INHIBITION OF RESVERATROL GLUCURONIDATION BY PROBENECID: *IN VITRO* EFFECTS AND CLINICAL RELEVANCE

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Abstract

Resveratrol is a naturally occurring antioxidant that is found in high density in sources such as red wine and grapes. Numerous experimental and *in vitro* studies reveal the therapeutic potential of resveratrol in many models of human disease, particularly in cancer chemoprevention. However, net clearance of resveratrol *in vivo* is very high, mainly due to glucuronide conjugation by Uridine diphosphate glucuronsyl transferases (UGT). Resveratrol undergoes significant conjugation by UGT enzymes, yielding resveratrol 3-*O*-glucuronide (R3G) and resveratrol 4′-*O*-glucuronide (R4G), which has less pharmacological activity than the parent compound. Using human liver microsomes, the *in vitro* effects of probenecid on resveratrol glucuronidation was evaluated in the present study. The results of the study show that probenecid inhibited R3G formation, with inhibition constant (*Ki*) value of 3.17 mM, and 47% inhibition at high concentration of probenecid. The data indicated a mixed competitive-noncompetitive mechanism of inhibition. The results suggest that co-administration of probenecid with resveratrol might increase concentrations of resveratrol in plasma. The relevance of the *in vitro* findings is to be tested in a randomized, two-way crossover study, which is needed to validate the potential value of probenecid as an agent to augment exposure of resveratrol in humans.

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List of Abbreviations

ADME	Absorption, distribution, metabolism and excretion
ANOVA	Analysis of variance
AUC	Area under the plasma concentration-time curve
CL	Clearance
C_{max}	Maximum plasma concentration
DDI	Drug-drug interaction
F	Bioavailability
FDA	Food and Drug Administration
HLM	Human liver microsome
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
IVIVE	*In vitro-in vivo* extrapolation
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
OAT	Organic anion transporter
R3G	Resveratrol 3-*O*-glucuronide
R4G	Resveratrol 4′-*O*-glucuronide
SD	Standard deviation
SULT	Sulfotransferases
$T_{1/2}$	Plasma elimination half-life
UDPGA	UDP-glucuronic acid
UGT	Uridine diphosphate glucuronsyl transferases
UV	Ultraviolet

Introduction

1.1 Potential Health Benefits of Resveratrol

Resveratrol (*trans*-resveratrol, 3,4′,5-trihydroxy-*trans*-stilbene; Figure 1) is a naturally occurring antioxidant that is found in high density in sources such as red wine and grapes.[1-3] A number of experimental and in vitro studies reveal that resveratrol has beneficial effects in models of lipid metabolism, glucose tolerance, cardiovascular diseases, and carcinogenesis.[1-5] The apparent beneficial effects of red wine in cardiovascular disease prevention observed in epidemiological studies is proposed to be attributable to the resveratrol content in red wine. To date extrapolation of these findings to humans is incomplete, most likely because of the complex pharmacokinetic properties of resveratrol.

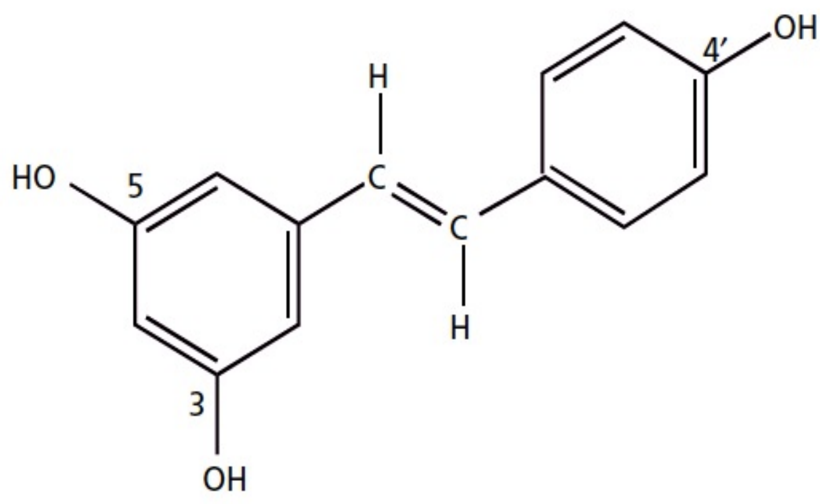

Figure 1: Chemical structure of *trans*-resveratrol (3,4′,5-trihydroxy-*trans*-stilbene)

1.2 Metabolism and Bioavailability of Resveratrol

As noted above, resveratrol has a complex metabolic pathway in human. It undergoes significant Phase II conjugation by uridine diphosphate glucuronsy ltransferases (UGT) and sulfotransferases (SULT) enzymes, to form glucuronide and sulphate conjugates.[6,7] Glucuronide conjugation by UGT enzymes occurs in the human liver and intestines, yielding resveratrol 3-*O*-glucuronide (R3G) and resveratrol 4′-*O*-glucuronide (R4G) which have less pharmacological activity than resveratrol itself.[8] S. Brill *et al.* screened different cDNA expressed UGT isoforms involved in resveratrol glucuronidation. UGT1A1, UGT1A9 and UGT1A7 were primarily involved in the formation of R3G, while R4G was formed mainly by UGT1A9, UGT1A1, UGT1A8 and UGT1A10 (Figure 2).[9]

