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Bioactivity and metabolism of *trans*-resveratrol orally administered to Wistar rats

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The purpose of this study was to investigate the implications of selected chemopreventive parameters and metabolic conversion of resveratrol *in vivo*. In two 8-week long feeding experiments with rats, a low-resveratrol diet containing 50 mg resveratrol per kg body weight (bw) and day and a high-resveratrol diet with 300 mg per kg bw and day were administered. For chemopreventive evaluation selected phase I and phase II enzymes of the biotransformation system, the total antioxidant activity, and the vitamin E status of the animals were determined. The level of resveratrol and its metabolites in the feces, urine, plasma, liver, and kidneys was identified and quantitated by high-performance liquid chromatography-diode array detection (HPLC-DAD) using synthesized resveratrol conjugate standards. Feeding of different dosages of resveratrol revealed no effect on the different chemopreventive parameters, except for the total antioxidant activity, which was elevated in plasma by 19% after feeding 50 mg resveratrol per kg bw and day. The formation of *trans*-resveratrol-3-sulfate, *trans*-resveratrol-4′-sulfate, *trans*-resveratrol-3,5-disulfate, *trans*-resveratrol-3,4′-disulfate, *trans*-resveratrol-3,4′,5-trisulfate, *trans*-resveratrol-3-*O*-β-D-glucuronide, and resveratrol aglycone was detected by HPLC analysis, depending on the biological material. Total resveratrol recovery in urine and feces of rats fed on 50 mg resveratrol per kg bw and day was 15% and 13%, respectively. For rats fed the higher dosage of 300 mg resveratrol per kg bw and day recovery was 54% and 17%, respectively. This is the first study performed with synthesized standards of relevant resveratrol conjugates. The lack of effect on the chemopreventive parameters is probably due to the formation of various resveratrol conjugates reducing its bioavailability in the rat.

Keywords: Chemoprevention / Metabolism / Resveratrol / Resveratrol glucuronide / Resveratrol sulfate

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1 Introduction

trans-Resveratrol (Fig. 1) is a naturally occurring phytoalexin produced by some spermatophytes in response to fungal attack, injury, or stress. The presence of *trans*-resveratrol (resveratrol) in plants relevant for human nutrition is limited. In western diets, the major sources of resveratrol include grapes, wine, peanuts, and peanut products. Analysis of red wine originating from diverse countries show large variations in ranges of concentrations up to 6.8 mg/L. Additionally, considerable amounts of piceid, one of the glycosidic forms of resveratrol, occur in red wine in varying amounts up to 50.8 mg/L [1–3]. Several *in vitro* studies describe various biological impacts of resveratrol, such as antioxidative, anti-inflammatory and estrogenic effects, as well as anticancer and chemopreventive activities [4–6]. The mode of functioning of resveratrol as a chemopreventive agent has also been evaluated in different *in vivo* studies and is described in depth in the literature and summarized in several reviews [7–9]. In doing so, Gescher and Steward [7] tried to relate results of *in vitro* experiments to those of *in vivo* studies, concluding that resveratrol elicits *in vivo* chemopreventive efficacy at very low doses (0.2–2 mg/kg). In *in vitro* experiments, concentrations of >5–100 μM are required to receive corresponding activity. Furthermore, the bioavailability of orally administered resveratrol *in vivo* is likely to be insufficient to furnish agent levels compatible with those which modulate carcinogenesis *in vitro* [7]. Up to now, only a few studies give insights into the bioavailability and metabolism of resveratrol *in vivo*, compared to the

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Abbreviations: bw, body weight; **CCR**, cytochrome *c* reductase; **CDNB**, 1-chloro-2,4-dinitrobenzene; **DAD**, diode array detection; **TE**, Trolox equivalents

numerous publications on the biological impacts of this polyphenol.

Initially, isolated rat small intestine perfusion models (jejunum and ileum) were used to study the absorption and metabolism of resveratrol. Kuhnle *et al.* [10] and Andlauer *et al.* [11] suggested that resveratrol is most likely to be in the form of a glucuronide conjugate after absorption by the small intestine. Smaller amounts of resveratrol sulfate were found on the luminal and vascular side of perfused rat small intestine while only minute amounts of resveratrol and its conjugates were found in the intestinal tissue [10, 11]. Marier *et al.* [12] showed that enterohepatic recirculation contributes significantly to the exposure of resveratrol and resveratrol glucuronide in rats. After oral or intraperitoneal administration of resveratrol to rats and mice, abundant resveratrol glucuronide and resveratrol sulfate were identified in rat urine and mouse serum by Yu *et al.* [13]. Virtually no unconjugated resveratrol was detected in these samples [13]. However, intact [^{14}C]resveratrol together with glucurono- and/or sulfoconjugates was found in the liver and kidneys of mice following oral administration of [^{14}C]resveratrol [14].

The absorption of resveratrol in humans was first examined by Goldberg *et al.* [15]. The authors tested the absorption efficiency of resveratrol when given orally to healthy human subjects in three different matrices: white wine, grape juices, and vegetable juice. Resveratrol was present in serum and urine predominantly as glucuronide and sulfate conjugates [15]. These findings were confirmed by studies of Meng *et al.* [16], in which bioavailability of resveratrol in humans after oral ingestion of grape juice preparations or the pure aglycone were examined. The study demonstrated that the glycosidic form of resveratrol in grape juice is absorbed to a lesser extent than the aglycone [16]. Beyond that, Walle *et al.* [17] administered [^{14}C]-resveratrol to six human volunteers. Only trace amounts of resveratrol aglycone could be detected in plasma. Most of the radioactivity after the oral dose was recovered in urine [17]. The results of these *in vivo* studies show that resveratrol is glucuronidated and sulfated in intestinal cells and enters the blood circulation predominantly in its converted forms. Further transport in the organism occurs mainly as resveratrol glucuronides and resveratrol sulfates [10 – 17]. The determination of the resveratrol glucuronides and sulfates in the presented literature was performed predominantly by enzymatic hydrolysis using β-glucuronidase or aryl-sulfatase. No resveratrol glucuronide or sulfate standard substances are currently commercially available. The synthesis of resveratrol glucuronides *via* a Heck reaction was described by Learmonth [18].

The purpose of this study was to simultaneously investigate the implications of selected chemopreventive parameters and metabolic conversion of resveratrol *in vivo*. In two 8-week long feeding experiments with rats, a low-resveratrol diet containing 50 mg resveratrol per kg body weight (bw) and day, and a high-resveratrol diet with 300 mg per kg bw and day were administered. For chemopreventive evaluation, selected enzymes of phase I and phase II of the biotransformation system were investigated. Additionally, the total antioxidant activity and the vitamin E status of the animals were determined. The level of resveratrol and its metabolites in the feces, urine, plasma, liver, and kidneys were analyzed by HPLC-diode array detection (DAD) using synthesized resveratrol conjugate standard substances.

2 Materials and methods

2.1 Chemicals

Reagents were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). Deuterated solvents were obtained from Eurisotop (Gif-sur-Yvette, France). Reagents used for protein isolation were acquired from Bio-Rad (Munich, Germany). *trans*-Resveratrol was purchased from Bio De Tek (Bensheim, Germany).

