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List of Abbreviations

AP-1	activator-protein 1
BCRP	breast cancer resistance protein
bw	body weight
CAT	catalase
CCR	C-C motif chemokine receptor
CXCR	C-X-C motif chemokine receptor
ec	<i>Escherichia coli</i>
eNOS	endothelial nitric oxide synthase
GPTx	glutathione peroxidase
HO-1	heme oxygenase
IFN- γ	interferon gamma
I κ B	inhibitor of kappa B
IKK	I κ B kinase
IL-6	interleukin 6
IL-8	interleukin 8
iNOS	inducible nitric oxide synthase
IRAK4	interleukin 1 receptor-associated kinase 4
IRF	interferon-regulatory factor
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharides
MAK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MIP-1 β	macrophage inflammatory protein 1 beta
MRP	multidrug resistance protein
MyD88	myeloid differentiation primary response protein 88
Nf κ B	nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
Nrf2	nuclear factor erythroid related factor 2
OATP	organic anion transport protein
PAMPs	pathogen associated molecular patterns

pg	<i>Porphyromonas gingivalis</i>
P-gp	P-glycoprotein
PRR	pattern recognition receptor
RNS	reactive nitrogen species
ROS	reactive oxygen species
Sirt1	Sirtuin-1
SOD	superoxide dismutase
TAK1	transforming growth factor- β -activated kinase 1
TIRAP	toll-IL-1 receptor domain-containing adaptor protein
TLR	toll like receptor
TNF- α	tumor necrosis factor alpha
TOPII	Topoisomerase II
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecules
TRIF	toll-IL-1 receptor domain-containing adaptor inducing interferon- β

1 Introduction

The phytoalexin Resveratrol, in its origin a secondary plant metabolite with antifungal properties, became of interest for scientists in Europe in the late 1980s and early 1990s, as part of the “French paradox” [1-3]. This term connected the moderate consumption of red wine with a lowered incidence of coronary heart disease, despite a diet high in saturated fatty acids in France. At first, the platelet-aggregation inhibiting properties of wine were attributed to the alcohol content [1], but this theory was soon replaced by studies with dealcoholized wine, and the beneficial effect accredited to red wine polyphenols [4], among those Resveratrol. Resveratrol was also found to be one of the main constituents of the roots of *Polygonum cuspidatum*, a widespread food and traditional medicinal plant in Japan and China [5]. Apart from being a common aliment, preparations of this root were and still are in use for external and internal treatment of liver, lung, skin, urinary tract, prostate, inflammation, pain, infection, hyperlipidemia and diabetes [5]. In the years following the upcoming of the “French paradox”, a large number of studies was published on the beneficial health effects of Resveratrol [6, 7]. Most of those results were obtained *in vitro*, *ex vivo* or in animal models, and until today, *in vivo* evidence for the attributed health benefits in humans is still sparse or partly contradictory [8, 9]. *In vivo* bioavailability studies with oral Resveratrol in humans and animals detected an eminently high metabolism rate of Resveratrol, forming mainly sulfated and glucuronated conjugates [10]. As the concentrations of metabolites in body fluids (blood, urine) and tissues (liver, kidney, brain) exceed those of the parent compound, studies on the bioactivity of these metabolites and their potential health effects have been conducted in the past few years, as they may account for the health effects originally attributed to the unconjugated stilbenoid.

This thesis presents *in vitro* findings on the bioactivity of the most physiologically abundant Resveratrol metabolites in cellular models of cancer, atherosclerosis and inflammation in general, as well as with focus on immune-modulation in the oral cavity. The parent compound Resveratrol has already been studied extensively and shown beneficial activity *in vitro* in all of those pathologies [11-13].

Additionally, this thesis addresses the widely postulated antioxidant capacity of Resveratrol [12]. Free radicals and oxidative stress are meanwhile strongly implicated in the pathology of cancer, cardiovascular diseases, ischemic injury, asthma, rheumatoid arthritis, diabetes, neurological disorders and ageing [14, 15]. Here, a comparison of two *in vivo* studies in healthy humans is also presented, focusing on the effect of low and high doses of dietary Resveratrol, as well as the likely impact of *in vivo* formed metabolites, on the blood oxidative state.

1.1 Resveratrol in Diet and Disease

Resveratrol is found in red grapes, and products thereof, with concentrations up to 14 mg/L (63 μ mol) in red wine [16-19], and in *Polygonum cuspidatum*, with 1 - 3 mg/g herb (4 – 13 μ mol). In a review from 2001 [20], the major biological activities of Resveratrol included cardioprotection (inhibited platelet aggregation, eicosanoid system, radical scavenging, vasorelaxation), cancer prevention and therapy (apoptosis, growth inhibition via suppression of cell-cycle phases S, G1 and G2) and anti-inflammatory properties (cytokine network, eicosanoid system, interaction with transcription factors). Bhat et al. [20] concluded that future challenges will be to extrapolate the obtained *in vitro* and *ex vivo/in vivo* animal data, encouraging as they may be, to *in vivo* in humans. The difficulties with this transfer is the imbalance between the concentrations that exert biological effects *in vitro* (up to several hundred μ M), and the doses actually ingested on a dietary scale. A Spanish cohort study by Zamora-Ros et al. [21] estimated the overall daily oral intake of Resveratrol in *cis* and *trans* form, as well as the Resveratrol glucoside piceid in *cis* and *trans* form, between 4 – 11 μ mol/70 kg body weight (bw) per day, which is already below concentrations of Resveratrol used in *in vitro/ex vivo* experiments. This comparison between intake and concentrations used *in vitro* is not considering plasma kinetics, which result in even lower concentrations of the parent compound readily available *in vivo*. Not surprisingly, although various health claims to the EFSA Panel on Dietetic Products for Resveratrol and *Vitis vinifera* extracts have been denied [22, 23], the low abundance of Resveratrol in the daily diet, combined with the numerous reports on especially lifespan extending and cancer preventive effects [11, 24], led to the production, marketing and sales of nutritional additives, some of