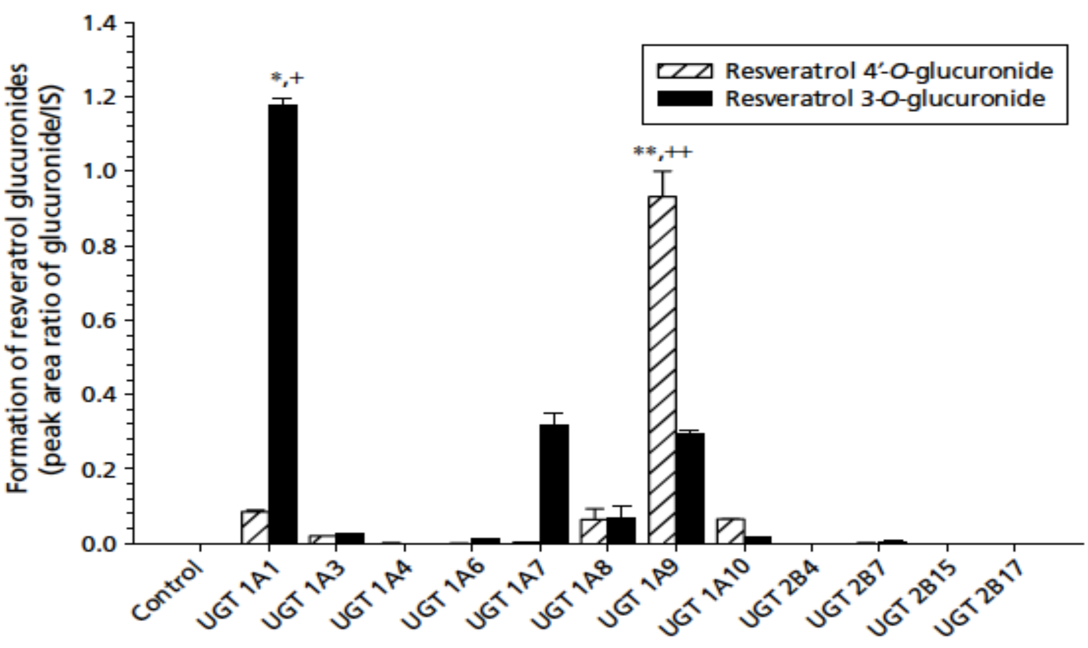

Figure 2: Glucuronidation of resveratrol by specific cDNA expressed human UGT isoforms (obtained from reference 9)

The UGT catalyzed glucuronidation of resveratrol leads to the formation of more hydrophilic compounds, which are more readily eliminated in bile and urine.[10] The high clearance and presystemic extraction of resveratrol lead to a low bioavailability. After oral administration of resveratrol to humans, even at doses of 5 grams, intact resveratrol is detectable in the systemic circulation only at very low concentrations, considerably below the exposure levels shown to have antineoplastic effects in the experimental models.[11-14] Some investigators have proposed a strategy of augmentation of resveratrol exposure via inhibition of metabolism leading to higher plasma levels.[15]

1.3 Probenecid as an Inhibitor of Glucuronidation

Probenecid has been in clinical use since the 1970s as a uricosuric agent in the treatment of gout, and as an agent to augment systemic exposure to penicillin derivatives via inhibition of renal tubular reabsorption.[16,17] Probenecid has the additional pharmacologic property of inhibiting the activity of human UGT enzymes.[18,19] In 1985 our laboratory reported that usual therapeutic doses of probenecid significantly impair clearance and elevate plasma levels of acetaminophen and lorazepam in humans (Figure 3).[20] Probenecid inhibition of clearance of acetaminophen, as well as temazepam, diflunisal, zomepirac, zidovudine, and naproxyn, has also been described by a number of research groups.[21-26] All of these drugs are substrates for biotransformation into glucuronide conjugates by UGT enzymes.

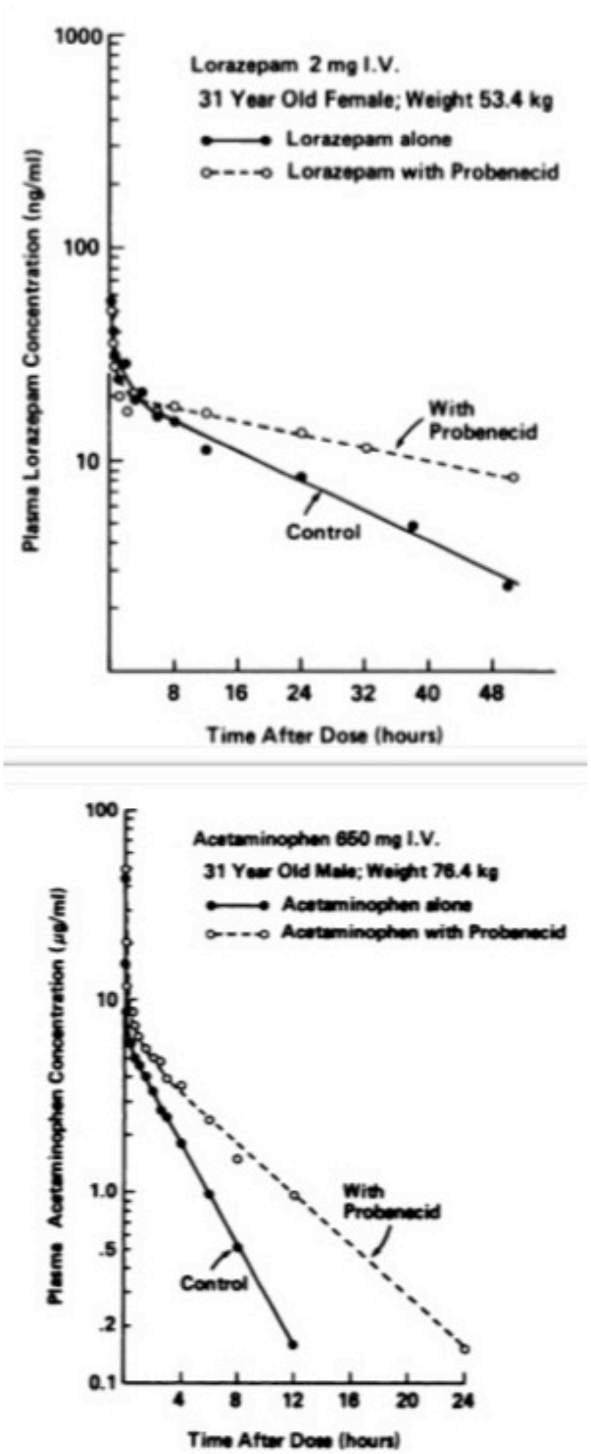

Figure 3: Plasma concentrations of lorazepam (top) and acetaminophen (bottom) in healthy voulnteers after i.v. doses in control state and during concurrent probenecid treatment (Obtained from reference 20).

