approved by the Institutional Animal Care and Use Committee.

**Catheterization:** Right carotid artery and jugular vein cannulation were performed under anesthesia with 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 (0.28-mm i.d. × 0.64 mm o.d., SCI, Lake Havasu City, Arizona) with heparin-saline (50 IU/ml, APP Pharmaceuticals, LLC, Schaumburg, IL) was inserted with the cannula tip in the right carotid artery. The cannula was tied into place, exteriorized at the back of the neck and the incision sutured. The right jugular vein was cannulated in a
similar manner. The cannulas were tied in place, exteriorized at the back of the neck and the incision was sutured. Animals were allowed to recover from the surgery. Animals regained full consciousness and started moving freely after 15 minutes of surgery.

**Drug administration and blood sampling:** RES was solubilized in 20% 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) in saline (Juan et al., 2010b). RES was administered by i.v. route at a dose of 15 mg/kg. Carotid artery cannula was used for blood sampling. Heparin-saline (20 µL, 50 IU/mL) was used to flush the cannula after systemic administration or blood sampling. Blood (20 µL) was serially sampled at 2.5, 5, 10, 15, 45, 90, 180, 300, 420 and 600 min. 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. Experiment details of RES i.a. administration at dose of 15 mg/kg have been previously published (Sharan et al., 2012).

**In vitro pulmonary glucuronidation:** RES glucuronidation was determined in pooled mouse lung S9 fraction and in pooled human lung microsomes. For mouse lung S9 glucuronidation assay, conditions of protein and time linearity were optimized in preliminary studies. Preliminary experiments showed that the reactions were linear up to 60 min and 2.5 mg/ml of protein. The incubation mixture consisted of mouse lung S9 fraction (final concentration, 0.5 mg/ml), substrate RES (concentration ranging from 0.01 µM to 5 mM) solubilized in HP-β-CD (final HP-β-CD concentration 2%), alamethicin (final concentration 10 µg/mL), MgCl₂ (final concentration 5 mM), and made up to final incubation volume of 500 µL with Tris-HCl buffer (100 mM, pH 7.4, 37°C). The reaction
mixture was preincubated for 3 min in a shaking water bath at 37°C. The reaction was started by adding appropriate volume of the cofactor UDPGA (uridine 5’-diphosphoglucuronic acid, final concentration 5 mM). To 20 µL of reaction mixture, 5 µL of ascorbic acid and 60 µL of ice cold methanol containing acetaminophen (APAP) as internal standard (IS) were added at the end of 60 min to stop the reaction. All reactions were performed in triplicate. Appropriate negative control experiments were performed under the same conditions but without UDPGA.

For human lung glucuronidation assay, the incubation was performed at 0.1, 0.5 and 2.5 mg/mL (final concentration) of human lung microsomes with substrate RES (0.5 mM) solubilized in HP-β-CD (final HP-β-CD concentration 2%). Preliminary studies showed minimal glucuronidation in human lung microsomes, hence kinetic assays were not conducted.

**In vitro pulmonary sulfation:** RES sulfation activity was determined in pooled mouse lung S9 fraction and in pooled human lung S9 fraction. Conditions of protein and time linearity were optimized in preliminary studies. Preliminary experiments showed that the reactions were linear up to 60 min and 2.5 mg/mL of total protein for each protein source. The incubation mixture consisted of mouse lung S9 fraction (final concentration, 1 mg/mL) or, human lung S9 fraction (final concentration, 0.5 mg/mL), the substrate RES (0.01 µM to 5 mM) solubilized in HP-β-CD (final HP-β-CD concentration 2%), MgCl2 (5 mM final concentration), and made up to final incubation volume of 500 µL with potassium phosphate buffer (10 mM, pH 6.5, 37°C). The reaction mixture was
preincubated for 3 min in a shaking water bath at 37°C. The reaction was started by adding appropriate volume of the cofactor PAPS (3’-phospho-adenosine-5’-phosphosulphate, final concentration 1 mM) and incubated in a shaking water bath for 60 min at 37°C. To 20 µL of reaction mixture, 5 µL of ascorbic acid and 60 µL of ice cold methanol containing APAP (IS) were added at the end of 60 min to stop the reaction. All reactions were performed in triplicate. Appropriate negative control experiments were performed under the same conditions but without PAPS.