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them containing high amounts of Resveratrol [25]. Nutritional additives do not underlie the same strict regulations as pharmaceuticals regarding, for example, stability, content and purity, and most importantly, safety. For example, the analysis of 14 nutritional additives, differing in prize and alleged Resveratrol content, showed that only five products reached near label values, whereas two additives were even under the limit of detection for Resveratrol [25]. Regarding safety of high dose oral Resveratrol, Boocock et al. [26] conducted a single dose human acute toxicity study (n = 40) with 0.5 to 5 g oral Resveratrol. No serious adverse events, neither clinical, biochemical nor hematological were reported and Resveratrol was deemed well tolerable in healthy individuals [26]. For repeated doses in rodents (once daily for three months), the reported no-observed-adverse-effect-levels was determined to be 750 mg/kg bw daily [27], for an adult with an average bw of 70 kg that would correspond to a very high daily dose over 50 g. In the same study by Williams et al. [27], Resveratrol did not show any potential for skin irritation, sensitization, subchronic and embryo/fetal toxicity or genotoxicity. In a repeated dose human pharmacokinetic study with 25 to 150 mg oral Resveratrol six times a day, for 13 consecutive days, Almeida et al. [28] reported mild side effects, mainly consisting of headaches. Brown et al. [29] conducted a repeated dose study with 0.5 to 5 g oral Resveratrol daily in healthy humans for a duration of 29 days. Here, the main adverse events were reported to be nausea, flatulence, abdominal discomfort, and diarrhea 30 minutes to one hour after intake of 1 g, 2.5 g and 5 g doses [29]. In the same study, Brown et al. [30] also determined the impact of repeated dose oral Resveratrol on the insulin-like growth factors axis, which is crucial for the development of colorectal cancer. In said study, doses of 1 g and 2.5 g oral Resveratrol had a significant effect on the parameters measured, suggesting a chemopreventive effect. Pinning down such an effect in humans proves to be very difficult, because so far, no predictive biomarkers have been recognized, neither in healthy humans nor in people with cancer [29]. To assess a *therapeutic* effect of Resveratrol supplementation *in vivo* in humans, the relevance of Resveratrol supplementation in cancer, diabetes, coronary vascular diseases, inflammatory diseases and neurological diseases has been established in human intervention studies in the last few years. Oral Resveratrol has been given from 8 mg to 3 g per

day, for durations between one week and 1 year. The general outcome was that effects in cancer could not be substantiated yet, mainly due to too small samples sizes ($n = 6 - 20$), but Resveratrol did have an effect on glucose regulation and insulin sensitivity in patients with manifested type II diabetes, though not in healthy subjects [31, 32]. Inflammation markers were reduced in patients with high cardiovascular risk, and obesity was reduced in patients with metabolic syndrome, corroborating *in vitro* findings [33, 34]. Resveratrol supplementation also seemed to have positive effects on cerebral blood flow, but not on cognitive function in healthy individuals [35]. Taken together, so far, oral Resveratrol has been shown to improve parameters in already manifested diseases, but its preventive effects have yet to be determined in healthy humans [9, 36-38].

1.2 Bioavailability and Metabolism of Resveratrol *in vivo*

Research on absorption, distribution, metabolism and elimination processes (ADME) of dietary compounds is very challenging. Food contents and the average intake have to be considered and both are either unknown or differ between climate zones, countries and cultures. The assessment of oral dietary intake of Resveratrol is also facing those problems. The already mentioned Spanish cohort study by Zamora-Ros et al. [21] estimated an average daily intake of total Resveratrol from grapes, wine, juices and must, of 1 - 2.5 mg (4 - 11 μmol). In early pharmacokinetic studies in rodents, bolus oral or intraperitoneal doses of Resveratrol exceeded the estimated dietary intake, ranging from 5 up to 300 mg/kg bw [39-41]. Summarizing the results of those early studies, Resveratrol was not found to be subjected to substantial phase I metabolism, but there was extensive phase II metabolism, resulting in glucuronidation and sulfation, leaving little to no Resveratrol unconjugated. Using ^{14}C -labeled Resveratrol to assess organ distribution, radioactivity was predominantly found in duodenum, kidney and liver, but also in lung, brain, heart, liver, spleen, colon and testis of rats [39]. Kuhnle et al. [42] demonstrated the immediate metabolism of Resveratrol upon absorption in the gut using isolated rat jejunum models *ex vivo*, where 99 % of the absorbed compound were glucuronated during the transfer from luminal to basolateral side. In a model of *in vivo* perfused rat jejunum, Juan et al. [43] could also demonstrate a

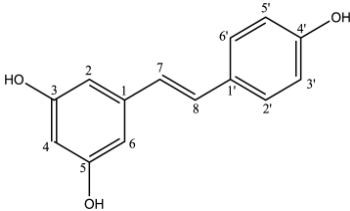
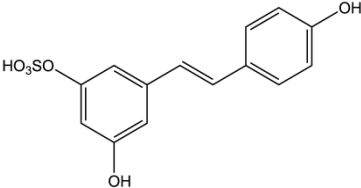
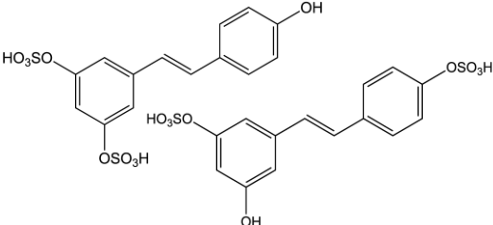
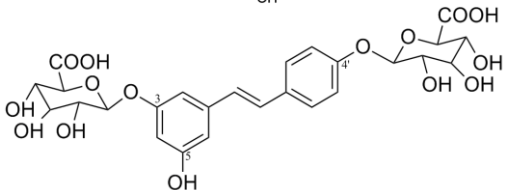
passive absorption of 70 % of the applied dose, with the extensive formation of glucuronated and sulfated conjugates. One of the first long-term *in vivo* absorption study in rodents (300 mg/kg bw per day over 8 weeks) confirmed the formation of sulfated and glucuronated metabolites, whereas Resveratrol could not be detected in plasma, and appeared only in small amounts in urine and feces (4% and 17% of the administered dose, respectively) [41]. The metabolites found in rat urine were mainly glucuronides, whereas rat feces showed excretion of Resveratrol as sulfated conjugates [41]. In humans, after a single oral dose of 25 mg (110 μ M) ^{14} C-Resveratrol, similar results have been obtained by Walle et al. [44]. In their study, maximum plasma concentrations of Resveratrol only reached about 2 μ M 60 min post dose, and the overall absorption of the administered Resveratrol was up to 70 % of the applied dose, based on urinary excretion data [44]. After a considerably higher oral bolus of 5 g oral Resveratrol, maximum plasma levels of Resveratrol (2.4 μ M), did not reach a relevant level for chemoprevention (5 μ M), as determined by Boocock et al. [26]. Burkon and Somoza [45] identified and quantified human metabolites over 48 h in plasma and urine after a dietary scale oral bolus dose of piceid (85,5 mg/70 kg bw), corresponding to 50 mg/70 kg bw Resveratrol (220 μ M), or about 1.5 L of red wine. The main metabolites and their maximum plasma concentrations ($c_{\max}$) were found to be Resveratrol-3-sulfate (0.95 μ M), Resveratrol-3,4'-disulfate (0.33 μ M), Resveratrol-3,5-disulfate (0.94 μ M), Resveratrol-3-glucuronide (0.16 μ M) and Resveratrol-4'-glucuronide (0.19 μ M) and Resveratrol-diglucuronides (0.35 μ M), with times of $c_{\max}$ ($t_{\max}$) of 1 h for Resveratrol-3-sulfate, and between 6 to 8 h for Resveratrol-disulfates and -glucuronides (Table 1) [45]. Neither piceid nor Resveratrol were detectable in plasma and urine. Comparably to this low dose study, in a repeated dose human study conducted by Brown et al. [29] with doses of 0.5, 1.0, 2.5 and 5 g Resveratrol for 29 days, Resveratrol-3-sulfate was also found to be the main plasma metabolite, besides disulfates and glucuronides. In their study, the $t_{\max}$ value of 1 h for Resveratrol-3-sulfate corresponds to previous findings, the glucuronides had $t_{\max}$ values of about 1.5 h. Resveratrol was also found in plasma, but in 4 – 17-fold lower concentrations than the metabolites, also corroborating the findings by Burkon and Somoza [29, 45]. In the studies by Almeida et al. [28] and Brown et al. [29], plasma levels indicated an accumulation of