The exact mechanism of action for probenecid's effect on the clearance of drugs is not well established. However, it may prevent the biotransformation of drugs to glucuronide conjugates by diminishing the activity of UGT enzymes, or it may decrease the renal tubular secretion and intestinal efflux of glucuronide metabolites followed by deconjugation back to the parent drug.[27 28]

1.4 Clinical Studies and Pharmacokinetics of Resveratrol

Following the numerous papers showing the *in vitro* therapeutic potential of resveratrol, a number of research groups have evaluated the *in vivo* pharmacokinetics (PK) of resveratrol in human subjects. *Goldberg et al.* have tested the absorptive efficiency of three different polyphenols, including resveratrol, in twelve healthy subjects.[29] The highest measured serum level of four subjects given 25 mg of resveratrol in various matrices ranged from 416 to 471 μg/L for total resveratrol (free and conjugated) in this study, whereas for free resveratrol alone, the peak measured serum concentration was at 30 minutes after consumption and ranged from 6 to 8.5 μg/L.[29] *Walle et al.* examined the bioavailability of ^{14}C-resveratrol after oral and i.v. doses in six human subjects, the highest equivalent plasma concentration mean was 491μg/L reached about one hour after an oral dose of 25mg.[13] The unmetabolized resveratrol observed in this study was less than 5 μg/L. However, *Boocock et al*. employed a different approach, running a dose escalation study ranging from 0.5 to 5 g of resveratrol in healthy volunteers.[30] The highest plasma concentration (C_{max}) across the four doses fell in a range between 73 to 539 μg/L for the parent compound, and higher values for the metabolites. Nevertheless, all theses studies suggest a very low bioavailability of resveratrol, with a low C_{max} concentration after oral doses, which are much less than the concentration tested in *in vitro* experiments. Table 1 summarizes some of the PK parameters in these clinical trials. [13,29,30]

Table 1: Summary of maximal plasma concentration (C_{max}) and area under the plasma concentration curve (*AUC*) of resveratrol after oral dosing

Dose	C_{max} µg/L (nM)		*AUC* µg.h/L	Reference
25 mg	Unchanged	8.5 (~37)	-	28
	Total	471 (~2065)	-	
25 mg	Unchanged	<5 (~22)	-	13
	Total	491 (~2150)	6240	
1 g	Unchanged	117 (~513)	544 (AUC_{inf})	29
2.5 g	Unchanged	268 (~1175)	786 (AUC_{inf})	
5 g	Unchanged	538 (~2360)	1319 (AUC_{inf})	

1.5 Research Objectives and Clinical Relevance

The objective of this dissertation is to determine the ability of probenecid to inhibit the hepatic glucuronidation of resveratrol both *in vitro* and *in vivo*. This approach will presumably reduce the pre-systemic extraction of resveratrol and allow an augmentation of the oral bioavailability. First, we evaluated the inhibitory role of probenecid in resveratrol glucuronidation, using an *in vitro* model of human liver microsomes (HLM). Subsequently, the clinical relevance of the *in vitro* findings was intended to be tested in a randomized, two-way crossover, clinical trial. Due to time constraints, the *in vivo* study has not yet been initiated. Only the study protocol and bioanalytic procedures are discussed in this thesis.

Materials and Methods

2.1 In Vitro Inhibition of Resveratrol Glucuronidation by Probenecid

2.1.1 Human Liver Microsomes

Liver microsomes were prepared from human liver tissue samples as described previously.[31,32] Human liver tissue from individual donors with no known liver disease were obtained from the International Institute for the Advancement of Medicine (Exton, PA); the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN); or the National Disease Research Interchange (Philadelphia, PA). Briefly, microsomes were prepared by ultracentrifugation; microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and were stored at -80°C until use. One set of pooled HLMs and three individual HLMs were used in this experiment.

2.1.2 Chemicals and Reagents

Trans-resveratrol, alamethacin, phenacetin (internal standard), uridine 5′-diphosphoglucuronic acid (UDPGA) and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile and phosphoric acid were purchased from Thermo Fisher Scientific (Waltham, MA).

2.1.3 *In Vitro* Inhibition Study with Probenecid

Incubation mixture – final volume of 100 μl – contained 5 mM magnesium chloride, 0.0125 mg/ml alemethacin, 0.25 mg/ml human liver microsomes and 50 mM phosphate buffer (pH=7.4). The mixture is pre-incubated for three minutes, and then added to tubes containing resveratrol. Thereafter, probenecid at varying concentration is added to the mixture and 10 mM UDPGA is added to initiate the reaction. Tubes are incubated for 20 minutes in a water bath at 37°C. After incubation for 20 minutes, reactions are stopped by the addition of 100μl of the stop solution (containing 100μM phenacetin in acetonitrile), and the samples are put on ice for three minutes. Tubes are centrifuged at 14000 rpm for five minutes, and the supernatant is transferred to HPLC autosampling vials for analysis.

First, enzyme kinetic experiments were performed using increasing concentrations of resveratrol without inhibitor, and the peaks height ratios (resveratrol-3-*O*-glucuronide (R3G) to phenacetin) were calculated. After establishing the V_{max} and K_m, 0.1 mM of resveratrol was incubated with varying concentrations of probenecid, and inhibition of metabolism was calculated by assessing the metabolite formation rate in relation to probenecid concentration.

2.1.4 HPLC Analysis of *In Vitro* Inhibition Samples

The analytes were measured using a Thermo Finnigan Surveyor HPLC. ChromQuest version 4.0 (Thermo Finnigan) was used for data acquisition and analysis. The analytic column was Nova-Pak® C18, 3.9×150 mm, from Waters Associates (Milford, MA, USA). The UV wavelength detector was set at 295 nm. The separation of analytes was achieved isocratically at a flow rate of 1.0 ml/minute. The mobile phase contained 70% deionized water and 30% methanol (containing 0.5% glacial acetic acid). The injection volume was 10 μl and the total run time was 20 minutes. The heights of the peaks were measured for the two glucuronides (R4G, R3G), internal standard (phenacetin) and resveratrol, respectively (Figure 4).