2.2 Animal feeding experiments

Male Wistar rats (Unilever HsdCpb:WU) were obtained from Harlan-Winkelmann (Borchen, Germany). Adult animals weighing 330 ± 12 g were individually housed in Macrolon cages. The temperature was maintained at 21 – 22°C and the humidity at 53 – 56%. A 12-h dark/light cycle was kept with artificial lighting. The animals were fed daily at 10 am with an average intake of 18 ± 1.1 g of the diet *per capita*. The animals were allowed free access to drinking water. Two experiments (EX-I and EX-II) were performed, comprising two randomized groups of ten rats each. The first group received a control diet without resveratrol, the second group received either a resveratrol dosage of 50 mg per kg bw and day (resveratrol-50) in the first experiment or of 300 mg per kg bw and day (resveratrol-300) in the second experiment (diets are detailed in Table 1). In both studies, the commercial diet C1056 low-fat (Altromin, Lage, Germany) served as stock diet. Each feeding experiment lasted 8 weeks. For collection of urine and feces samples, animals were housed in metabolic cages in the 3rd and in the 7th week for a period of 10 days, respectively. Urine and feces samples of both periods were pooled and stored at –40°C until further analysis. After the 8th week rats were fasted for 24 h. Afterwards, animals were anesthetized with a CO_2/air mixture and decapitated. Blood was collected and immediately separated by centrifugation into plasma and erythrocytes. The abdominal cavity was opened, liver and kidneys were removed rapidly, weighed, and washed in ice-cold 0.9%

Table 1. Composition of experimental diets

Experiment	Group	*n*	Resveratrol	Altromin diet[a)]	Oil	Cellulose
			($g \cdot kg^{-1}$)			
I	Control	10	–	860	120	20
	Resveratrol-50	10	0.75	849	120	20
II	Control	10	–	860	120	20
	Resveratrol-300	10	4.50	856	120	20

a) Altromin C1056 low-fat

NaCl. All samples were stored at −80°C until further analysis. The experimental protocols and procedures were approved by the Animal Care and Use Committee at the University of Kiel.

2.3 Activity of selected enzymes of phase I and phase II of the biotransformation system

Prior to the analysis of the NADPH-cytochrome-*c* reductase (CCR) and the glutathione-*S*-transferase (GST) activity, the liver and kidney samples were homogenized and centrifuged as described earlier [19]. The microsomal activity of CCR in liver and kidneys was analyzed according to the method of Masters *et al.* [20]. The activity of liver and kidney cytosolic GST with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was determined as described by Habig *et al.* [21]. Protein was analyzed by the method of Lowry *et al.* [22] as modified by Markwell *et al.* [23], using bovine serum albumin as reference substance.

2.4 Protein isolation, western blot, and expression of GST isoforms α, μ and π

Cytosolic fractions of liver and kidney samples were lysed with 3 × sample buffer (New England Biolabs, Frankfurt, Germany) followed by a 5 min denaturation step at 95°C. In doing so, the protein content of the samples were adjusted to 6 μg/μL. The samples were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad) *via* electroblotting. The expression of the respective GST isoforms α, μ, and π was determined by use of anti-GST A1-1, anti-GST M1-1, and anti-GST P1-1 specific antibodies (Calbiochem, La Jolla, CA, USA), respectively. Detection was performed by the use of a horseradish peroxidase (HRP)-linked secondary antibody and a chemiluminescence-sensitive gel documentation and quantitation system (Cell Signal Technology, Beverly, MA, USA; Kodak Image Station Biostep GmbH, Jahnsdorf, Germany).

2.5 Determination of the total antioxidant activity

The antioxidant activtiy of plasma samples was determined *in vitro* by measuring their inhibitory effect on linoleic acid peroxidation closely following the procedure reported recently by Lindenmeier *et al.* [24]. An aliquot (120 μL) of the plasma samples was diluted with ethanol (30 μL) and then used for antioxidant analysis. The results were related to the absorption of a standard solution of Trolox (1 mmol/L) in water/ethanol (50 : 50 v/v) and were expressed as Trolox equivalents (TE values).

2.6 Quantitation of tocopherol equivalents in plasma and erythrocytes

Concentrations of tocopherol equivalents in plasma were determined by HPLC with UV/VIS detection according to a method reported by Jacob and Elmadfa [25]. For quantitation of tocopherol equivalents in erythrocytes the method was modified as follows. Erythrocytes were diluted (1 : 2) with water, 100 μL were mixed with 1 mL ethanol and vortexed. Tocol (300 μL) as internal standard and 6 mL *n*-hexane (containing 0.0005% butyl hydroxytoluene (BHT) per mL) were added and vortexed. The *n*-hexane phase was then removed *in vacuo*, and the residue obtained was dissolved in a mixture (90/10 v/v; 400 μL) of methanol and dichloromethane. The extraction procedure was repeated twice. α- and γ-Tocopherol were separated isocratically using methanol/dichloromethane (85/15 v/v) as the mobile phase (flow rate of 0.8 mL/min) and a SpheriGROM ODS-1.5 μm analytical column (250 mm length, 4.6 mm internal diameter, 5 μm particle size; GROM, Rottenburg-Hailfingen, Germany). Detection of α- and γ-tocopherols was performed by UV/VIS (λ = 295 nm). Both tocopherol isomers were quantified by external standard calibration. Tocopherol equivalents were calculated, taking into account that the bioactivity of γ-tocopherol *in vivo* is about 25% of that of α-tocopherol (mg α-tocopherol + mg γ-tocopherol × 0.25 = mg tocopherol equivalents). The content of tocopherol equivalents in plasma and erythrocytes were adjusted to the content of total lipids, performed with a Randox® testkit [26].

2.7 Synthesis of resveratrol conjugates

The resveratrol sulfates were synthesized following a standard procedure reported in the literature by Kawai *et al.*

[27], with one equivalent of *trans*-resveratrol reacting with two equivalents of sulfur-trioxide-pyridine complex in dry pyridine. The resveratrol glucuronides were synthesized following an adapted procedure reported by Brandolini *et al.* [28], with one equivalent of resveratrol reacting with two equivalents of acetobromo-α-D-glucuronic acid methyl ester in anhydrous methanol. The resulting resveratrol glucuronic acid methyl esters were saponified to form the target compounds [18]. The reaction mixtures containing the corresponing resveratrol conjugates in different degrees of substitution were preseparated by preparative column chromatography on RP-18 material. Final purification was conducted by automated preparative HPLC-collection. Structure determination was performed by LC-MS in ESI$^+$-mode and 1-D and 2-D nuclear magnetic resonance measurements.