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Resveratrol after repeated doses of 0.9 g and 5 g respectively, albeit with high interindividual variability. Despite repeated doses, no apparent steady-state was reached with the administration of oral Resveratrol once daily, due to short elimination half lives of 5 – 10 hours, but Patel et al. [46] found high concentrations of up to 674 μM of the parent compound in colon tissue after 8 consecutive days of 1.0 g oral intake, whereas plasma levels were not detectable, indicating tissue accumulation. In the same study, Patel et al. [46] identified Resveratrol-sulfate-glucuronide to be the major metabolite in plasma (c_{max} 36 μM) and Resveratrol-3-sulfate in colon tissue ($c_{\text{max}} = 366 \mu\text{M}$).

In their earlier mentioned single dose study, additionally to identification and quantification of metabolites, Burkon and Somoza [45] also established plasma protein binding for Resveratrol and its metabolites (Table 1).

Table 1 Mean plasma protein binding (PPB) for Resveratrol and metabolites. *PPB for Resveratrol has been established with reference plasma, values for metabolites were determined with samples from Burkon and Somoza [45]; **representative structure of Resveratrol-3,4'-diglucuronide

Structure	Common name	mean PPB
	Resveratrol *	94 %
	Res-3-sulfate	34 %
	Res-disulfates	44 %
	Res-diglucuronide **	46 %

Interestingly, the metabolites were found to be highly bound to plasma proteins, albeit with a lower percentage than Resveratrol. The extent of plasma protein binding is a crucial factor in the disposition and elimination of substances, and in consequence for their effects, for mostly only the unbound compound can be transported and exerts biological activities [47].

According to Burkon and Somoza [45], as well as Walle et al. [44], Resveratrol, in form of its metabolites, is eliminated from the human body mainly via urinary excretion (53 – 85 %) within 48 hours.

Taken together, Resveratrol is well-absorbed (>70 %), but immediately metabolized in intestinal cells and liver tissue, resulting in a very high first-pass effect, and elimination was determined to be *via* the urinary and intestinal tract. Recently, Bode et al. [48] also observed the dehydrogenation of Resveratrol by gut bacteria, presenting further physiological metabolites. Overall, the bioavailability of the parent compound Resveratrol is very low, leading the authors of the above-mentioned studies to the conclusion that the concentrations of oral Resveratrol in plasma and tissues, reached either through standard diet or nutritional additives, is not sufficient for exerting the profound biological effects observed *in vitro*. In this thesis, it was hypothesized, that physiologically formed metabolites could also be bioactive, maybe even more so than the parent compound, thus being equally or even more important for effects originally attributed to Resveratrol alone.

1.3 Cellular Transport of Resveratrol and its Metabolites

1.3.1 Principles of Cellular Transport

For absorption and elimination, nutrients and drugs have to pass cellular barriers between the “outside” (luminal or apical side) and the “inside” of the body (interstitium, or basolateral side), or *vice versa*. This transfer through the selective phospholipid cellular bilayers largely depends on substance characteristics like size, solubility in biological fluids, stability, lipophilicity, ionization or plasma protein binding, all of which influence the mode of transport [49, 50]. Other factors are of course gastric emptying, motility, intestinal pH or diseases. Cells in tissues involved in absorption and elimination from the body can be seen as bidirectional interfaces,

consisting of polarized cells. Examples would be cells of intestinal epithelia (intestine ↔ blood), the proximal tubules in the kidney (urine ↔ blood), hepatocytes (bile ↔ blood) or the blood-brain barrier [51]. Basic transport mechanisms through, into or out of these polarized cells are passive diffusion, vesicle trafficking, carrier-mediated transport without energy expenditure, following a gradient or *via* uniport/symport/antiport activity, and active transport with energy expenditure [49, 52]. Membrane transporter proteins are expressed on either side of the polarized cells, with directional specificity (influx or efflux) [50]. Among the most studied membrane transporters for drugs and nutrients nowadays are ATP binding cassette transporters (ABC), especially the active efflux transporters P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance proteins (MRP). Among influx transporters, the solute carriers organic anion transport polypeptides (OATP) are the most studied [50, 53]. A polarized cell with the prevalent location of selected transporters is shown in Figure 1.

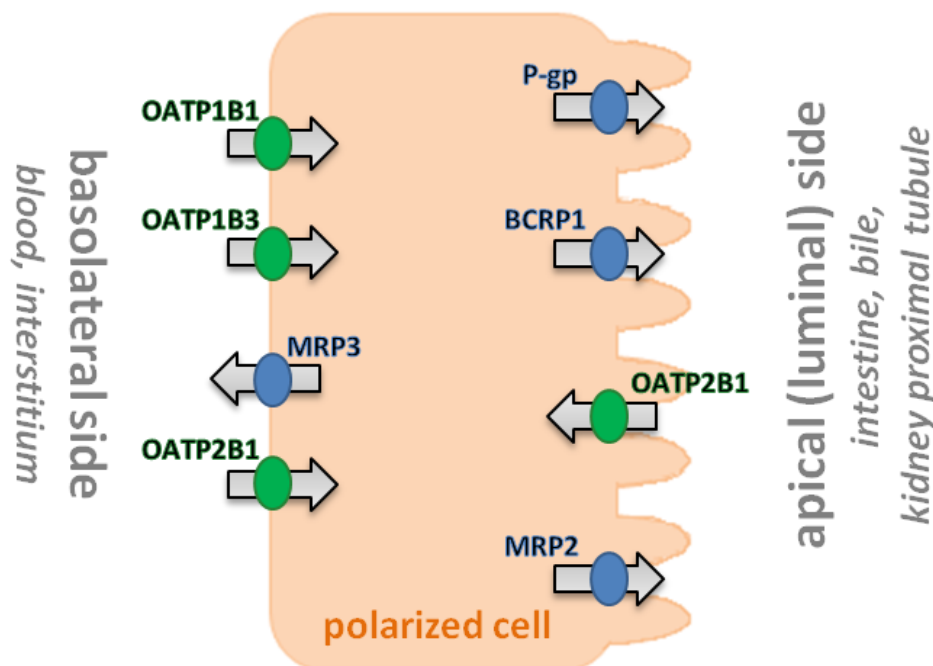


Figure 1 Polarized cell with representative transporters. Efflux transporters are depicted in blue, influx transporters in green