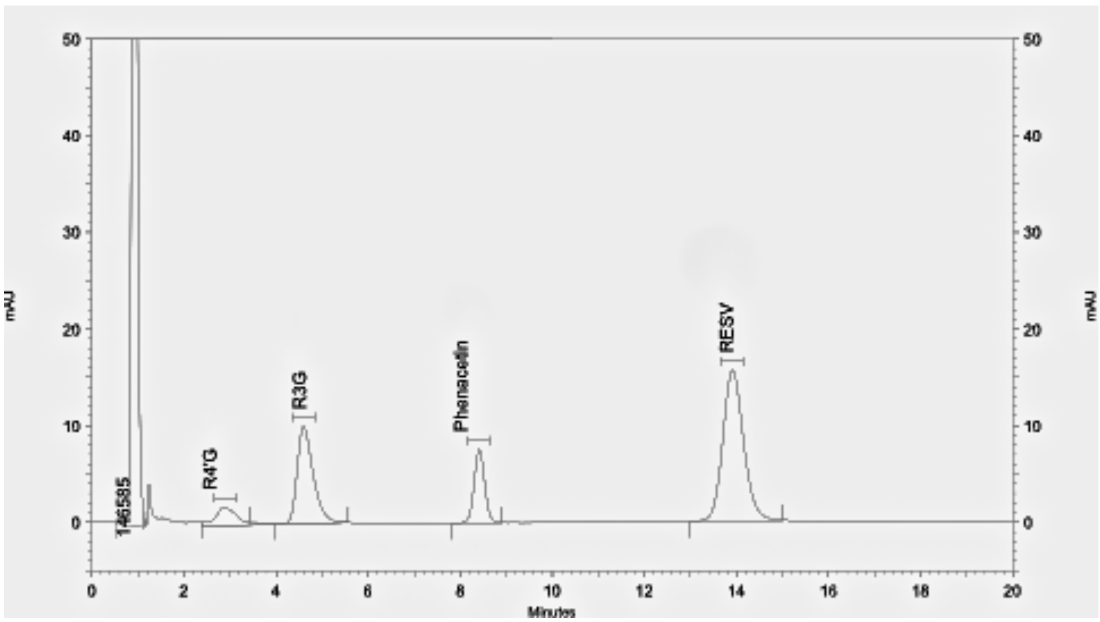

Figure 4: Representative retention peaks of HPLC separation of the two metabolites (R4G and R3G), internal standard (phenacetin) and resveratrol; peaks were eluted at 2.5, 4.5, 8 and 14 minutes, respectively.

2.1.5 Analysis of the *In Vitro* Data

2.1.5.1 Determination of Michaelis-Menten Parameters

Enzyme kinetic experiments with varying concentration of substrate were performed to determine UGT enzymes' K_m (substrate concentration that yields a half-maximal velocity) and V_{max} (maximum velocity). Nonlinear regression analysis was performed to fit a Michaelis-Menten model to data points using GraphPad Prism 6 or SigmaPlot 9. Both programs yielded identical results.

2.1.5.2 Determination of IC_{50} values for probenecid

Reaction velocities with coaddition of inhibitor were expressed as a percentage ratio versus the corresponding velocity with no inhibitor (*Rv*). The relationship of *Rv* to the inhibitor (probenecid) concentration (*I*) was analyzed by nonlinear regression using the following equation:

$$R_v = 100(1 - \frac{E_{\max} \cdot [I]^b}{[I]^b + IC^b})$$

Iterated variables were E_{max}, *IC*, and *b*. In this equation, E_{max} represents the maximum degree of inhibition, *IC* represents the inhibitor concentration producing a 50% decrease in R_v for a given E_{max} (100-E_{max}), and *b* is an exponent. The actual IC_{50} value was calculated with the following equation:

$$IC_{50} = IC / (2E_{\max} - 1)^{1/b}$$

2.1.5.3 Inhibition Constant (Ki) for Probenecid

For determination of inhibition constant for probenecid versus R3G metabolite formation, the relative metabolite formation velocities (*V*) were calculated at different concentrations of substrate ([*S*]) and inhibitor ([*I*]). Nonlinear regression analysis was performed using GraphPad Prism 6.

Metabolite formation kinetics in the inhibition study were analyzed using a mixed competitive-noncompetitive inhibition model[33] derived from the Michaelis-Menten model, as follows:

$$V = \frac{V_{\max} \cdot [S]}{K_m(1 + \frac{[I]}{K_i}) + [S](1 + \frac{[I]}{\alpha \cdot K_i})}$$

Iterated variables were: V_{max}, the maximum metabolite formation velocity; K_m, the Michaelis-Menten constant; K_i, the inhibition constant for the inhibitor; α, a factor which represents the mix of competitive and noncompetitive inhibition mechanisms.

2.2 Proposed Clinical Study Protocol

2.2.1 Specific Aims and Outcomes

To evaluate the effect of co-administration of probenecid, or matching placebo, on the kinetics of a single dose of resveratrol in a two-way crossover study of 12 healthy volunteers. The principal outcome measure is the total area under the plasma concentration curve (AUC) for resveratrol in Trial 2 compared to Trial 1. An increase in AUC due to probenecid indicates inhibition of resveratrol clearance.