2.8 Spectroscopic data

The corresponding chemical structures are displayed in Fig. 1; the assignment of the NMR-signals refers to the numbering given in Fig. 1. Monosodium-*trans*-resveratrol-3-sulfate (**1**); yield 17%: UV/VIS: λ_{max} 291 nm (pH 8.2); LC-MS (ESI$^+$): *m/z* 353 (100, [M+Na]$^+$); ^{1}H NMR (400 MHz; MeOD-d$_4$, COSY): δ 6.66 [t, 1H, *J* = 2.2 Hz, CH, C(2)], 6.74 [m, 1H, CH, C(4)], 6.76 [d, 2H, *J* = 8.6 Hz, 2 × CH, C(11) and C(13)], 6.86 [d, 1H, *J* = 16.2 Hz, CH, C(7)], 6.97 [m, 1H, CH, C(6)], 7.03 [d, 1H, *J* = 16.2 Hz, CH, C(8)], 7.37 [d, 2H, *J* = 8.6 Hz, 2 × CH, C(10) and C(14)]; ^{13}C NMR (90 MHz; MeOD-d$_6$; HMBC, HMQC, DEPT-135): δ 107.6 [CH, C(2)], 109.6 [CH, C(4)], 110.4 [CH, C(6)], 115.6 [2 × CH, C(11) and C(13)], 125.6 [CH, C(7)], 128.0 [2 × CH, C(10) and C(14)], 128.5 [CH, C(8)], 129.2 [C, C(9)], 140.2 [C, C(5)], 154.2 [C, C(1)], 157.5 [C, C(12)], 158.4 [C, C(3)]. Monosodium-*trans*-resveratrol-4′-sulfate (**2**); yield 9%: UV/VIS: λ_{max} 291 nm (pH 8.2); LC-MS (ESI$^+$): *m/z* 353 (100, [M+Na]$^+$); ^{1}H NMR (400 MHz; MeOD-d$_4$, COSY): δ 6.18 [t, 1H, *J* = 2.2 Hz, CH, C(2)], 6.48 [d, 2H, *J* = 2.2 Hz, 2 × CH, C(4) and C(6)], 6.93 [d, 1H, *J* = 16.3 Hz, CH, C(7)], 7.02 [d, 1H, *J* = 16.3 Hz, CH, C(8)], 7.28 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(11) and C(13)], 7.48 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(10) and C(14); ^{13}C NMR (90 MHz; MeOD-d$_6$; HMBC, HMQC, DEPT-135): δ 102.3 [CH, C(2)], 105.2 [2 × CH, C(4) and C(6)], 121.6 [2 × CH, C(11) and C(13)], 127.4 [2 × CH, C(10) and C(14)], 127.9 [CH, C(8)], 128.6 [CH, C(7)], 134.5 [C, C(9)], 139.9 [C, C(5)], 152.5 [C, C(12)], 158.9 [C, C(1) and C(3)]. Disodium-*trans*-resveratrol-3,4′-disulfate (**3**); yield: 23%: UV/VIS: λ_{max} 297 nm (pH 8.2); LC-MS (ESI$^+$): *m/z* 455 (100, [M+Na]$^+$); ^{1}H NMR (400 MHz; MeOD-d$_4$, COSY): δ 6.70 [t, 1H, *J* = 2.2 Hz, CH, C(2)], 6.81 [m, 1H, CH, C(4)], 7.00 [d, 1H, *J* = 16.2 Hz, CH, C(7)], 7.01 [m, 1H, CH, C(6)], 7.10 [d, 1H, *J* = 16.2 Hz, CH, C(8)], 7.28 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(11) and C(13)], 7.51 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(10) and C(14)]; ^{13}C NMR (90 MHz; MeOD-d$_6$; HMBC, HMQC, DEPT-135): δ 109.3 [CH, C(2)], 111.3 [CH, C(4)], 111.9 [CH, C(6)], 122.9 [2 × CH, C(11) and C(13)], 128.6 [2 × CH, C(10) and C(14)], 129.3 [CH, C(7)], 129.7 [CH, C(8)], 135.7 [C, C(9)], 140.9 [C, C(5)], 153.7 [C, C(12)], 155.3 [C, C(1)], 159.7 [C, C(3)]. Disodium-*trans*-resveratrol-3,5-disulfate (**4**); yield: 15%: UV/VIS: λ_{max} 297 nm (pH 8.2); LC-MS (ESI$^+$): *m/z* 455 (100, [M+Na]$^+$); ^{1}H NMR (400 MHz; MeOD-d$_4$, COSY): δ 6.78 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(11) and C(13)], 6.93 [d, 1H, *J* = 16.2 Hz, CH, C(7)], 7.10 [d, 1H, *J* = 16.2 Hz, CH, C(8)], 7.14 [t, 1H, *J* = 2.2 Hz, CH, C(2)], 7.29 [d, 2H, *J* = 2.2 Hz, 2 × CH, C(4) and C(6)], 7.39 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(10) and C(14)]; ^{13}C NMR (90 MHz; MeOD-d$_6$; HMBC, HMQC, DEPT-135): δ 114.6 [CH, C(2)], 116.8 [2 × CH, C(4) and C(6)], 116.9 [2 × CH, C(11) and C(13)], 125.8 [CH, C(7)], 129.4 [2 × CH, C(10) and C(14)], 130.2 [C, C(9)], 131.4 [CH, C(8)], 141.5 [C, C(5)], 154.7 [2 × C, C(1) and C(3)], 159.0 [C, C(12)]. Trisodium-*trans*-resveratrol-3,4′,5-trisulfate (**5**); yield: 24%: UV/VIS: λ_{max} 291 nm (pH 8.2); LC-MS (ESI$^+$): *m/z* 557 (100, [M+Na]$^+$); ^{1}H NMR (400 MHz; MeOD-d$_4$, COSY): δ 7.08 [d, 1H, *J* = 16.3 Hz, CH, C(7)], 7.15 [t, 1H, *J* = 2.2 Hz, CH, C(2)], 7.19 [d, 1H, *J* = 16.3 Hz, CH, C(8)], 7.29 [d, 2H, *J* = 8.7 Hz, 2 × CH, C(11) and C(13)], 7.34 [d, 2H, *J* = 2.2 Hz, 2 × CH, C(4) and C(6)], 7.54 [d, 2H, *J* = 8.8 Hz, 2 × CH, C(10) and C(14)]; ^{13}C NMR (90 MHz; MeOD-d$_6$; HMBC, HMQC, DEPT-135): δ 113.8 [CH, C(2)], 115.8 [2 × CH, C(4) and C(6)], 121.7

	Compound	R_1	R_2	R_3
Resveratrol sulfates	**1**	SUL	OH	OH
	2	OH	OH	SUL
	3	SUL	OH	SUL
	4	SUL	SUL	OH
	5	SUL	SUL	SUL
Resveratrol glucuronides	**6**	OH	OH	GLU
	7	GLU	OH	OH

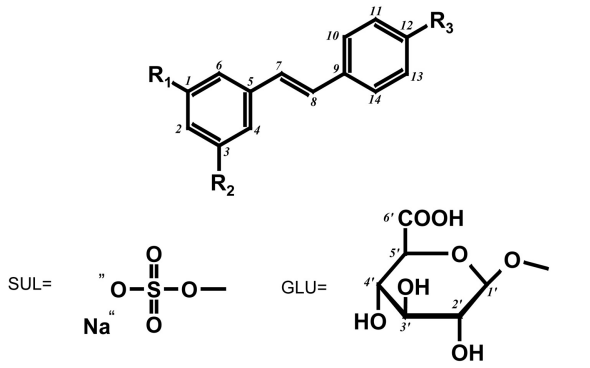

Figure 1. Chemical structures of resveratrol and different resveratrol sulfates and resveratrol glucuronides.

[2 × CH, C(11) and C(13)], 127.4 [CH, C(7)], 127.5 [2 × CH, C(10) and C(14)], 129.5 [CH, C(8)], 134.0 [C, C(9)], 139.6 [C, C(5)], 152.7 [C, C(12)], 153.8 [2 × C, C(1) and C(3)]. *trans*-resveratrol-4′-*O*-β-D-glucuronide (**6**); yield 17%: UV/VIS: λ_{max} 297 nm (pH 8.2); LC-MS (ESI^+): *m/z* 405 (100, $[M+1]^+$); ^1H NMR (400 MHz; MeOD-d_4, COSY): δ 3.53 [m, 3 H, 3 × CH, C(2′)–C(4′)], 3.78 [m, 1 H, CH, C(5′)], 4.93 [d, 1 H, *J* = 7.4 Hz, CH, C(1′)], 6.18 [t, 1 H, *J* = 2.2 Hz, CH, C(2)], 6.47 [d, 2 H, *J* = 2.2 Hz, 2 × CH, C(4) and C(6)], 6.87 [d, 1 H, *J* = 16.2 Hz, CH, C(7)], 6.99 [d, 1 H, *J* = 16.2 Hz, CH, C(8)], 7.10 [d, 2 H, *J* = 8.8 Hz, 2 × CH, C(11) and C(13)], 7.43 [d, 2 H, *J* = 8.8 Hz, 2 × CH, C(10) and C(14)]; ^{13}C NMR (90 MHz; MeOD-d_6; HMBC, HMQC, DEPT-135): δ 73.9 [CH, C(2′), C(3′) or C(4′)], 75.0 [CH, C(2′), C(3′) or C(4′)], 76.9 [CH, C(5′)], 78.1 [CH, C(2′), C(3′) or C(4′)], 102.7 [CH, C(1′)], 103.3 [CH, C(2)], 106.2 [2 × CH, C(4) and C(6)], 118.4 [2 × CH, C(11) and C(13)], 128.8 [2 × CH, C(10) and C(14)], 128.9 [CH, C(7)], 129.2 [CH, C(8)], 133.5 [C, C(9)], 141.3 [C, C(5)], 159.1 [C, C(12)], 159.9 [2 × C, C(1) and C(3)], 176.7 [COO, C(6′)]. *trans*-resveratrol-3-*O*-β-D-glucuronide (**7**); yield 16%: UV/VIS: λ_{max} 297 nm (pH 8.2); LC-MS (ESI^+): *m/z* 405 (100, $[M+1]^+$); ^1H NMR (400 MHz; MeOD-d_4, COSY): δ 3.52 [m, 3 H, 3 × CH, C(2′)–C(4′)], 3.76 [m, 1 H, CH, C(5′)], 4.90 [d, 1 H, *J* = 7.2 Hz, CH, C(1′)], 6.50 [t, 1 H, *J* = 2.2 Hz, CH, C(2)], 6.62 [m, 1 H, CH, C(4)], 6.76 [m, 3 H, 3 × CH, C(6), C(11) and C(13)], 6.84 [d, 1 H, *J* = 16.2 Hz, CH, C(7)], 7.00 [d, 1 H, *J* = 16.2 Hz, CH, C(8)], 7.43 [d, 2 H, *J* = 8.6 Hz, 2 × CH, C(10) and C(14)]; ^{13}C NMR (90 MHz; MeOD-d_6; HMBC, HMQC, DEPT-135): δ 73.7 [CH, C(2′), C(3′) or C(4′)], 74.8 [CH, C(2′), C(3′) or C(4′)], 76.7 [CH, C(5′)], 77.8 [CH, C(2′), C(3′) or C(4′)], 102.6 [CH, C(1′)], 104.3 [CH, C(2)], 107.6 [CH, C(6)], 108.3 [CH, C(4)], 116.5 [2 × CH, C(11) and C(13)], 126.8 [CH, C(7)], 128.9 [2 × CH, C(10) and C(14)], 129.9 [CH, C(8)], 130.4 [C, C(9)], 141.4 [C, C(5)], 158.5 [C, C(12)], 160.6 [C, C(1)], 175.5 [COO, C(6′)].