**Protein binding Assay:** Equilibrium dialysis was performed using a 96-well equilibrium dialyzer with MW cutoff of 5K (Harvard Apparatus, Holliston, MA) and placed in dual-plate rotator set to maximum speed (Harvard Apparatus, Holliston, MA) placed in a 37°C incubator with 10% CO2 atmospheric environment. Frozen mouse plasma was thawed and its pH was adjusted to 7.4. RES, R3S and R3G plasma protein binding was determined at a concentration of 20 µM. The protein binding assay was performed with a published protocol (Kochansky et al., 2008).

**LC-MS/MS Analysis:** RES, R3S and R3G concentrations in plasma and in the in vitro reaction mixture 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 µL of a 15% solution) was added to 10 µL plasma samples and vortexed for 1 min. Then 30 µL 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. For in vitro studies,
samples were prepared as described earlier. Supernatant (10 µL) was injected into the liquid chromatography tandem mass spectrometry system. The chromatographic separation system consisted of a guard column (Zorbax SB-C18, 5µm, 4.6 × 12.5 mm; Agilent Technologies), an analytical column (Zorbax SB-C18, 5µm, 4.6 × 150 mm; Agilent Technologies) and a gradient mobile phase of A 5mM ammonium acetate and B methanol. The elution started with 90% A at 0 min to 80% at 2 min, 65% at 10 min, 40% at 12 min to 17 min and 90% at 19 min. Flow rate of the mobile phase was 1mL/min and the flow from the column was split 1:3 into an 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 IS (APAP), 403→113 for R3G and 307→227 for R3S with a dwell time of 400 ms for each ion transition. The retention time was ~ 5 min for IS (APAP), ~ 5.9 min for R3G, ~ 9.2 min for R3S and ~ 14.2 min for RES. The lower limit of quantification was 2.4 ng/mL for R3S and 10 ng/mL for R3G and RES.

Noncompartmental pharmacokinetic analysis:
Pharmacokinetic parameters of RES, R3G and R3S were analyzed by noncompartmental analysis with Phoenix, WinNonlin (version 6.1, Pharsight Corporation, Palo Alto, CA). The area under the plasma concentration-time curve (AUC) was calculated with the linear trapezoidal method; clearance (CL) was calculated as CL = Dose/AUC_{0-inf}; volume of distribution at steady state (Vss) was calculated as Vss = CL×MRT_{0-inf}; the terminal half-life (t_{1/2}) was calculated as 0.693/λ, where λ is the slope of the terminal regression line.
AUC₀-inf is the area under the curve from time zero to infinity and MRT₀-inf is the mean residence time from time zero to infinity. The bioavailability (fL) of RES after i.v. and i.a. administration was calculated with the following equation (Cassidy and Houston, 1980):

\[ fL = \left( \frac{\text{mean AUC}_0-\text{inf, i.v.}}{\text{mean AUC}_0-\text{inf, i.a.}} \right) \times 100 \]  

(1)

**Data analysis for enzyme kinetics:** All data were initially transformed and Eadie-Hofstee curves were plotted before nonlinear regression analysis. The Michaelis-Menten model was fit only to data which showed linear Eadie-Hofstee plots. The following equation (equation 2) was used to fit the data showing linear Eadie-Hofstee plots and Michaelis-Menten parameter estimates were determined (Segel, 1993).

\[ v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \]  

(2)

Where \( v \) is the rate of the reaction, \( V_{\text{max}} \) is the maximum velocity estimate, \( [S] \) is the substrate concentration, and \( K_m \) is the Michaelis-Menten constant.

The following equation (equation 3) was used to fit the data exhibiting partial substrate inhibition profile (Hutzler and Tracy, 2002; Tracy and Hummel, 2004):

\[ v = \frac{V_{\text{max}} \cdot [S]/(K_m + [S] + \left( \frac{[S]^2}{K_i} \right))}{K_m \cdot [S] + [S]} \]  

(3)

where \( K_i \) is the partial substrate inhibition constant. Nonlinear regression was performed with GraphPad Prism for Windows (version 4.03; GraphPad Software Inc., San Diego, CA).