1.3.2 Efflux Transporters

P-gp is predominantly present at the apical side of liver, kidney and intestinal cells, but also at very high cellular levels in various cancers, leading to an increased efflux

of chemotherapeutics from cancer cells, resulting in highly problematic drug resistance [54, 55].

BCRP was first found in the breast cancer cell line MCF-7 (hence the name), presenting a new important factor for drug resistance [56]. In normal tissue, BCRP is expressed in the epithelia of colon and small intestine, in vein endothelium, bile canaliculi, the placenta and mammary tissue, emphasizing its role in elimination and enterohepatic recirculation, but also in protecting the fetus from xenobiotics and enriching breast milk with essential vitamins [57]. Evidence was found that the BCRP encoding gene is more efficiently transcribed in cancerous than in normal tissue, possibly explaining the enhanced ability of cancer cells for efflux of chemotherapeutics via BCRP transporters, thereby, as already mentioned for P-gp, also resulting in drug resistance [58].

The MRP family consists of nine different members, five of which have been implicated in drug transport [50]. The location of MRPs is not limited to one side of polarized cells, MRP2 was found in the apical membrane of enterocytes, whereas MRP1, MRP3, MRP4 and MRP5 seem to be located mainly in the basolateral membrane [50]. All MRPs are efflux transporters, but due to their location, play a role in elimination *and* entering of substances into circulation [50].

Substrates for above-mentioned transporters are widely-spread, including endogenous compounds like vitamin B₂, bilirubin, bile salts, steroid hormones, leukotriens or prostaglandins, but also drugs from the groups of chemotherapeutics, antibiotics, steroids, statins, antihelminthics, diuretics as well as drugs conjugated to glutathione, sulfate or glucuronic acid, and natural compounds like digoxin, quercetin, genistein, and sulfate and glucuronide conjugates [50, 51]. Substance specificity and affinity for those membrane transporters, but also competition for, and inhibition or stimulation of transporters, as well as substrate overlap are crucial determinants for pharmacokinetics, drug safety and efficacy. Therefore, drug-drug and drug-nutrient interactions are very important points to consider during drug development [51].

1.3.3 Influx Transporters – Organic Anion Transport Polypeptides

OATP transporters form a complex family of solute carriers, with subfamilies from OATP 1 to 6 [59]. The broad spectrum of substrates (in general organic anions) includes endogenous substances (e.g. bilirubin, prostaglandins, leukotriens, bile salts, thyroid hormones, substance P, somatostatin, neuropeptide Y), drugs (e.g. statins, antibiotics, steroid hormones, methotrexate), and natural compounds (digoxin, quercetin sulfate conjugates) [59-61]. In humans, members of the OATP family were shown to be situated mainly in the liver (basolateral), the brain, colon and small intestine, but also the retina, kidney, lung, placenta, testes or spleen [62-65]. The ever growing list of substrates for OATPs makes them important players in drug and nutrient disposition [60]. For OATP1A2, OATP1B3 and OATP6A1 an aberrant expression in breast and brain cancers could be shown, presenting them as targets for drug delivery in cancer therapy [66]. OATP4A1, OATP5A1 and OATP6A1 were found to be differentially expressed in various subtypes and stages of small cell lung cancer [67]. Those findings of variable expression patterns could on the one hand be used as biomarkers for tumor progression, as proposed by Brenner et al. [67], but on the other hand it could also have an impact on drug and nutrient concentrations in cells, due to increased influx, possibly making chemotherapeutics more effective. The competition for transport proteins between drugs and nutrients can cause unwanted changes in the pharmacokinetic profile of drugs. An animal study by Qiang et al. [68] demonstrated that the simultaneous oral application of the antihistamine fexofenadine and the statin fluvastatin caused a decrease in the maximum plasma concentration of fluvastatin by 50 %, and attributed these pronounced changes to competition for OATP transporters. Not only drug-drug interactions but also those of drugs and nutrients are of significance in pharmacokinetics, as established for the diminished absorption of the antihistamine fexofenadine, in combination with grapefruit juice or orange juice [69]. Therefore, it is important to identify substrates, inhibitors and affinities for OATP transporters among nutrients, as the pharmacokinetic profile of drugs could be largely altered by the simultaneous intake of nutritional compounds, especially that of highly concentrated nutrients in nutritional additives.

1.3.4 Transport of Resveratrol and its Conjugates

Resveratrol is known for its anti-cancer activities [70]. Recently, Resveratrol-3-sulfate was shown to decrease colon cancer cell growth *in vitro* at 30 μM (SW480 and metastatic derived SW620 cell lines), whereas the Resveratrol-4'- and 3-glucuronide were not active [71]. In contrast, both glucuronides showed anti-proliferative activity in Caco-2 cells [72]. Babich et al. [73] demonstrated, that immortal gingival cell lines were 1-3 times more sensible to the effects of Resveratrol than non-tumorigenic cell lines, with IC_{50} -values (24h exposure) for tumor-derived cell lines and non-carcinogenic cells of about 200 μM and 400 μM , respectively. Also, colorectal cancer patients who received 0.5 or 1 g oral Resveratrol daily for 8 days presented high levels of RSV conjugates in colorectal tissues [46]. The mentioned effects could well be due to differential expression of influx and efflux transporters in the cell lines used and in *in vivo* colon cancer tissue. For Resveratrol, fitting its manifold biological activities, various and concomitant modes of interaction with cellular membranes and transporters have been identified, such as endocytosis mediated by lipid rafts, passive diffusion, and active transport [52, 74]. Regarding passive diffusion and active transport, in *in vitro* and rodent experiments, Resveratrol was found to cross human hepatic cellular membranes via a combination of passive diffusion and active transport, the latter accounting for most of the uptake [75]. Maier-Salamon et al. [76] conducted *in vitro* transport experiments across human CaCo-2 monolayers, showing an extensive uptake of 90-99% of Resveratrol into human colon cancer cells, but also a saturation in metabolism with increasing doses (12% native compound versus 61% inside the cells for incubations with 10 μM and 200 μM , respectively). Further experiments by the same group in rat liver cells confirmed that P-gp is not involved in active transport of neither Resveratrol nor conjugates [77]. Maier-Salamon et al. [77] could further show that the efflux of glucuronides of Resveratrol was dependent on the efflux transporter MRP2, transporting metabolites from liver cells into bile, entering recirculation. In a study on *in vivo* perfused rat jejunum, Juan et al. [43] measured uptake by passive diffusion, but also used inhibitors for known efflux transporters for drugs and nutrients. In their study, over 60% of the absorbed Resveratrol was secreted back into the gut lumen in form of conjugates, with