2.9 Chemical identification of resveratrol conjugates by HPLC

The HPLC apparatus (Jasco, Groß-Umstadt, Germany) consisted of an HPLC pump system PU 1580 with an in-line degasser (DG-1580-53), a low-pressure gradient unit (LG-1580-02), and a diode array detector (DAD) type MD 1515. Analytical separations were performed using 250 × 4.6 mm ID Phenomenex Hyperclone 5µ ODS columns (Phenomenex, Aschaffenburg, Germany) with a flow rate of 0.8 mL/min. Standard chromatography was carried out using mixtures of aqueous ammonium formate (10 mmol/L; pH 8.2) and methanol as the mobile phase. Starting with aqueous ammonium formate, the methanol content was increased to 75% within 45 min and then to 100% within 10 min. Thereafter, the column was reset to the initial conditions within 5 min. For the analytical separation of the resveratrol disulfate isomers (**3** and **4**) the same stationary phase was used with a mixture of aqueous magnesium acetate (7.5 mmo/L; the pH-value was adjusted to 5.0 with acetic acid) and methanol as the mobile phase. Starting with a mixture of aqueous magnesium acetate (90%) and methanol (10%), the methanol content was increased to 15% within 60 min and then to 100% within 5 min. Thereafter, the column was reset to the initial conditions within 5 min.

2.10 Automated HPLC purification of resveratrol conjugates

The HPLC apparatus (Jasco) consisted of an HPLC pump system PU 1580 with an in-line degasser (DG-1580-53), a low-pressure gradient unit (LG-1580-04), a UV/VIS detector type UV 1575, and a Jasco 4-port fraction collector. Preparative separations were performed using 250 × 10 mm ID Phenomenex Hyperclone 5 µ ODS columns (Phenomenex) with a flow rate of 3.0 mL/min. Liquid chromatography was carried out using mixtures of aqueous ammonium formate (10 mmol/L; pH 8.2) and methanol as the mobile phase. The resveratrol conjugates were separated and collected simultaneously using the following conditions: Sulfates: starting with a mixture of aqueous ammonium formate (90%) and methanol (10%), the methanol content was increased to 35% within 35 min and then to 90% within 5 min. Thereafter, the column was reset to the initial conditions within 5 min and equilibrated for another 15 min. Glucuronides: starting with a mixture of aqueous ammonium formate (95%) and methanol (5%), the methanol content was increased to 10% within 15 min, to 55% within 20 min and then to 90% within 5. Thereafter, the column was reset to the initial conditions within 5 min and equilibrated for another 15 min.

2.11 LC-MS of resveratrol conjugates

The analytical HPLC column was coupled to a LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) operating in positive ESI mode (ESI^+). The samples were separated using the standard HPLC gradient with aqueous ammonium formate (10 mmol/L, pH 8.2) or aqueous formic acid (0.1%, pH 2.5) and methanol as the mobile phase.

2.12 NMR spectroscopy of resveratrol conjugates

^{13}C and DEPT-135 experiments were performed on a Bruker-AV-360 spectrometer (Bruker, Bremen, Germany). ^1H, COSY, HMQC as well as HMBC spectroscopy was performed on a Bruker-AMX 400-III spectrometer (Bruker). The samples were dissolved in MeOD-d_4 and inserted into

NMR-tubes (Schott, Mainz, Germany) with tetramethylsilan (TMS) as the internal standard. The measurements were performed at room temperature (298 K). Evaluation of the experiments was carried out using 1D- and 2D-WIN-NMR as well as UX-NMR software (Bruker).

2.13 Preparation of feces, urine, plasma, liver, and kidney samples for HPLC analysis

After lyophilization, 50 mg of the pooled feces samples were extracted with 2 mL acetonitrile (30%), vortexed, and gently shaken for 24 h. The samples were then centrifuged at 4°C for 5 min at 4000 rpm (Jouan, Unterhaching, Germany). A second extraction was perfomed with the residue, to which 2 mL acetonitrile (30%) was added, vortexed, shaken for 2 h, and centrifuged at 4°C for 5 min at 4000 rpm. The residue was extracted for a third time as described. The supernatants obtained from the three extraction procedures were pooled, filtered through a PVDF membrane filter (0.45 μm), and 20 μL was used for the HPLC analysis. The pooled urine samples (1000 μL) were extracted with 400 μL acetonitrile (100%), mixed, and centrifuged at 4°C for 3 min at 10 000 rpm with a microfuge (Eppendorf, Hamburg, Germany). The supernatant was filtered through a PVDF membrane filter (0.45 μm). Samples (20 μL) of EX-I were injected undiluted onto the HPLC column, whereas samples of EX-II were diluted with bidistilled H_2O (1 : 3) before injection of 5 μL sample solution. Plasma samples (100 μL) were extracted with 250 μL acetonitrile (100%), mixed, and centrifuged at 4°C for 3 min at 10 000 rpm. The supernatant was evaporated under a nitrogen stream, redissolved in 200 μL methanol (25%), and 80 μL was used for the HPLC analysis. Minced liver and kidney samples were defrosted and 1 g was homogenized (1000 rpm) with 2 mL NaCl (0.9%). The homogenate was extracted with an equal amount of acetonitrile (100%), vortexed, gently shaken for 20 min, and centrifuged at 4°C for 3 min at 10 000 rpm. The extraction procedure was repeated for kidney samples using diluted acetonitrile (50%). The supernatant was evaporated under a nitrogen stream, redissolved in 200 μL methanol (25%), and 80 μL was injected onto the HPLC column.

2.14 HPLC analysis of resveratrol and resveratrol conjugates in rat samples

The HPLC apparatus "Elite La Chrom" (Merck Hitachi, Darmstadt, Germany) consisted of an HPLC pump system L-2130 with an in-line degasser and a low-pressure gradient unit, an HPLC autosampler type L-2200, and a DAD type L-2450. Separations were performed using 250 × 4.6 mm ID SpheriGROM 1.5 μm ODS columns (Grom) with precolumns of the same material (Phenomenex) and a flow rate of 0.8 mL/min. Standard chromatography was carried out as described above. A stock solution of 100 μg/mL resveratrol was prepared in 25% methanol. Stock solutions of resveratrol conjugates containing 100 μg/mL *trans*-resveratrol-3-*O*-β-D-monoglucuronide, monosodium-*trans*-resveratrol-3-monosulfate, monosodium-*trans*-resveratrol-4′-monosulfate, and trisodium-*trans*-resveratrol-3,4′,5-trisulfate, respectively, were prepared in bidistilled water. The stock solution of disodium-*trans*-resveratrol-3,4′-disulfate and disodium-*trans*-resveratrol-3,5-disulfate was a 60 : 40 – mixture of the two isomers, containing 100 μg/mL of total *trans*-resveratrol-disulfate (Fig. 2). Identification and characterization of resveratrol and its metabolites in feces, urine, plasma, liver, and kidney samples was performed by retention times according to retention times of standards, addition of standards, characteristic UV spectra as well as LC-MS analysis. LC-MS analysis was performed as described above. For the quantitation of resveratrol and its metabolites in feces, urine, plasma, liver, and kidney samples, standard curves of five relevant concentrations for each standard substance were performed. The curves were characterized by regression coefficients of $R^2 = 0.99$ or above. The coefficient of variation of a sixfold replicate was less than 5%. Recovery of resveratrol in feces, urine, and plasma was 110%, 94%, and 100%, respectively. The extraction efficiencies was lower in liver (32%) and kidney (34%) samples. The detection limits for resveratrol and its metabolites in feces, urine, and liver samples was less than 8 mg/g, 4 μg/mL, and 3 μg/g, respectively.