**Statistics:** Student’s unpaired t test was used, with \( p < 0.05 \) set as the significance level. GraphPad Prism for Windows (version 4.03; GraphPad Software Inc., San Diego, CA) was used to perform statistical analysis.
Results:

Non-compartmental pharmacokinetic analysis of RES: The concentration-time profile of RES and its metabolites after administration of RES via i.v. route is shown in Figure 2. RES (15 mg/kg) i.a. data have been previously published (Sharan et al., 2012). Both i.v. and i.a. experiments were conducted in parallel under the same laboratory conditions, therefore we were able to compare the data. RES was metabolized into two major metabolites i.e. R3S and R3G. The results of the noncompartmental pharmacokinetic analysis are summarized in Table 1.

RES exposure (294.98 ± 137.87 min*µM) and half life (101.30 ± 43.41 min) after i.v. administration were significantly lower than its exposure (591.08 ± 167.29 min*µM) and half life (190.58 ± 69.65 min) after i.a. administration (AUC: p = 0.01, t_{1/2}: p = 0.04). The bioavailability (f_L) of RES after i.v. administration was found to be 49.90 %. The clearance and volume of distribution at steady state of RES after i.v. administration were not statistically significantly different compared to i.a. administration (CL: p = 0.05, Vss: p = 0.85). Interestingly, it was observed that the exposure of R3G (2268.35 ± 517.00 min*µM) after i.v. administration of RES increased significantly compared to R3G exposure (921.23 ± 457.07 min*µM) after RES i.a. administration (p = 0.004). No significant change was observed in the exposure of R3S after RES administration by both routes (p = 0.67).

The plasma protein binding of RES, R3G and R3S in mouse plasma was found to be 91.95 ± 0.99, 66.75 ± 1.56 and 87.24 ± 4.89 % (mean ± SD, n=3) respectively.
In vitro pulmonary metabolism: The glucuronidation of RES was studied in mouse and human lung fractions. Figure 3A shows the formation rate of R3G in mouse lung fraction with its Eadie-Hofstee (E-H) plot shown as inset. The R3G profile exhibited partial substrate inhibition. This was determined by fitting the data to the partial substrate inhibition equation (equation 3) and by visual examination of E-H plot. The E-H plot showed a hook in the upper quadrant typical of partial substrate inhibition kinetic profile (Figure 3A inset) (Hutzler and Tracy, 2002). Enzyme kinetic parameters are presented in Table 2. When RES was incubated with 0.1 and 0.5 mg/mL human lung microsomes, no R3G was observed above LOQ (10 ng/mL for R3G) at the end of 60 min incubation. Therefore no further RES glucuronidation kinetic studies were performed with human lung microsomes.

Figure 3B and 3C show the formation kinetics of R3S in mouse and human lung fractions with Eadie-Hofstee (E-H) plots as insets respectively. R3S formation in both mouse and human lung fractions showed partial substrate inhibition (Hutzler and Tracy, 2002).
Discussion:

RES is known to be extensively metabolized into its conjugates, mainly R3G and R3S (Yu et al., 2002; Meng et al., 2004; Hoshino et al., 2010; Iwuchukwu et al., 2012)).

Lungs are the third in a series of three potential biotransformation sites (along with the gut and liver) which orally ingested RES must cross before entering the general circulation. First pass metabolism by gut, liver and lungs in series can synergistically increase total body clearance of RES. Although the role of gut and liver in the metabolism of RES is known (Miksits et al., 2005; Brill et al., 2006; Iwuchukwu and Nagar, 2008), contribution of lungs to RES metabolism has not been evaluated. In the present study, contribution of lungs in the metabolism of RES was evaluated using multiple site of administration and single site of sampling design (Cassidy and Houston, 1980) in a mouse model. The in vivo study clearly demonstrated the contribution of lungs in the glucuronidation of RES to R3G in mice. In vivo results were corroborated by in vitro studies in mouse lung fractions.