involvement of MRP2 and BCRP1 [43]. MRP2 was also shown to transport Resveratrol and Resveratrol conjugates in human colon carcinoma cells (CaCo-2 cell line) [78]. In an mouse study, conducted by Wenzel et al. [41], the Resveratrol metabolite profiles in plasma, liver, kidneys, urine and feces determined the urinary excretion of mainly glucuronides, whereas sulfated metabolites were found in feces, and also showed higher half-lives, suggesting enterohepatic recirculation. The involvement of (the above-mentioned) specific efflux and influx transporters in the enterohepatic recirculation and excretion of Resveratrol and its conjugates was tested in knockout mice for BCRP1 and MRP3 by van de Wetering et al. [79] and in BCRP1 deficient mice by Alfaras et al. [80]. Both studies clearly showed that plasma, tissue, urine and fecal metabolite profiles changed dramatically with the knockout of either efflux transporter, strongly implicating them in excretion and enterohepatic recirculation of parent compound and metabolites [79].

In three publications included in this thesis, (1) *Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells*, (2) *Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells* and (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages*, the uptake of selected Resveratrol metabolites was studied.

(1) *Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells* identifies and characterizes substrates for selected OATP transporters (transfected into chinese hamster ovary cells) among Resveratrol metabolites, by determining uptake patterns and transport kinetics for Resveratrol, Resveratrol-3-sulfate, Resveratrol-disulfate and Resveratrol-3- and -4'-glucuronide, as well as the impact of Resveratrol-disulfate on the breast cancer cell line ZR-75-1.

The publications (1) *Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells* and (2) *Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells* also show the further metabolization of Resveratrol conjugates in the respectively used cell lines.

Additionally, publications (2) *Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells* and (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages* link the observed effects to the substance uptake.

1.4 Targeting Topoisomerase II and Macrophages in Cancer

Cancer is a very complex disease with a multitude of cellular pathways and signalling molecules involved in initiation, promotion, progression and metastasis. The role of topoisomerase II and macrophages in manifested cancer will be detailed in this chapter.

1.4.1 Topoisomerase II in Cancer and Chemotherapy

The essential function of topoisomerases is to cause DNA single- (Topoisomerase I) and double- (Topoisomerase II, TOPII) strand breaks, thereby uncoiling the DNA double helix. Controlled strand breaks are necessary for reducing the massive strain on supercoiled DNA strands in proximity to strand separations in cellular transcription and replication [81, 82]. Double strand breaks are also required for the decatenation of DNA strands, which is substantial for completion of replication, and enables separation of chromosomes towards the end of the mitotic cycle [82]. To this end, the TOPII enzyme is covalently bound to helical DNA, forming a so-called “cleavage complex” [83]. Inside this complex, complementary DNA strands are ATP dependently cut at the same place, and left free to uncoil and reduce the strain on the DNA molecule, or separate catenated newly copied chromosomes. With the release of TOPII from the complex, both strands are resealed by the enzyme [81]. The abundance of cleavage complexes in cells is normally very low, and changes in the number of complexes can be dangerous or even lethal, due to the accumulation of strand breaks and the possibility of mutations and chromosomal aberrations [84]. Nevertheless, because of its importance in cell proliferation, the TOPII enzyme has become an important target in highly proliferating cancer cells. There are two kinds of TOPII targeting drugs, TOPII poisons, and TOPII catalytic inhibitors [85]. TOPII poisons stabilize the “cleavage complexes” on the DNA strands, causing the accumulation of double-strand breaks, leading to apoptosis or resulting in cell cycle

arrest [83, 85]. Catalytic inhibitors of TOPII deplete the enzyme of its activity, by interfering with binding to DNA or blockage of the ATP-binding site, consequently impairing transcription and also leading to cell death [83]. TOPII directed chemotherapeutics do not specifically target topoisomerase enzymes in cancer cells, but in highly proliferating cells in general, explaining the deleterious side-effects on the gastrointestinal tract, and the bone marrow, the latter even leading to malignancies like secondary leukemia [83].

To enhance uptake into cancer tissue and reduce side-effects on healthy tissues, efforts are made to develop target-oriented TOPII poisons and inhibitors, by linking the chemotherapeutic drug to tumour- or receptor-specific peptides [83]. Given the deleterious side-effects, the increasing incidence of cancer, as well as the multitude of cancers known, the research and evaluation of novel chemotherapeutic compounds is ongoing, both on synthetic and natural compounds [83].

1.4.2 Macrophages in Tumor Environment

Macrophages possess an immense adaptive capacity, regulated by the huge amount of known recognition receptors for a multitude of potential pathogens [86]. Macrophages react to exogenous (bacteria, viruses, parasites, fungi), but also endogenous (adipokines, interferons, interleukins) influences with specifically attuned reactions (polarization phenotypes M1 and M2) [87, 88]. The polarization to M1 macrophages happens in reaction to interferons and bacteria (LPS), generating a proinflammatory, microbicidal and anti-tumoral phenotype [88]. M2 macrophages, on the other hand, are polarized in response to interleukins that are normally associated with attenuating inflammation (e.g. IL-4, IL-10, IL-13), the resulting phenotype characterized by anti-inflammatory, tissue remodeling & proliferation and pro-tumoral activities [89]. It has been shown by Clark et al. [90] that even in the earliest stages of pancreatic cancer, an infiltration of immune cells takes place, surprisingly not for protection from tumor growth, as would be expected, but to form an *immunosuppressive* environment. This effect was demonstrated to be countered with the activation of those macrophages, changing their phenotype and leading to tumor recession [90]. In other cancers, macrophages surrounding solid cancerous tissues, so-called tumor associated macrophages

(TAMs), have been identified to be mainly M2 phenotype and play a role in tumor vascularization, thereby driving cellular growth [89]. TAMs originate from the bone marrow as monocytes and are generally associated with a poor prognosis [89]. The chemokine monocyte chemoattractant protein 1 (MCP-1/CCL2, for chemokine nomenclature see 1.5.3), which is primarily excreted by monocytes and macrophages, has been highly implicated in promotion of breast, ovarian and prostate cancer [91]. In a tumor environment in mice, it has been shown that the attraction of bone marrow derived TAMs *via* MCP-1 leads to enhanced tumor development and angiogenesis. MCP-1 knockout mice on the other hand showed necrotic areas inside the primary tumor and markedly reduced the levels of vascular endothelial growth factor, presenting MCP-1 as a possible macrophage-derived target in cancer therapy [92].