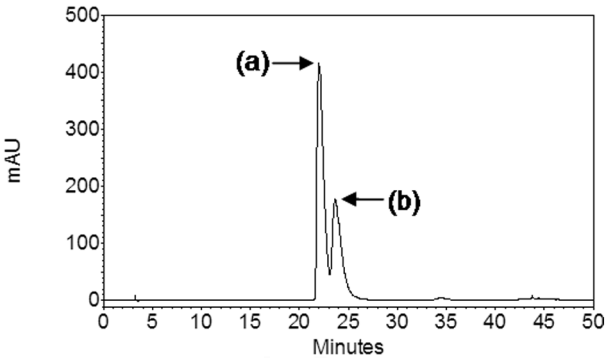

Figure 2. Representative HPLC-DAD chromatogram (λ = 300 nm) of *trans*-resveratrol disulfat standard solution (100 μg/mL) with (a) *trans*-resveratrol-3,4′-disulfate and (b) *trans*-resveratrol-3,5-disulfate.

2.15 Statistical analysis

The results are presented as mean values and standard deviations. The data presented in the paragraphs "parameters of phase I and phase II of the biotransformation system" and "parameters on antioxidative properties" are given as percent change related to the mean value analyzed

for control animals. Statistical analysis was performed using Student's *t*-test at a level of significance of $p < 0.05$.

3 Results

3.1 Animal feeding experiments

All groups had an average body weight of 330 ± 3.1 g at the beginning and 409 ± 4.8 g at the end of the feeding experiments. The increase in body weight during the 8-week-long studies is due to the relatively high fat content of the diets (12%). As the four different diet groups showed this development in body weight gain, an effect on the analyzed parameters can be excluded. Furthermore, there were no differences in the dietary intake between the different groups. The average food consumption during the experimental period was 18.6 ± 0.19 g. In both experiments, rats fed on resveratrol were drinking significantly more water compared to the control groups of EX-I and EX-II (22.8 ± 4.8 and 21.7 ± 3.8). However, there were no differences between the different resveratrol dosages with 26.6 ± 3.6 mL for the resveratrol-50 and 26.6 ± 3.8 mL for the resveratrol-300 dosage. According to the water consumption, the average daily urinary excretion of rats fed on the resveratrol diets, was slightly ($p > 0.05$) increased (11.5 ± 4.14 g resveratrol-50 and 10.0 ± 2.59 g resveratrol-300, respectively) in comparison to the control groups (7.79 ± 2.12 g EX-I and 7.46 ± 1.41 g EX-II, respectively).

3.2 Parameters of phase I and phase II of the biotransformation system

The CCR enzyme activity in liver and kidney microsomes was not affected by the administration of resveratrol, neither after the resveratrol-50 nor after the resveratrol-300 dosage (data not shown). The activity of GST, shown in Table 2, also indicated no significant differences in liver and kidney cytosol after administration of resveratrol. A slight ($p > 0.05$) increase was measured in liver and kidney cytosol of animals fed 300 mg resveratrol per kg bw and day. The data presented in Table 2 are given as percent change relative to the mean value analyzed for control animals. In EX-I the GST activity in the liver and kidney samples of control animals were 378 ± 56 and 52 ± 9.7 nmol CDNB per mg protein per minute, respectively. In EX-II the enzyme activity was 611 ± 178 and 75 ± 15.4 nmol CDNB per mg protein per minute for liver and kidney cytosol, respectively. The slight increase of GST activity could be confirmed by Western blot studies on the expression of the GST-α for both liver and kidney samples in EX-II, with 121 ± 41% and 114 ± 22% relative signal intensity of GST-α expression related to control animals (= 100%), respectively (Table 2). The administration of resveratrol showed no effect on the expression of GST-μ in either animal study.

Table 2. Relative cytosolic liver and kidney GST activity and GST-α expression in rats fed for 8 weeks with 50 or 300 mg resveratrol per kg bw and day related to control animals[a)]

	GST activity (%)		Signal intensity of GST-α expression (%)	
	Liver	Kidney	Liver	Kidney
Resveratrol-50	125 ± 25	96 ± 17	80 ± 21	79 ± 25
Resveratrol-300	136 ± 34	110 ± 23	121 ± 41	114 ± 22

a) No statistical differences *vs.* means of controls

Interestingly, the expression of the GST-π isoenzyme could not be detected in any of the analyzed samples, suggesting the occurrence of a polymorphism in this Wistar strain.

3.3 Parameters of antioxidative properties

Plasma total antioxidant activity, calculated on the basis of TE values, was significantly elevated to 119 ± 15% in animals fed the resveratrol-50 diet compared to animals of the control group (100%), shown in Fig. 3. However, this result could not be confirmed by the administration of the high resveratrol dosage in EX-II. Tocopherol equivalents in plasma and erythrocytes were unchanged in rats fed resveratrol containing diets (Fig. 3).

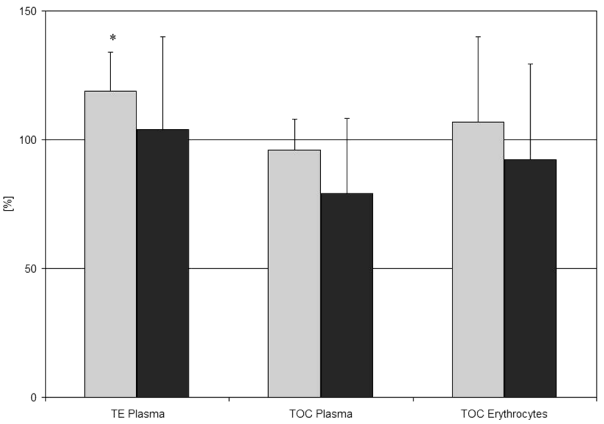

Figure 3. Relative content of TE and tocopherol equivalents adjusted to total lipids (TOC) in plasma and erythrocytes in rats fed with 50 or 300 mg resveratrol per kg body weight and day for 8 weeks related to control animals. Asterik (*) indicates that the content of TE of rats fed with resveratrol was significantly different from that of control animals ($p < 0.05$). Left columns, resveratrol-50; right columns, resveratrol-300.