Because species dependent differences in metabolism are known, in vitro studies were also conducted in human lung fractions to see if reliable extrapolation of data can be made between mice and humans. Interestingly, no significant glucuronidation of RES was observed in human lung fractions, implying that contribution of pulmonary glucuronidation in the metabolism of RES might be quantitatively less important in humans. The species difference in RES glucuronidation at its 3-OH position can be explained by differential expression of uridine 5’-diphosphogluconosyltransferase (UGT) isoforms in mouse and human lungs. RES has been reported to be glucuronidated
at its 3-OH position via UGT1A1, UGT1A7, and UGT1A9, with minor contribution from UGT1A8, UGT1A10 and UGT2B7 (Brill et al., 2006). Ugt1a6 has been shown to be expressed well in mouse lung (Buckley and Klaassen, 2007), and might be responsible for RES glucuronidation in mouse lungs. There are conflicting reports about the presence of UGT enzymes in human lung. UGT1A1 and UGT1A10 have been reported in lung cancer samples (Oguri et al., 2004). Several UGT2B isozymes are reportedly present in human lungs (Turgeon et al., 2001). UGT expression as well as activity has been reported in the upper respiratory tract but not in lungs in humans (Zheng et al., 2002), which becomes important for inhaled compounds. Other studies have shown low or no UGT expression in normal human lung tissue (Zheng et al., 2002; Somers et al., 2007; Nakamura et al., 2008). In the present study, the absence of R3G formation in human lung fraction is consistent with low or absent expression of UGT isoforms in normal human lung tissue (Nakamura et al., 2008) which are responsible for RES glucuronidation (Brill et al., 2006).

Sulfation experiments showed that RES is sulfated by both mouse and human lungs. RES sulfation at its 3-OH position has been reported via sulfotransferase (SULT) 1A1, SULT1A2, SULT1A3 and SULT1E1 isoforms (Miksits et al., 2005). Mouse lungs express sult1a1, with very low expression of sult1e1 (Alnouti and Klaassen, 2006). SULT1A1, SULT1A3, SULT1E1, SULT2A1 and SULT1B1 have been found to be expressed in human lungs, and SULT1A1, SULT1A3 and SULT1E1 account for around 80 percent of all SULTs expressed in human lungs (Riches et al., 2009). Therefore, sult1a1 and sult1e1 in mouse lungs and SULT1A1, SULT1A3 and SULT1E1 in human
lungs might be responsible for R3S formation. Steroid sulfatase activity has been reported in human (Milewich et al., 1983) and mouse lung tissue (Milewich et al., 1984), with highest activity in microsomal fraction of human lung tissue homogenate (Milewich et al., 1983). Steroid sulfatase can desulfate R3S to give RES locally in the lung cells. This futile cycling of RES/R3S by the combined activity of sulfatase and sulfotransferase enzyme can lead to increase in the retention of the RES/R3S within the lung. This can be important since RES and R3S both have been shown to have pharmacological activity in vitro (Hoshino et al., 2010).

Transporters in conjunction with metabolizing enzymes play an important role in the disposition of drugs and metabolites. R3G and R3S disposition is known to be influenced by transporters. R3G has been shown to be a high affinity substrate for multidrug transporter protein (MRP) 2 (ABCC2), MRP3 (ABCC3) and breast cancer resistance protein (BCRP; ABCG2) transporters (Maier-Salamon et al., 2008; van de Wetering et al., 2009; Juan et al., 2010a). Although the role of MRP1 (ABCC1) specifically in R3G transport has not been evaluated, there are reports of MRP1-mediated transport of glucuronides such as 17β-estradiol-glucuronide (Jedlitschky et al., 1996), etoposide glucuronides, SN-38 glucuronide (Deeley and Cole, 2006) and β-O-glucuronide conjugate of the tobacco specific carcinogen 4-(methyl-nitrosamo)-1-(3-pyridyl)-1-butanol (NNAL) (Leslie et al., 2001). BCRP and MRP2 are involved in the transport of R3S (van de Wetering et al., 2009; Juan et al., 2010a). MRP4 (ABCC4) and MRP1 (ABCC1) have not been studied for R3S transport but both MRP4 and MRP1 are reportedly involved in the transport of sulfo-conjugates such as dehydroepiandrosterone.
sulfate (Zelcer et al., 2003) and estrone 3-sulfate (Qian et al., 2001). Mouse lungs have been shown to express Mrp1, Mrp3 and Mrp4 transporters (Maher et al., 2005). It is interesting that mouse lungs did not express Mrp2 and Bcrp transporter (Scheffer et al., 2002; Maher et al., 2005).