1.4.3 Resveratrol in Clinical Cancer Therapy

Despite the undisputable promising effects of Resveratrol *in vitro* and in animal studies, human clinical trials with oral supplementation of Resveratrol are still inconclusive, the major factors being a small study population size, varying (pre-)medication, (pre-)treatments, compliance and unexpected complications due to the malignancy itself.

A clinical trial with colon cancer patients, Resveratrol containing oral formulations (4 – 16 mg/day for two weeks, n = 8) had no effect on the promotion-associated marker Wnt, neither in normal colonic mucosa cells nor in cancer cells [93]. The administration of a comparable dose of 5 mg/day for 3 months, n = 12), decreased aberrant gene methylation in women with high risk for breast cancer [94]. Resveratrol supplementation in both trials was very well tolerated. In yet another trial, Patel et al. [46] could show a reduction of colon tumor cell proliferation by 5 % after Resveratrol intervention (1 g/day for eight days, n = 20), with no serious adverse events observed. At higher doses, in micronized form with increased oral bioavailability, intake of 5 g Resveratrol for 14 days showed an increase only in the apoptosis marker cleaved caspase-3 in hepatic metastases, whereas other markers in plasma and tumor tissue were not altered by the treatment, as compared to healthy liver tissue (n = 6) [95]. Adverse effects were mainly of gastrointestinal

nature (nausea, diarrhea), but also rash and skin irritation [95]. In another human clinical trial with multiple myeloma patients, monotherapy with micronized Resveratrol (5 g/day for 20 days in repeated cycles), very common adverse events were nausea, diarrhea, vomiting and anaemia [96]. More alarmingly, renal failure occurred in 50 % of treatments with bioavailability enhanced Resveratrol, leading to discontinuation of patient recruitment [96].

The topoisomerase II inhibition shown in (2) *Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells*, combined with the attenuating effect of Resveratrol and metabolites, especially on MCP-1, IL-6 and TNF- α , shown in (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages* and (4) *Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations*, as well as the growth inhibition observed in breast cancer cells in (1) *Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells* may present the first results on the activity of Resveratrol metabolites in cancer and cancer related mechanisms. Clearly, further research is indicated in various cancer models *in vitro* and *in vivo* to estimate the pharmacodynamics of high concentrations of Resveratrol and metabolites in humans after intake of bioavailability-enhanced formulations.

1.5 Impact of Resveratrol Metabolites on the Innate Immune Response to Bacterial Challenges

Besides their role in cancer, as elucidated above, macrophages, cytokines and chemokines are a vital part of the innate host immune reaction to bacteria, by triggering protective immunological reactions and phagocytosing bacteria [97].

1.5.1 Bacteria in Periodontal Diseases

Negligence of oral hygiene and the resulting accumulation of dental plaque can lead to an excessive immune response in gum tissue of the oral cavity, triggered by

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lipopolysaccharides (LPS), an endotoxin present in huge amounts in the outer membrane of gram-negative plaque bacteria [98].

This immune response can result in the formation of an inflammatory infiltrate of activated monocytes, macrophages, neutrophils and fibroblasts, within about a week [98]. The infiltrating and proliferating cells excessively produce inflammatory and matrix degrading agents (MMPs, ROS, phagocytosis), leading to the formation of periodontal pockets. The ongoing destruction of connective and attaching tissue leaves the root and alveolar bone unprotected. Those processes finally peak in alveolar bone resorption and tooth loss [99].

Human dental plaque is a complex mixture of matrix-enclosed bacterial species and as shown by 16s ribosomal DNA sequencing of the human oral bacterial community, consists of over 400 different species [100]. In human plaque samples, the presence of certain bacterial species could be associated with the presence of others, forming reoccurring bacterial complexes (Figure 2), which in turn could be associated with healthy and diseased oral sites [101].

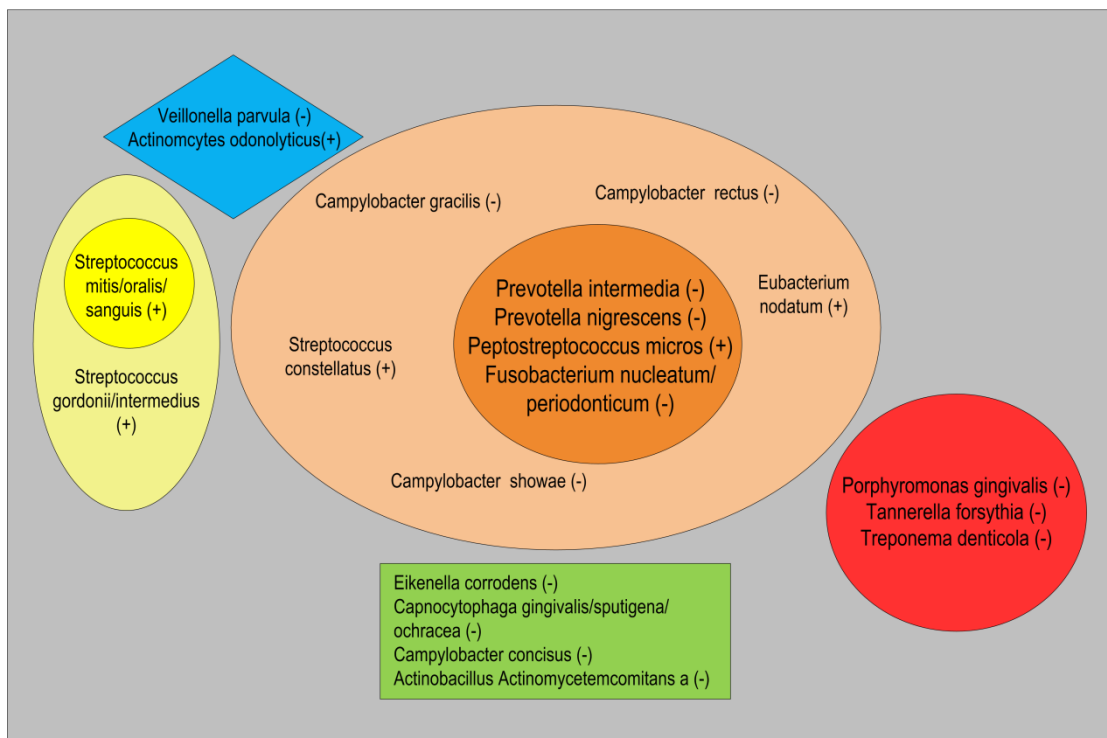


Figure 2 Microbial complexes in subgingival plaque. Relationships among complexes are depicted by proximity of coloured clusters. (+) gram positive, (-) gram negative; Adapted from Socransky et al., J Clin Per, 1998 [101].