3.4 HPLC analysis of resveratrol and resveratrol conjugates in rat samples

Figure 4 shows representative chromatograms of the HPLC-DAD analysis of different urine extracts from rats

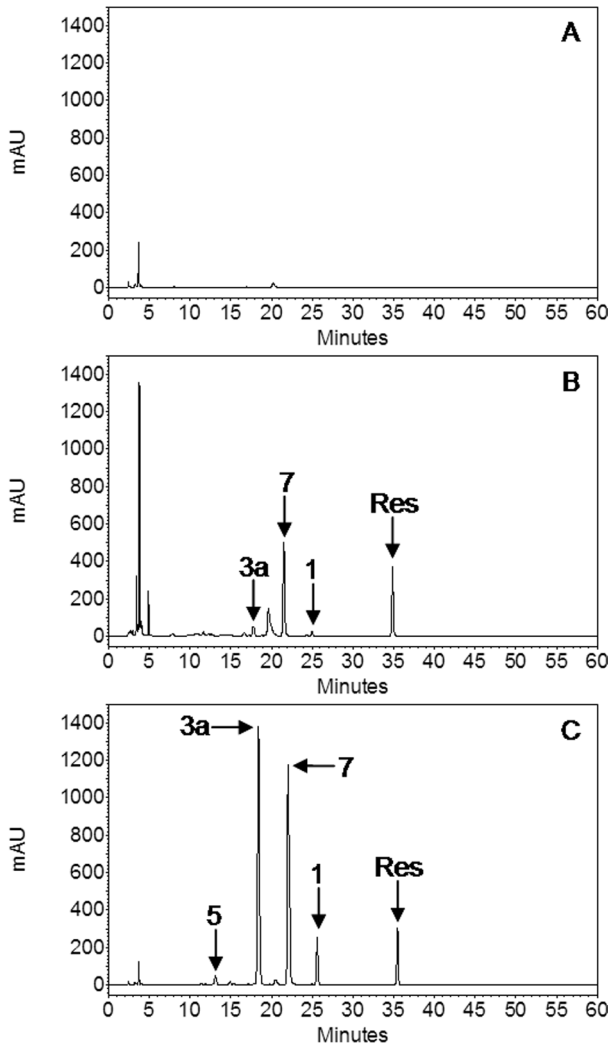

Figure 4. Representative HPLC-DAD chromatograms (λ = 300 nm) of urine extracts from rats fed for 8 weeks (A) a resveratrol free control diet, (B) a diet containing 50 mg or (C) 300 mg resveratrol per kg body weight and day. **1**, *trans*-resveratrol-3-sulfate; **3a**, *trans*-resveratrol-disulfate (the stereoisomers *trans*-resveratrol-3,5-disulfate and *trans*-resveratrol-3,4′-disulfate were coeluting, using the standard HPLC-DAD procedure); **5**, *trans*-resveratrol-3,4′,5-trisulfate; **7**, *trans*-resveratrol-3-*O*-β-D-glucuronide, and Res (resveratrol).

fed a control diet (A), the resveratrol-50 (B), or the resveratrol-300 (C) diet. Chromatogram (A) shows neither resveratrol nor resveratrol conjugates. The administration of 50 mg resveratrol per kg bw and day resulted in the formation of *trans*-resveratrol-3-sulfate (**1**), *trans*-resveratrol-disulfat (**3a**), *trans*-resveratrol-3-*O*-β-D-glucuronide (**7**), and resveratrol (Res). Furthermore, a sixfold increase of the resveratrol dosage resulted in the additional formation of *trans*-resveratrol-3,4′,5-trisulfate (**5**). With the standard HPLC procedure, the two stereoisomers *trans*-resveratrol-3,5-disulfate and *trans*-resveratrol-3,4′-disulfate were co-eluting, and were designated as *trans*-resveratrol disulfate. For separation, HPLC conditions were modified as described in Section 2 and Fig. 2. The content of resveratrol conjugates detected in urine samples are displayed in Table 3. In both experiments, *trans*-resveratrol-3-*O*-β-D-glucuronide was the main metabolite of resveratrol with 0.19 ± 0.09 mg and 3.46 ± 1.29 mg/mL, respectively. After feeding resveratrol-50, the amount of *trans*-resveratrol-3,5-disulfate and *trans*-resveratrol-3-sulfate was approximately 100- and 50-fold less, in relation to the 300 mg dosage, respectively. In EX-I 5% of the dose administered could be detected as resveratrol, whereas, after feeding the high resveratrol dosage, only 4% of the aglycone could be detected in the urine. Total resveratrol recovery in urine of rats fed on 50 and 300 mg resveratrol per kg bw and day was 15% and 54%, respectively. In feces samples (Table 3) of rats of the EX-I resveratrol group only *trans*-resveratrol-3-sulfate and resveratrol could be determined. The administration of 300 mg resveratrol per kg bw and day resulted in the formation of both resveratrol monosulfates, both resveratrol disulfates, and *trans*-resveratrol in which *trans*-resveratrol-3-sulfate and *trans*-resveratrol-4′-sulfate were the major conjugates with 5.68% and 3.83% of the dose administered. Total resveratrol recovery in feces was 15% and 31% for EX-I and Ex-II, respectively.

After feeding of 50 mg resveratrol per kg bw and day, neither resveratrol nor resveratrol conjugates could be detected in plasma, liver, and kidney samples. In the second animal study, the resveratrol-300 dosage resulted in the formation of several resveratrol conjugates, as shown in Table 4, but interestingly these resveratrol conjugates could only be determined in 50% of the animals. In the samples of the remaining 50% neither resveratrol aglycone nor any resveratrol conjugate could be detected. Chromatograms of plasma samples showed no resveratrol aglycone (Table 4). By far, the main metabolite in these samples was *trans*-resveratrol-3,4′-disulfate (7.46 ± 2.34 μg/mL). Followed by *trans*-resveratrol-3,4′,5-trisulfate and *trans*-resveratrol-3-*O*-β-D-glucuronide with 3.28 ± 1.22 μg/mL and 3.13 ± 0.88 μg/mL, respectively. *Trans*-resveratrol-3,5-disulfate and *trans*-resveratrol-3-sulfate could also be detected in plasma. In liver *trans*-resveratrol-4′-sulfate could be analyzed predominantly with 1.77 ± 0.65 μg/g. Otherwise, this monosulfate could only be detected in feces samples. Furthermore, *trans*-resveratrol-3-sulfate and *trans*-resveratrol-3-*O*-β-D-glucuronide were generated by the liver with similar amounts of 0.59 ± 0.24 μg/g and 0.64 ± 0.24 μg/g, respectively. In contrast to plasma and kidneys, free resveratrol could also be determined in the liver samples (Table 4). The main metabolite in kidneys was *trans*-resveratrol-3-*O*-β-D-glucuronide with 2.71 ± 0.60 μg/g. Unfortunately, the total amount of kidney samples was insufficient, therefore it was impossible to separate the *trans*-resveratrol disulfate in the appropriate HPLC procedure. As a minor metabolite, *trans*-

Table 3. Resveratrol metabolites in urine and feces of rats fed for 8 weeks different dosages of 50 mg and 300 mg resveratrol per kg bw and day

	Urine		Feces	
	Resveratrol-50	Resveratrol-300	Resveratrol-50	Resveratrol-300
	($mg \cdot mL^{-1}$)		($mg \cdot g^{-1}$)	
trans-Resveratrol-3,4′,5-trisulfate	n. d.[a)]	0.20 ± 0.11	n. d.	n. d.
	–	(1.42)[b)]	–	–
trans-Resveratrol-3,4′-disulfate	0.01 ± 0.01	0.41 ± 0.13	n. d.	1.76 ± 0.70
	(0.56)	(3.50)	–	(3.42)
trans-Resveratrol-3,5-disulfate	0.01 ± 0.00	1.08 ± 0.33	n. d.	0.41 ± 0.25
	(0.26)	(10.0)	–	(0.80)
trans-Resveratrol-3-sulfate	0.01 ± 0,00	0.53 ± 0,2	0.14 ± 0.01	2.32 ± 0.91
	(0.60)	(5.93)	(1.73)	(5.68)
trans-Resveratrol-4′-sulfate	n. d.	n. d.	n. d.	1.56 ± 0.69
	–	–	–	(3.83)
trans-Resveratrol-3-*O*-β-D-glucuronide	0.19 ± 0.09	3.46 ± 1.29	n. d.	n. d.
	(8.90)	(28.6)	–	–
Resveratrol	0.06 ± 0.02	0.27 ± 0.12	0.76 ± 0.25	5.24 ± 1.27
	(5.03)	(3.99)	(13.1)	(17.2)

a) Not detected
b) Values put in parentheses indicate the percentage of the administered dose.