2.2.2 Overall Design

Up to 18 subjects will enroll to assure 12 individuals completing the two-way crossover study. The two trials (in a random sequence) are:

Trial	Co-treatment	Index substrate administration
1	Placebo	Resveratrol, 2 grams
2	Probenecid, 500 mg (2 doses)	Resveratrol, 2 grams

The trials are separated by at least one week, the washout period between each administration of resveratrol. Within each trial, the co-treatments are administered twice, once on the afternoon (between 4 and 6 PM) of the day prior to resveratrol dosage, and again 60 minutes prior to resveratrol. Resveratrol itself is given with 8 oz. water at 8:30 AM. Ten venous blood samples are drawn during 8 hours after resveratrol administration. The schedule of each study day is as follows:

Time	Procedure
Day prior to resveratrol	
4-6 PM	Co-treatment (placebo or probenecid, 500 mg)
Day of resveratrol dosage	
7 AM	Arrive at study site; Light breakfast
7:15 AM	I.V. catheter placed in forearm vein; pre-dose blood sample
7:30 AM	Co-treatment (placebo or probenecid, 500 mg)
8:30 AM	Resveratrol, 2 grams, with 10 oz. tap water
8:30 AM - 5 PM	Blood samples (7 ml each) at 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 hours
12 Noon	Lunch provided
5 PM	Discharge from study unit after final blood sample

2.2.3 Sample Size Determination

The principal outcome measure is total AUC for resveratrol. A change in AUC of 35% (Trial 2 vs. Trial 1) is considered to be clinically important. Based on prior DDI studies, the relative standard deviation of the mean difference in AUC is estimated to be 40% of the mean difference itself. Under these conditions, a sample size of n=12 allows power of 0.80 with alpha=0.05.

2.2.4 Recruiting and Screening of Subjects

Subjects are initially recruited through advertising or word of mouth. They undergo a telephone screening interview. Potential candidates are then invited for on-site screening and evaluation. After signing informed consent, they complete a standardized questionnaire requesting demographic information, habits of alcohol and tobacco use, medical history, medication use (prescription, over-the-counter, or herbal/natural products), and allergies.

2.2.4.1 Subject Inclusion Criteria

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

- Male and female volunteers aged 18 to 55 years
- Active ambulatory adults with no history of significant medical disease and taking no prescription medications
- In good general health as evidenced by medical history and physical examination
- Laboratory results within normal range

- Women of reproductive potential must agree to use oral contraceptives or barrier method of contraception
- Ability to understand and the willingness to sign a written consent document

2.2.4.2 Subject Exclusion Criteria

An individual who meets any of the following criteria based on the screening information, laboratory data, medical history, and physical examination will be excluded from participation in this study:

- Evidence of disease involving the heart, liver, or kidneys
- Extremes of body weight
- Cigarette smoking
- Actual or suspected excessive use or abuse of alcohol or other substances
- Significant medical or psychiatric history
- Use of prescription medications, or potentially interfering over-the-counter drugs or herbal/nutrient products
- Allergy or sensitivity to any of the study medications
- Recent participation in other clinical research studies.
- Anything that would place the individual at increased risk or preclude the individual's full compliance with or completion of the study

2.2.5 Effect of Probenecid on the Pharmacokinetics of Resveratrol

The terminal log-linear phase of each resveratrol plasma concentration curve is identified visually, and the slope (beta) determined by log-linear regression analysis. This is used to calculate the elimination half-life ($T_{½}$). Area under the plasma concentration curve from time zero until the last non-zero concentration is calculated by the linear trapezoidal method, and extrapolated to infinity by addition of the final concentration divided by beta. This yields the total area under the curve from time zero to infinity (AUC). Apparent oral clearance (*CL*) is calculated as the administered dose divided by AUC. Observed maximum plasma concentration (C_{max}) and time of maximum concentration (T_{max}) are used as indicators of the rate of absorption.

For each kinetic variable for each trial, values are arrayed in tabular form, with calculation of arithmetic mean, SD, median, geometric mean, and 90% confidence interval. The principal statistical procedure will be analysis of variance (ANOVA) for repeated measures, with comparison of Trial 2 versus Trial1. Also calculated are ratios (Trial 2 divided by Trial 1) of individual values for C_{max} and AUC. The arithmetic mean, SD, geometric mean, and 90% confidence intervals are determined for these ratios.[34,35]

2.3 Determination of Resveratrol in Serum

2.3.1 Chemicals and Reagents

Trans-resveratrol and resveratrol-(4-*hydroxyphenyl*-$^{13}C_6$) (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade methanol and DI water were purchased from Thermo Fisher Scientific (Waltham, MA). Blank bovine serum was used in the method development and validation.

2.3.2 Instrumentation

The LC-MS/MS analysis was performed using an Agilent 1100 series HPLC coupled with an Applied Biosystems API3000 instrument. The mass spectrometer was equipped with a turbo electrospray ionization source operated in negative ion mode. Nova-Pak® C18, 3.9×150 mm, from Waters Associates (Milford, MA, USA) was used as an analytical column. The mobile phase A consisted of water and mobile phase B was methanol. The following gradient was employed at a constant flow rate of 0.5 mL/min:

Time (minutes)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
12	20	80
17	20	80
18	80	20
22	80	20

The following parameters were utilized for the MS analysis: curtain gas, 8 psi; nebulizer gas, 12 psi; CAD gas, 6 psi; Turbo Ion Spray (IS) voltage, −5000 V; collision exit potential (CXP), −17 V; declustering potential (DP), −36 V; collision energy (CE), -28 V; and source temperature, 300 °C. For quantification purposes, the following transitions were monitored in the Multiple Reaction Monitoring (MRM) mode, resveratrol *m/z* 227→184, internal standard m/z 233→191. Data were acquired and analyzed by Analyst software, version 1.4.2 (AB Sciex, Toronto, Canada).

2.3.3 Samples preparation and Extraction

Stock solution of resveratrol and internal standard were prepared in methanol and stored away from light at -20°C until use. A liquid-liquid extraction method was developed to extract resveratrol from serum. Various solvents were tried, but ethyl acetate had the best extraction recovery and reproducibility. In brief, 100 μl of drug-free bovine serum was added to tubes containing dried resveratrol and internal standard, followed by adding 300 μl of ethyl acetate to the serum. The samples were shaken for 15 minutes, and then followed by separation of the phases with centrifugation for 15 minutes. The organic layer was transferred to new test tubes and the solvent was evaporated to dryness. Samples were reconstituted with mobile phase and transferred to vials for analysis. Calibration curves were achieved by plotting series of concentration (5nM to 500 nM) to the peaks area and height ratio of resveratrol to IS.