Cellular distribution and localization of transporters in mouse lungs is unknown, although cellular distribution and localization of MRP1, MRP2 and BCRP in human lungs are reported. MRP1 is expressed on basolateral membrane whereas MRP2 and BCRP are expressed towards apical membrane in human lungs (Bosquillon, 2010). Although cellular localization of MRP3 and MRP4 transporters is unknown in lungs, they are expressed on the basolateral side in hepatocytes (Zamek-Gliszczynski et al., 2006).

Based on present results and previous reports (Maher et al., 2005; Bosquillon, 2010), a simplified scheme for disposition of RES, R3S and R3G in mouse lung cells is proposed (Figure 4A). RES when administered i.v., can diffuse into mouse lung cells and can either get metabolized by sult enzymes present in the cytosol or ugt enzymes present in the endoplasmic reticulum. Additionally, R3S formed from RES can get further desulfated by sulfatase enzymes in the endoplasmic reticulum to give RES, which can further get glucuronidated to R3G. This R3G can diffuse into the cytoplasm and be transported into the blood by Mrp3 and possibly Mrp1. This correlates well with our observation that even after sulfation of RES in lungs (based on our in vitro results) we did not observe any significant difference in the plasma exposure of R3S, when RES was administered by i.v. route compared to i.a. route. Similarly, a schematic pathway for disposition of RES in
human lungs has been proposed (Figure 4B). RES when presented to human lung cells gets sulfated to R3S in the cytoplasm. R3S formed can be eliminated in the pulmonary lumen by MRP2 and BCRP or into blood by MRPI and MRP4. Additionally it can be desulfated by steroid sulfatase in endoplasmic reticulum to give back RES. Since no activity of UGT was observed in human lung tissue, RES formed in endoplasmic reticulum can act as a depot, or can diffuse back into cytoplasm to again get sulfated to R3S. This futile cycling of RES/R3S can prolong the presence of RES/R3S in human lung tissue.

The pulmonary metabolism of RES can have implications for local tissue levels as well as systemic concentrations of parent and metabolites. The absence of pulmonary glucuronidation in human lung as compared to extensive pulmonary glucuronidation in mouse lungs is a possible reason for the observed difference in pharmacokinetics of RES in rodents versus humans. In rodents R3G has been observed as the quantitatively major metabolite (Juan et al., 2010b; Colom et al., 2011; Iwuchukwu et al., 2012; Sharan et al., 2012) as compared to R3S in humans (Boocock et al., 2007; Brown et al., 2010).

Our results might have implications in the role of RES as a cancer chemopreventive in the lung. As a substrate for SULTs, RES might interfere with the metabolism of cigarette smoke toxicants. For example, sulfation of benzo[a]pyrene-7,8-catechol has been suggested to be a detoxification pathway for benzo[a]pyrene-7,8-dione by limiting its ability to undergo redox cycling (Zhang et al., 2012a). Also, increased RES and/or R3S in the lung can lead to enhanced quenching of unstable free radicals and can lead to reduced
DNA damage by reactive oxygen species (ROS). ROS are known to be produced by cigarette smoke toxicants. Interestingly, RES and R3S have been shown to have comparable ability to quench the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical (Hoshino et al., 2010).

In summary, significantly higher R3G exposure was observed in mouse when RES was administered by i.v. route compared to i.a. route. Extensive glucuronidation and sulfation of RES was observed in mouse lung fractions. Human lung fractions on the other hand showed only sulfation of RES and a potential for futile cycling of RES/R3S in lung, which can have pharmacological significance. In conclusion, pulmonary metabolism of RES might play an important role in the pharmacokinetics and activity of RES.
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Authorship Contributions

Participated in research design: Sharan, Nagar

Conducted experiments: Sharan

Performed data analysis: Sharan, Nagar

Wrote or contributed to the writing of the manuscript: Sharan, Nagar
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References


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Footnotes

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Figure Legends

**Figure 1:** Structure of RES and its major monoconjugated metabolites R3S and R3G.

**Figure 2:** Mean plasma concentration-time profile after administration of RES i.v. 15 mg/kg. Data are represented as mean ± SD (n = 5).