Table 4. Resveratrol metabolites in plasma, liver and kidney of rats fed for 8 weeks 300 mg resveratrol per kg bw and day

	Resveratrol-300[a)]		
	Plasma ($\mu g \cdot mL^{-1}$)	Liver ($\mu g \cdot g^{-1}$)	Kidney ($\mu g \cdot g^{-1}$)
trans-Resveratrol-3,4′,5-trisulfate	3.28 ± 1.22	n. d.[b)]	n. d.
trans-Resveratrol-disulfat[c)]	–	–	1.94 ± 0.51
trans-Resveratrol-3,4′-disulfate	7.46 ± 2.34	n. d.	–
trans-Resveratrol-3,5-disulfate	1.28 ± 0.31	n. d.	–
trans-Resveratrol-3-sulfate	0.37 ± 0.09	0.59 ± 0.24	0.34 ± 0.11
trans-Resveratrol-4′-sulfate	n. d.	1.77 ± 0.65	n. d.
trans-Resveratrol-3-*O*-β-D-glucuronide	3.13 ± 0.88	0.64 ± 0.24	2.71 ± 0.60
Resveratrol	n. d.	0.73 ± 0.23	n. d.

a) Recovery in plasma, liver, and kidneys was 100%, 32%, and 34%, respectively. Detection limit between 0.3 and 3 µg/g, depending on the respective metabolite.
b) Not detected
c) Coeluting stereoisomers *trans*-resveratrol-3,5-disulfate and *trans*-resveratrol-3,4′-disulfate

resveratrol-3-sulfate was formed in kidneys of rats fed the resveratrol-300 diet, also shown in Table 4.

4 Discussion

To date, no information was available on biological effects of resveratrol in long-term feeding experiments. The studies published were mainly short-term experiments with the administration of single-doses [29, 30]. Only the recent investigations of Breinholt *et al.* [31] included a 10-week feeding experiment with rats, in which a very low dosage of 0.08 mg resveratrol per kg bw and day was orally administered. The uptake of resveratrol in humans depends particularly on the composition of the diet and the wine consumption. As dietary habits vary intra- and interindividually as well as interculturally, it is difficult to estimate the average dietary intake of resveratrol in humans. In our experiments dosages were chosen guided by the literature on other polyphenolic substances. Biologically active dietary doses of different polyphenols administered daily over a long time period, have been between 0.01% and 0.5%. These dietary concentrations translate into doses of 10–500 mg per kg bw in the rat [7]. Furthermore, in a very recent publication on resveratrol-associated renal toxicity, the no observed adverse effect level was set at 300 mg resveratrol per kg bw per day in rats [32].

Besides several *in vitro* studies on the chemopreventive activities of resveratrol, the mode of functioning of resvera-

trol as a chemopreventive agent has also been evaluated in different *in vivo* studies. So far, little is known about the influence of resveratrol on the phase II enzyme GST. Yen *et al.* [33] reported that the activity of the GST in human lymphocytes was induced by resveratrol (10 – 100 μM). Only Breinholt *et al.* [31] performed an experiment on the GST activity in rats after administration of resveratrol. According to our findings, the authors could not observe any effect of resveratrol on the GST activity in liver or colon cytosol of rats fed 0.08 mg resveratrol per kg bw and day for 10 weeks [31]. Asensi *et al.* [34] indicated that glutathione conjugation seems not to be involved in the metabolism of resveratrol since no formation of conjugates could be observed after *in vitro* incubation of glutathione and resveratrol in the presence of GST [34], which might be a good explanation for the unchanged GST activity. The antioxidative capacity of resveratrol, especially *in vitro*, has been widely proven and summarized within the literature [4, 6, 8]. The ambiguous results of the total antioxidant activity of the presented data might primarily be due to the deviations caused by interindivdual variations in the second animal experiment. According to the results of the antioxidant activity, feeding of either 50 or 300 mg resveratrol per kg bw and day had also no effect on the content of tocopherol equivalents in plasma and erythrocytes of the rats in both studies. Apparently, the administered resveratrol dosages did not yield in an elevated vitamin E status in the rats, due to the additional antioxidant source of resveratrol. In the literature no data can be found in which the vitamin E status is associated with the administration of resveratrol.

The lack of effect of resveratrol concerning the chemopreventive as well as the antioxidative parameters examined in our experiments gave rise to the assumption that the bioavailability of the parent compound was likely to be insufficient to provide effective levels in the corresponding organs. Up to now, the determination of resveratrol-metabolites in biological samples (*e.g.*, urine, plasma, or liver) is performed by enzymatic hydrolysis using β-glucuronidase or aryl-sulfatase. The use of a synthesized standard was first described by Yu *et al.* [13], though it was limited to *trans*-resveratrol-3-sulfate and *trans*-resveratrol-4′-sulfate. So far, no resveratrol glucuronide or sulfate reference compounds are commercially available. In the presented study, the resveratrol metabolites could be determined for the first time directly by HPLC-DAD using synthesized standard substances of the respective conjugates.

Two studies on rat model systems with isolated small intestines already suggested that, in the small intestine, the majority of absorbed resveratrol is conjugated to resveratrol glucuronide and enters the blood circulation as such. Only smaller amounts of resveratrol sulfate could be detected in these publications [10, 11]. The determination of resveratrol and its metabolites in the feces of rats has not been reported so far. In contrast to the other biological samples analyzed in the presented experiments, no *trans*-resveratrol-3-*O*-β-D-glucuronide could be detected in the feces, neither after feeding the resveratrol-50 nor after feeding the resveratrol-300 diet. A possible explanation could be that part of the administered resveratrol is not absorbed and passes the gut without metabolic conversion. Another alternative would be the presence of bacterial enzymes, namely β-glucuronidase in the gut, resulting in the large amount of free resveratrol aglycone detected, especially in feces samples of EX-II, where 17% of the dose administered was recovered as resveratrol aglycone. The only publication dealing with resveratrol in feces samples of rats is the study of Soleas *et al.* [29], who performed competition experiments with tritium-labeled and unlabeled resveratrol in rats. After administration of a single dose of max. 1 mM by gavage, around 20% of the dose of tritium-labeled resveratrol was accounted for the radioactivity of the feces after a period of 24 h [29]. These results are in good accordance to the findings presented here.

Several studies in the literature deal with the analysis of urine after administration of resveratrol to different species [13, 15 – 17, 35, 36]. But unfortunately, in most of these publications, no identification of resveratrol metabolites is provided and in none of the cases an adequate quantitation has been performed. Walle *et al.* [17] tried to identify some metabolites by LC-MS and described the formation of two *trans*-resveratrol monoglucuronide isomers as well as a *trans*-resveratrol monosulfate after administration of a single dose of 100 mg resveratrol per kg bw to one person [17]. The experiments of Yu *et al.* [13] included *in vivo* studies with intraperitoneal administration of 25 mg resveratrol per kg bw to rats, resulting in the excretion of *trans*-resveratrol-3-*O*-β-D-glucuronide with the urine [13]. In our experiments, the oral administration of different resveratrol dosages to rats also gave rise to the generation of *trans*-resveratrol-3-*O*-β-D-glucuronide as the main metabolite in EX-I and EX-II with 0.19 ± 0.09 mg/mL and 3.46 ± 1.29 mg/mL, respectively. Free resveratrol aglycone could be detected in urine as well in considerable amounts in both animal studies. But, especially the feeding of the higher resveratrol-300 dose lead to the formation of *trans*-resveratrol-3,5-disulfate (1.08 ± 0.33 mg/mL), *trans*-resveratrol-3-sulfate, *trans*-resveratrol-3,4′-disulfate, and to a lesser extent *trans*-resveratrol-3,4′,5-trisulfate (0.20 ± 0.11 mg/mL). A possible explanation for these findings could be that elimantion of resveratrol occurs in a two-rate reaction of phase II biotransformation enzymes. The first step of the eliminantion pathway might be the glucuronidation by UDP-glucuronosyl-transferases. The second step would only occur if a specific resveratrol level in the organism is exceeded and resveratrol is metabolized by sulfation reactions *via* sulfotransferases. This two-rate reaction could also be an explanation for the higher excretion (recovery

5%) of free resveratrol aglycone after administration of the lower resveratrol-50 dosage compared to the feeding of the resveratrol-300 diet (recovery 4%). It is conceivable that on the basis of the second elimination pathway, the level of circulating free resveratrol aglycone is reduced by sulfotransferases. However, Soleas *et al.* [29] concluded that metabolic conversion of resveratrol is saturable, so that as the dose is increased, a higher percentage is excreted unchanged in the urine. But, possibly the administered (gavage) bolus doses (10 nM, 100 nM, or 1 mM) were not high enough to activate the proposed second elimantion pathway [29]. Furthermore, Soleas *et al.* could show that after a period of 24 h around 50% of the dose of tritium-labeled resveratrol administered was accounted for the radioactivity in urine [29], what confirms our results of a recovery of 53% of total resveratrol in the urine of rats fed the resveratrol-300 diet.