Results

3.1 Kinetics of Resveratrol Glucuronidation, and Inhibition by Probenecid

First, enzyme kinetics results were utilized by incubating various concentration of resveratrol without the inhibitor. Rate of metabolites (R3G) formation in relation to resveratrol concentration were analyzed using non-linear regression (Figure 5). The experiment yielded a V_{max} (maximum velocity) of 6.3 and K_m (substrate concentration at half maximal velocity) of 0.0429.

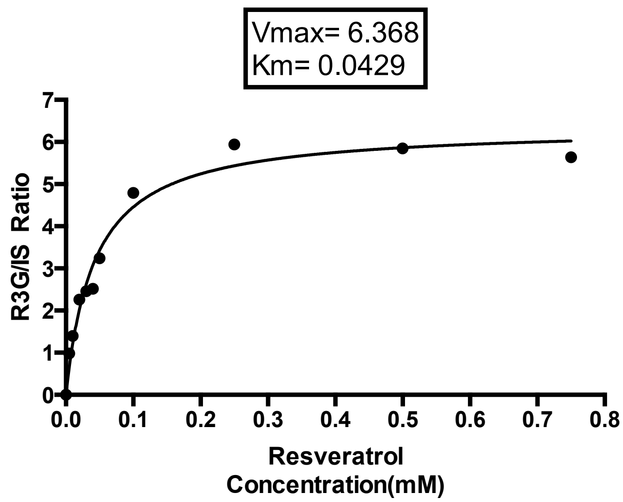

Figure 5: Rate of formation of the R3G metabolite in relation to resveratrol concentration. Solid line is the function of best fit consistent with the Michaelis-Menten model.

Time-dependent inhibition by probenecid was tested with another substrate, 2-methoxyestradiol.[36] The observed IC_{50} values were similar in samples pre-incubated with probenecid and those without probenecid pre-incubation. The results indicate that the inhibition by probenecid is reversible and not time-dependent. In the resveratrol inhibition study, the calculated IC_{50} of probenecid was 2.14 with a resveratrol concentration of 0.05 mM.

In vitro inhibition of resveratrol glucuronidation was tested first by incubating 0.1 mM of resveratrol with 0, 0.25, 1.0, 2.5, 5.0 and 7.1 mM of the inhibitor. Probenecid reduced the formation rates of both glucuronides. However, since the R3G is the major metabolite, and due to the low reproducibility of R4G data, only results of the R3G inhibition are reviewed. The study revealed that more than 46% inhibition of resveratrol glucuronidation was achieved in both 5 and 7.1 mM (Figure 6).

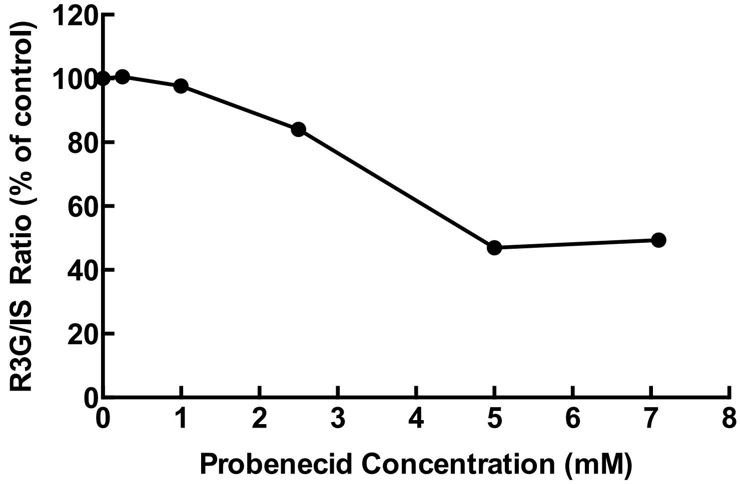

Figure 6: In vitro inhibition of resveratrol R3G metabolite formation by probenecid. Results shown are the means, n=2.

In vitro incubation of various concentrations of resveratrol (0 to 0.25 mM) and probenecid (0 to 7.1 mM) were tested to assess the inhibition of R3G metabolites formation (Figure 7). The mixed model for enzyme inhibition was used as mentioned previously. Increasing the inhibitor concentration lowered the rate of resveratrol glucuronidation. Probenecid inhibited R3G formation with kinetic parameters shown in Table 2.