Healthy sites are predominantly associated with gram-positive *Streptococcus* species, whereas diseased sites, as for example periodontal pockets, are occupied by anaerob, gram-negative species like *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella sp.* and *Treponema denticola* [100, 102, 103].

Highly used in *in vitro* experiments, *Porphyromonas gingivalis* and *Escherichia coli* LPS (pgLPS, ecLPS) have been shown to trigger transcription and translation of inflammatory cytokines, chemokines, matrix degrading agents and adhesion factors in fibroblasts, epithelial cells, as well as immune cells like monocytes, macrophages, dendritic cells and B- and T-lymphocytes (Figure 3) [86, 99].

1.5.2 The Innate Immune Response to Bacterial Challenges

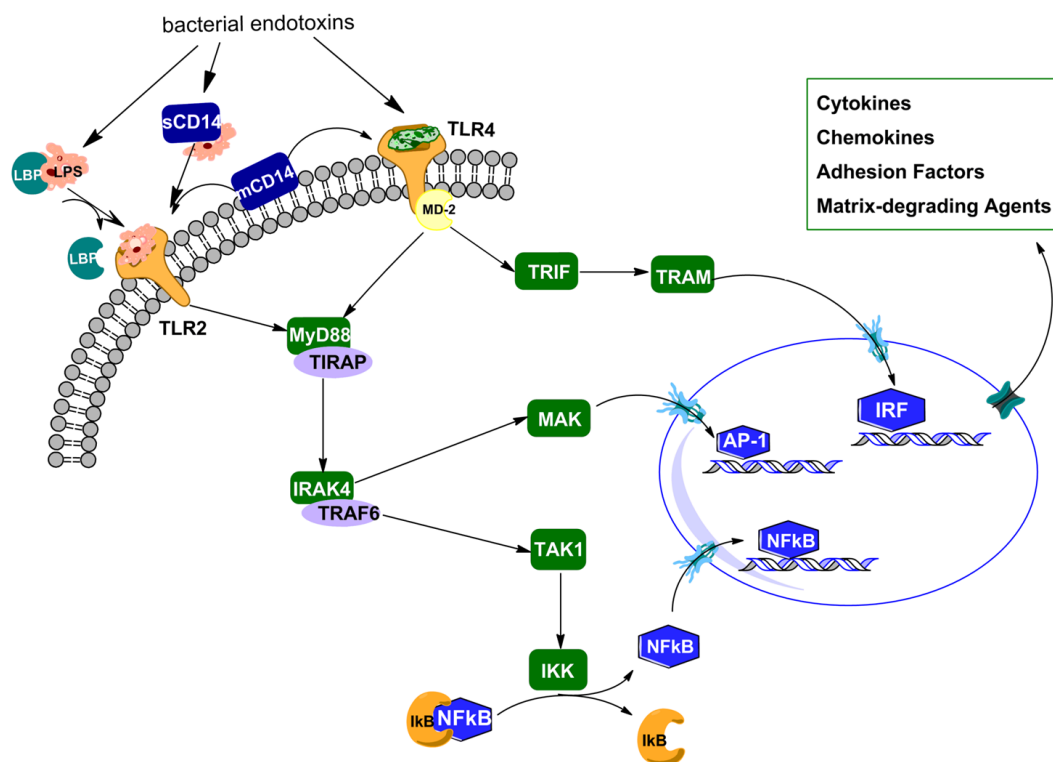


Figure 3 Pathways of TLR-2 and TLR-4 activation. LPS is recognized by lipopolysaccharide binding protein (LBP) or soluble and membrane bound accessory proteins (s/mCD14, MD-2). The binding of LPS to the receptor/accessory protein complex activates a cascade involving various enzymes and transcription-factors. The activation of transcription factors (dark blue) results in transcription of inflammatory cytokines, chemokines, adhesion factors and matrix degrading agents.

The innate immune system has long been thought to be unspecific, but that has been revised by the finding of pattern recognition receptors (PRRs), mainly expressed on immune cells like macrophages, monocytes, dendritic cells, mast cells and lymphocytes, but also on fibroblasts or endothelial cells [86].

For the innate immune response to bacteria, toll-like receptors (TLR) are the most important receptors among PRRs. So far, ten human TLRs have been identified, which recognize pathogen associated molecular patterns (PAMPs) [86]. PAMPs generally are distinct conserved microbial structures, like lipopolysaccharides, lipoproteins, peptidoglycans, dsRNA, ssRNA or bacterial DNA [86]. Pathways for two PRRs (TLR-2 and TLR-4) are depicted in Figure 3, being activated by lipoproteins, peptidoglycans and the already mentioned pgLPS and eLPS.

1.5.3 Function of Cytokines and Chemokines

Cytokines are involved in regulating growth, cell proliferation, differentiation & survival, hematopoiesis, satiety, immune cell activation, immune response, chemokine and cytokine induction, antigen presentation and antibody production as well as allergic reactions [104]. Chemokines are important factors in cell adhesion and the innate immune response, and not only enable localized immune cell trafficking from the bone marrow and blood stream into tissues via chemotaxis, but also enhance localized cell activation and proliferation of tissue and immune-competent cells [105]. The chemokine and cytokine networks are crucial determinants in development and progression of a multitude of human pathologies: oral diseases, cardiovascular diseases, allergies, dermatitis, multiple sclerosis, rheumatic diseases, inflammatory bowel diseases, viral and bacterial infections, fever, cancer, hematopoietic disorders or transplant rejection but also in conditions that are not obviously associated with inflammation and immune response like metabolic syndrome, obesity, sarcopenia, diabetes, schizophrenia, bipolar disorders or Alzheimer's disease [104-107].

The cytokine interleukin 6 (IL-6) plays key roles in the inflammatory response (fever, acute phase proteins), in host defense (B-cell activation, immunoglobulin production), and in bone metabolism (osteoclast function). IL-6 levels are acutely elevated during infections or trauma [104]. Chronically elevated levels of IL-6 have

been found in patients with autoimmune diseases, and IL-6 may also be involved in cancer development [104]. Besides IL-6, tumor necrosis factor alpha (TNF- α) majorly contributes to inflammation, induction of fever, coagulation and the induction of cytokine and chemokine production. Whereas during host defense, those mechanisms contribute to counter bacterial infection, an overactivation of TNF- α can lead to septic shock, severely injuring the host [104].