In our experiments, no resveratrol conjugates were detected in plasma, liver, and kidney samples of rats receiving the resveratrol-50 diet. The group of Bertelli *et al.* [30, 35] was one of the first who described the absorption and distribution of resveratrol after administration to rats. In their studies, rats were given a single dose of red wine, containing 28.24 μg resveratrol by an intragastric tube. It was shown that resveratrol plasma and liver levels had its maximum 1 h after administration of the red wine (around 0.03 μg/mL) and decreased rapidly within 4 h to its basic level. In the kidneys, peak concentration of resveratrol was achieved 2 h after administration, but declined much slower, and returned not to its basic level within 6 h. Juan *et al.* [37] even reported that the resveratrol concentration of rat plasma 15 min after an oral dose of approximately 0.5 mg was 0.175 μg/mL. Slightly different results are presented by Meng *et al.* [16]. After i.g. administration of resveratrol to rats (2 mg/kg), up to 1.2 μM resveratrol was observed in the plasma. Thereby, the plasma level increased more than 2-fold at 1.5 h and further rose moderately at 4 h. However, the findings of Bertelli and Juan were confirmed by several research groups [13 – 15, 17, 30, 34, 35, 37, 38]. Their findings might give an explanation for the absence of resveratrol conjugates after administration of the low resveratrol-50 dosage in the here presented study, as rats were fasted 24 h prior to sacrifice. By now, there is no adequate explanation why in 50% of the plasma, liver, and kidney samples of rats of the resveratrol-300 group of EX-II no resveratrol conjugates were observed. The most likely is that the amount of the resveratrol conjugates was below the detection limit which was between 0.3 and 3 μg/g, depending on the respective metabolite. Even in the samples where the metabolites were identified, the quantity of them was quite low (Table 4). It seems reasonable that due to interindividual differences, the amount of resveratrol conjugates in these animals was just too low for detection. Considering the low recovery in liver and kidney samples of 32% and 34%, respectively, the determined quantities of the resveratrol metabolites identified in these tissues should be evaluated carefully. Sale *et al.* [38] also reported very low recovery (50%) for resveratrol conjugates and therefore even abstained from quantitation [38].

With 7.46 ± 2.34 μg/mL *trans*-resveratrol-3,4′-disulfate was the main metabolite in plasma samples of EX-II. Furthermore, the sulfates *trans*-resveratrol-3,4′,5-trisulfate, *trans*-resveratrol-3,5-disulfate and *trans*-resveratrol-3-sulfate could be detected, as well as *trans*-resveratrol-3-*O*-β-D-glucuronide. Interestingly, no resveratrol aglycone occurred in plasma samples. Also Walle *et al.* [17] could not detect any measurable levels of free resveratrol in plasma samples after oral administration of 25 mg resveratrol to healthy volunteers. However, evidence of both sulfate and glucuronic acid conjugates could be found in their study as well as in the experiments presented here. The work performed by Sale *et al.* [38] gave some indications on the distribution of resveratrol and its metabolites in different organs. The authors identified a resveratrol monoglucuoronide and *trans*-resveratrol-3-sulfate in liver and kidney samples of mice, which received 240 mg resveratrol per kg bw *via* the intragastric route [38]. These results confirm our findings, in which *trans*-resveratrol-3-*O*-β-D-glucuronide was the main metabolite in rat kidneys. The lack of free resveratrol aglycone in kidney samples indicates that apparently resveratrol does not accumulate in the kidneys, but is rather excreted with the urine directly. Decreasing concentrations of radioactivity in kidneys of mice, as shown by Vitrac *et al.* [14], also suggests that renal excretion might be one of the major ways of elimination of resveratrol [14]. This hypothesis is also confirmed by our results on the high recovery (53%) of total resveratrol in urine samples of rats after administration of the resveratrol-300 diet.

In contrast to plasma and kidney samples, free resveratrol aglycone could be detected in the liver of rats in EX-II. Probably, small amounts of resveratrol accumulate in the liver prior to further metabolism. This assumption could be supported by microautoradiography of mice liver samples performed by Vitrac *et al.* [14], who suggested that hepatocytes in particular were able to incorporate [^{14}C]*trans*-resveratrol derived radioactivity 3 h after administration. Additionally, the authors stated a relatively high concentration of unidentified radioactive glucuronide or sulfate conjugates, what might reflect some active metabolic accumulation process in the liver, which possibly can be due to a metabolism *in situ* [14]. Yu *et al.* [13] also described the formation of *trans*-resveratrol-3-*O*-β-D-glucuronide, *trans*-resveratrol-4′-*O*-β-D-glucuronide, and *trans*-resveratrol-3-sulfate in incubations with human and rat hepatocytes [13]. In the same way, in human liver microsomes, only small amounts of resveratrol sulfates occur, while glucuronidation of this stilbene remains the preferred reaction [37]. In the experiments presented here, the predominant metabo-

lites in the liver were also monosubstituted resveratrol conjugates. In conclusion, liver seems subject to an accumulation of resveratrol and its metabolites.

The presented results indicate that after feeding of different resveratrol dosages to rats over a period of 8 weeks, resveratrol is absorbed, conjugated, and excreted by the rat. The overall resveratrol recovery in feces and urine after feeding 50 and 300 mg resveratrol per kg bw and day was 30% and 84%, respectively. At this time, we cannot explain the low recovery in EX-I. It is conceivable that resveratrol is metabolized *via* other still unidentified, *e.g.*, microbial or endogenous metabolic pathways, depending on its administered dosage. Soleas *et al.* [29] suggested in their study that the lack of 25% of the radioactivity administered in urine and feces could be accounted for excretion *via* sweat and respiratory water as well as by metabolism of CO_2. Due to its moderate lipid solubility, resveratrol might also be deposited in adipose tissue. Skeletal muscle is another possible tissue of resveratrol accumulation that by now has not been examined [29]. Possibly, the administration of the high resveratrol dosage, in our experiments, overstrained these pathways resulting in the higher recovery (84%) in feces and urine of the rats fed on the resveratrol-300 diet. Virtually no unconjugated resveratrol was detected in plasma and kidney samples, which might have implications regarding the significance of *in vitro* studies that used only unconjugated resveratrol. Recently, Rimbach *et al.* [39] described the effect of sulfation of the isoflavone genistein on its antioxidative properties. The results showed that with increasing sulfation of the parent compound, the antioxidative capacity is reduced to a minimum [39]. The biological efficacy of resveratrol metabolites is yet unknown. If resveratrol metabolites are biological active compounds, they could conceivably contribute to, or account for, different effects of resveratrol *in vivo*. In that case, a lot of the extensive published data on the properties of resveratrol in cells *in vitro* would be rendered rather irrelevant with respect to explaining activity in animals and eventually in humans *in vivo* [7]. Similarly, Goldberg *et al.* [13] concluded that the voluminous literature reporting powerful *in vitro* anticancer and anti-inflammatory effects of resveratrol is irrelevant, given that they are absorbed as conjugates [13]. Further investigations have to be done to clarify these remaining questions. The previously described biological effects of *in vitro* experiments of resveratrol aglycone should be repeated using resveratrol conjugates formed *in vivo*. Additionally, absorption of resveratrol glycosides should be examined *in vivo*, as this is the main source of resveratrol ingested with the daily human diet.

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