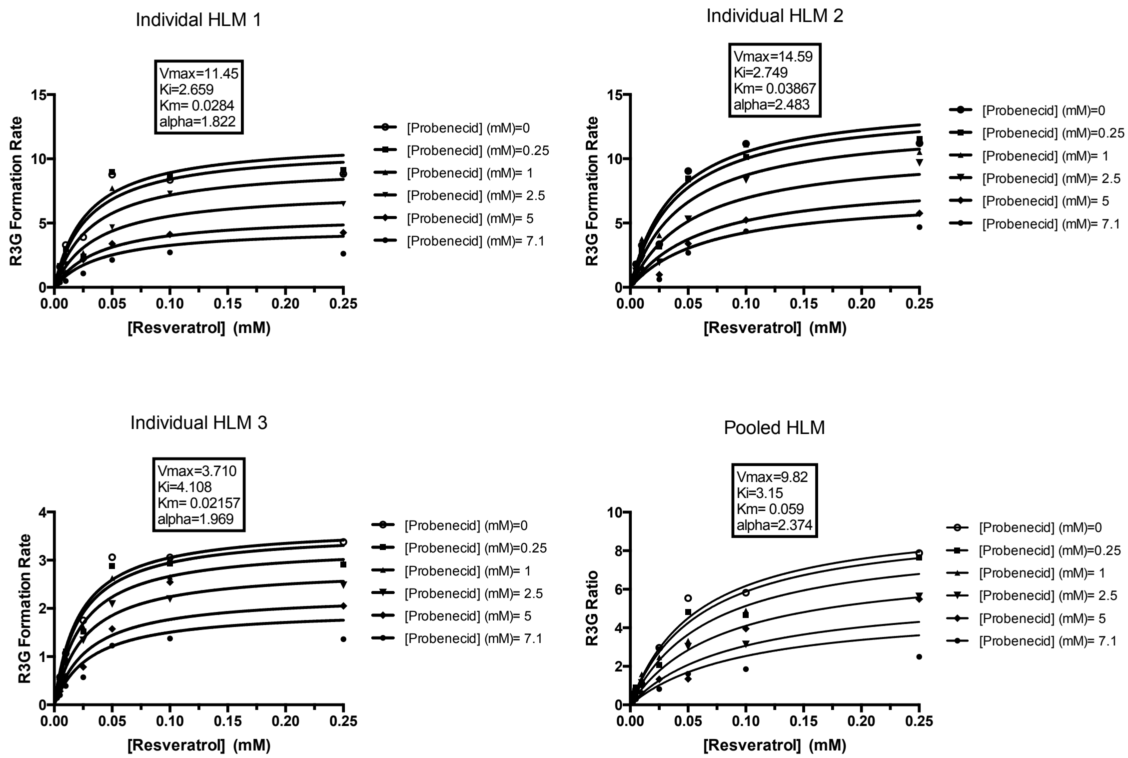

Figure 7: In vitro inhibition of resveratrol metabolite formation (R3G) by probenecid in three individual and pooled HLM.

Table 2: Enzyme kinetic parameters for R3G formation inhibition with probenecid

HLM	*Km* (mM)	*Ki* (mM)	*alpha*	*Vmax*	R^2
1	0.028	2.66	1.82	11.45	0.91
2	0.039	2.75	2.48	14.59	0.93
3	0.022	4.11	1.97	3.71	0.95
Pooled	0.059	3.15	2.37	9.82	0.94
Mean (±SD)	0.037±0.016	3.17±0.66	2.16±0.32	9.89±4.5	0.93±0.01

3.2 LC-MS/MS Analysis of Resveratrol

3.2.1 Mass Spectrometry

Product ion mass spectra was achieved in the negative ion mode for resveratrol and resveratrol-(4-*hydroxyphenyl*-$^{13}C_6$) (internal standard). The fragmentation conformed to previously published articles.[37,38] Representative MS chromatography for extracted resveratrol and IS are shown in Figure 8.

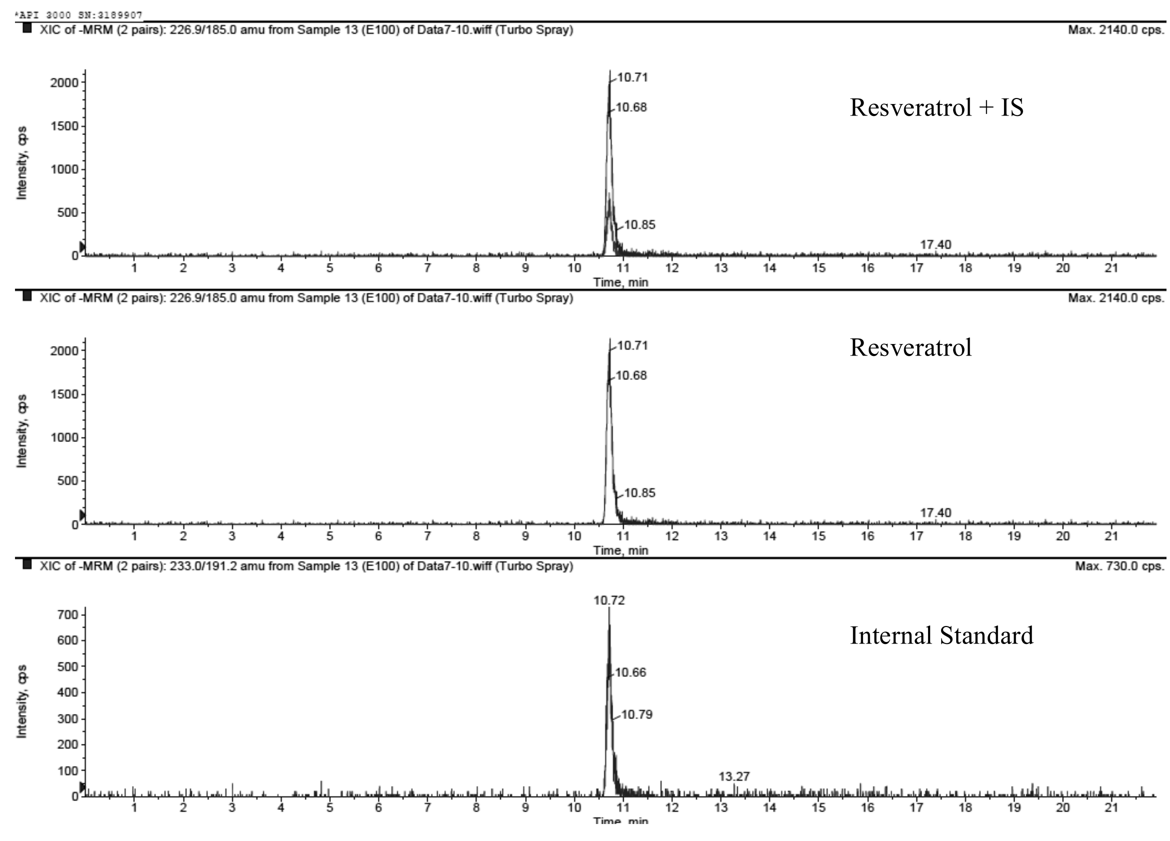

Figure 8: Representative LC-MS/MS chromatograph utilizing Multiple Reaction Monitoring (MRM) mode for resveratrol (100nM) *m/z* 227→184 and internal standard (100nM) m/z 233→191.

3.2.2 Linearity

Calibration curves were utilized by plotting concentration of resveratrol versus peak height ratio of resveratrol to IS (Figure 9). The calibration curves found linear within the range of 5 nM to 500 nM with correlation coefficients ≥ 0.99.