**Figure 3:** In vitro kinetics of A) R3G formation in mouse lung S9 fraction, B) R3S formation in mouse lung S9 fraction, C) R3S formation in human lung S9 fraction. Data are reported as mean ± standard deviation, (n = 3). The solid line represents curve fitting with the partial substrate inhibition equation (equation 3); the inset represents Eadie-Hofstee plots.

**Figure 4:** Proposed schematic representation of RES metabolism in A) mouse lung cell, B) human lung cell. Solid arrows represent pathways based on results in this manuscript and published literature reports for metabolism and/or transport of RES, R3S and R3G. Transporters followed by “?” indicates that the role of these transporters has not been established for transport of R3S and R3G, but is hypothesized based on literature detailed in the Discussion.
Tables

Table 1. Noncompartmental pharmacokinetic analysis upon a single 15 mg/kg (i.a.) RES and 15 mg/kg (i.v.) RES dose. Data is presented as estimate ± SD.

<table>
<thead>
<tr>
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<th>RES 15 mg/kg i.a.(^a)</th>
<th>RES 15 mg/kg i.v.</th>
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<td>(n = 5)</td>
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<tr>
<td>AUC(_{0-\infty})</td>
<td>591.08 ± 167.29(^b)</td>
<td>294.98 ± 137.87(^b)</td>
<td>min(^a)µM</td>
</tr>
<tr>
<td>Cl</td>
<td>118.77 ± 33.36</td>
<td>280.04 ± 158.25</td>
<td>mL/min/kg</td>
</tr>
<tr>
<td>Vss</td>
<td>37.59 ± 23.70</td>
<td>34.90 ± 20.10</td>
<td>L/kg</td>
</tr>
<tr>
<td>t(_{1/2})</td>
<td>190.58 ± 69.65(^b)</td>
<td>101.30 ± 43.41(^b)</td>
<td>min</td>
</tr>
</tbody>
</table>

**R3G**

| AUC\(_{0-\infty}\) | 921.23 ± 457.07\(^b\) | 2268.35 ± 517.00\(^b\) | min\(^a\)µM |

**R3S**

| AUC\(_{0-\infty}\) | 174.94 ± 45.75         | 157.21 ± 77.77       | min\(^a\)µM |

\(^a\) Data are from (Sharan et al., 2012).

\(^b\) Denotes statistically significant parameters between the i.a. versus i.v. groups, with an unpaired student t test, and p < 0.05 considered significant.
Table 2. Kinetic parameter estimates for the sulfation and glucuronidation of RES and R3S by human and mouse lung fractions. Data are expressed as estimates ± SD; n = 3. Estimate units are as follows: Vmax: nanomoles per minute per milligram total protein; Km, Ki: micromolar.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conjugation</th>
<th>Protein</th>
<th>Vmax (pmol/min/mg)</th>
<th>Km (µM)</th>
<th>Ki (µM)</th>
<th>Goodness of Fit (r²)</th>
<th>Type of Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>RES</td>
<td>R3G</td>
<td>Mouse lung</td>
<td>324.40 ± 13.05</td>
<td>7.34 ± 1.60</td>
<td>6632 ± 1198</td>
<td>0.93</td>
<td>Partial substrate inhibition</td>
</tr>
<tr>
<td>RES</td>
<td>R3S</td>
<td>Mouse lung</td>
<td>13.05</td>
<td>4.45 ± 0.79</td>
<td>7305</td>
<td>0.95</td>
<td>Partial substrate inhibition</td>
</tr>
<tr>
<td>RES</td>
<td>R3S</td>
<td>Human lung</td>
<td>16.15 ± 0.48</td>
<td>717.6</td>
<td>23238 ± 7305</td>
<td>0.96</td>
<td>Partial substrate inhibition</td>
</tr>
</tbody>
</table>

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Fig 1

RES

R3G

R3S

sulfate