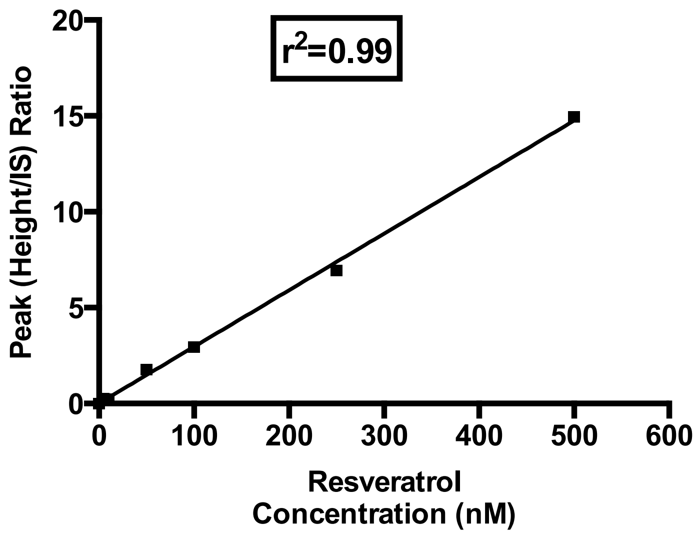

Figure 9: The calibration curve was linear within a range of 5 to 500 nM of resveratrol

3.2.3 Extraction Recovery

Recovery of resveratrol and internal standard from bovine serum was estimated by comparing the peaks height of extracted analytes with the peaks height obtained from unextracted known amount of standard solutions. Table 3 shows the extraction recovery for 100 nM of resveratrol and 200 nM of internal standard with results of 83% and 79.8%, respectively.

Table 3: Recovery of resveratrol and IS from bovine serum, n=5.

Analyte	**Concentration**	**Recovery of Extraction (%) (Mean ± SD)**
Resveratrol	100 nM (~23µg/ml)	83.03 ± 2
Internal Standard	200 nM	79.88 ± 3

3.2.4 Precision, Accuracy and Stability

Inter-day and intra-day precision and accuracy will be tested to validate the analytical method. Three different concentration of extracted resveratrol (low, medium and high) with n of 6 will be utilized to assess inter and intra-day precision and accuracy. Moreover, the bench-side stability will be evaluated by measuring the concentration of resveratrol after leaving the samples in room temperature for a period of time.

Discussion

Resveratrol has received a great deal of attention from investigators in recent years, particularly as a cancer chemopreventive agent in pre-clinical models.[1,39] However, several pharmacokinetics trials could not attain desirable plasma concentration of resveratrol, determined from *in vitro* chemoprevention studies, due to its high clearance and low bioavailability.[13,29,30,40-42] In the present study, we tested the ability of probenecid to inhibit resveratrol glucuronidation, aiming to increase its bioavailability.

Probenecid, a uricosuric agent, is capable of inhibiting the clearance of several drugs. Possible mechanisms include inhibition of hepatic glucuronidation as in lorazepam and acetaminophen study[18], reducing the efflux of resveratrol metabolites by inhibiting the intestinal multidrug resistance-associated protein (MRP2) transporters[28], or inhibiting the renal excretion of diflunisal glucuronides.[22]

The *in vitro* inhibition study showed considerable inhibition of R3G formation, reaching 46% at a concentration of 5 mM of probenecid. This suggests that coadministration of probenecid with resveratrol might augment concentrations of resveratrol in plasma. The data indicated a mixed competitive-noncompetitive mechanism of inhibition with a mean α of 2.16 and an *in vitro* inhibition constant Ki of 3.17 mM. According to the latest FDA guidance on drug interaction studies, *in vitro* metabolism-based interactions are evaluated using the inhibition constant (Ki) and the maximum inhibitor concentration $[I]$ encountered clinically. A drug's potential to inhibit an enzyme is considered low when I/Ki ratio is less than 0.1. This criterria is commonly adopted in ruling out the need for *in vivo* inhibition study.[43] PK studies of probenecid indicated that following single 0.5, 1.0 and 2.0 g oral doses, mean plasma peaks of 35.3, 69.6 and 148.6 μg/ml were attained, respectively.[17,44] The corresponding *I/Ki* ratios in our study are 0.06, 0.11 and 0.24, which predict the possibility of inhibiting resveratrol hepatic glucuronidation by probenecid, and are the basis for proceeding with a clinical study. It is also well-established that usual clinical doses of probenecid inhibit clearance of a number of drugs metabolized by glucuronide conjugation.[20-26]

Quantitative translation of *in vitro* metabolic data to predict in vivo DDIs is not straightforward. The case is more complex in this study because of the use of probenecid, which has an inhibitory effect on multiple UGT isoforms and organic anion transporters (OAT).[45] Translating the *in vitro* data to predict absorption, distribution, metabolism and excretion (ADME) can be achieved, but it requires detailed information about the kinetics of the inhibitor and other related factors (physical chemistry, biology, physiology and genetics). *In vitro –in vivo* extrapolation (IVIVE) of net oral bioavailability (F) depends on three components as used in the following equation: $F = fa \times F_G \times F_H$, where ($fa$) is the fraction of the dose which enters the gut wall, (F_G) is the fraction of the drug which escapes metabolism in the gut wall and enters the portal vein, and (F_H) is the fraction of the drug that enters the liver and escapes metabolism.[46] However, these three components are not easily estimated.[47,48]

Moreover, a number of experimental factors may influence the validity of the prediction, and introduce discrepancies between the *in vitro* and *in vivo Ki*.[49] One such factor is the enzyme source used in the method. Although HLMs are considered reliable as an enzyme source, hepatocytes are maybe more preferable for predictive models, due to lower extrapolation bias of the *in vivo* intrinsic clearance (CL_{int}), and because hepatocytes express uptake and efflux transporters.[50] The latter is a relevant aspect of this study since both probenecid and resveratrol may be substrates for transporters. Other potential sources of bias include the dependence of UGT activities on pH, buffer, and organic solvent; the nonspecific binding of substrate and inhibitor; and the inhibitory effect of endogenous fatty acids.

In summary, these *in vitro* results indicate a significant inhibition of resveratrol glucuronidation by probenecid. Precise prediction of the *in vivo* effect is challenging. Therefore, a clinical study is needed to validate the potential value of probenecid as an agent to augment exposure of resveratrol in humans.

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