The chemokine family is classified by the number and location of cystein residues located at the N-terminus of the proteins (C-C family, C-X-C family), whereas the suffix L stands for ligand, and R for receptor [108]. Chemokines majorly influence T-cell differentiation as well as monocyte and T-cell trafficking. MCP-1, but also macrophage inflammatory protein 1 beta (MIP-1 β /CCL4) and interleukin 8 (IL-8/CXCL8), as well as the multitarget receptors CCR2, CCR1 and CXCR2 are involved in progression of cancer, malaria, HIV, transplant rejection, and autoimmune responses, just to name a few [105].

Not surprisingly, the identification of novel compounds to influence cytokine and chemokine dependent pathways is a persistent scientific issue. Thinking about nutrition worldwide, it makes sense to emphasize on foods and beverages with a high distribution and accessibility, like coffee, tea, cocoa, wine and grapes, fruits/vegetables and spices. Of course, all of those aliments consist of complex mixtures of chemically different substances but nevertheless, many specific compounds with immune-modulatory potential *in vitro* and *in vivo* have already been identified, including Resveratrol [109].

1.5.4 Immune-modulatory and Anti-inflammatory Activity of Resveratrol and its Metabolites

Resveratrol is a known potent immune-modulatory and anti-inflammatory agent *in vitro*. In TNF- α stimulated mouse fibroblasts (3T3/NIH) was identified as a potent activator of Sirtuin-1 (Sirt1), an indirect inhibitor of transcription factor Nf κ B, resulting in the reduction of the inflammatory response triggered by this pathway [110]. Deng et al. [111] showed the reduction of the TNF- α mediated cell adhesion between monocytes and endothelial cells by 60 % after a two hour pretreatment with 10 μ M Resveratrol, and after pretreatment with 3 μ M Resveratrol a significant

inhibition of mRNA expression of adhesion molecules [111]. Moreover, Cullen et al. [112] could determine the down-regulation and inhibition of binding activity of MCP-1 receptor in THP monocytes by Resveratrol in a concentration (0.1 – 100 μ M) and time (0 – 24 h) dependent manner. Besides the Nf κ B pathway, Resveratrol is also known for targeting cyclooxygenase enzymes and the prostaglandin system [8]. *In vivo*, in an animal model of acute small intestine inflammation, daily oral doses of 20 mg/kg bw (87 μ M) Resveratrol for 10 days, significantly lowered the tissue mRNA-expression of interferon gamma (IFN- γ), TNF- α , IL-6 and MCP-1 [113]. This study is just one of many examples of the anti-inflammatory effect of Resveratrol in pathological animal models (obesity, diabetes, asthma) [8]. Despite the promising effects *in vitro* and *in vivo* in animals, showing a modulation of inflammatory markers *in vivo* in humans proves surprisingly difficult. It seems that modest positive health effects of Resveratrol treatment can mainly be seen in already compromised individuals, whereas in healthy subjects, a preventive effect has yet to be determined (also see chapter 1.1) [36].

Some *in vitro* studies have already been conducted to elucidate the anti-inflammatory potential of Resveratrol metabolites. Resveratrol-3-sulfate, -3-glucuronide and -4'-glucuronide show similar antioxidant activity as the parent compound, as also determined in (6) *Concentration dependent effects of resveratrol and metabolites on the redox status of human erythrocytes in single dose studies*. The metabolites more strikingly exhibit nearly the same antiproliferative effect on Caco-2 cells at concentrations of 10 – 50 μ M after 48 h of treatment [114]. Resveratrol-4'-sulfate inhibit COX-1 and COX-2 comparable to Resveratrol with IC₅₀ values of 5.1 μ M/2.5 μ M and 1.1 μ M/1.3 μ M, respectively [115].

The here presented publications (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages* and (4) *Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations* present data on the anti-inflammatory and immune-modulatory impact of physiological metabolite concentrations (1-10 μ M) on activated human immune cells *in vitro*.

1.6 Impact of Resveratrol and its Metabolites on the Redox System

In a healthy state, reactive oxygen and nitrogen species (ROS, RNS) exert physiological roles in cellular signaling and host defense mechanisms, and are in balance with physiological antioxidants in the human body (antioxidant enzymes and vitamins) [14, 116]. Stress, pollution, exposure to radiation, smoking, drug intake or malnutrition can lead to an excess of oxidants, also termed “oxidative stress” [14]. This imbalance has been implicated in ageing, but also in the development and progression of various diseases, like cancer, diabetes, ischemia, inflammation, or neurodegenerative diseases [14].

1.6.1 Physiological Redox Balance

ROS are physiologically formed in the mitochondrial electron transport chain, in iron homeostasis, during lipid peroxidation, or in enzyme systems like NADPH oxidase, xanthine oxidase, cyclooxygenase, cytochrome P450 and nitric oxide synthase [117]. The formed radicals exert important functions in cell growth signaling, activation and deactivation of proteins via cysteine and methionine oxidation, autophagy, host defense mechanisms, or the activation of redox sensitive transcription molecules (activator-protein 1, nuclear factor erythroid related factor 2 (Nrf2) pathways) [117].

RNS are produced by nitric oxide synthases (NOS). Three types of NOS have been identified, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [14]. The most important action of nitric oxide (NO) is the maintenance of endothelial function. NO is produced in endothelial cells, and activates the cyclic guanylate cyclase in neighbouring smooth muscle tissue (vasculature), leading to vasorelaxation of mainly venous vessels [118]. NO is therefore important in blood pressure regulation, but also has functions in neurotransmission and immune reactions [14].

To counter an excess of ROS, mainly produced by the multitude of enzymes, defensive mechanisms are in place, mostly in the form of the antioxidant enzymes glutathione peroxidase (GPx), heme oxygenase (HO-1), superoxide dismutase (SOD) and catalase (CAT) [14]. Other antioxidants are limited by nutritional intake, like the vitamin A family (retinol, retinoids, carotenoids), vitamin C (ascorbic acid) and the vitamin E family (tocopherols and tocotrienols) [116].

1.6.2 Consequences of Redox Imbalance

High levels of ROS have been shown to interfere with the regulation of various pathways, including mitogen activated protein kinase (MAPK), phosphoinositol 3 kinase (PI3K) or Nrf2 [119]. Dysregulation of these pathways is associated with ageing, cancer, ischemic disorders, rheumatoid arthritis, diabetes, Alzheimer's or Parkinson's disease [14]. An imbalance in NO is also strongly associated with cardiovascular diseases [118]. The term "endothelial dysfunction" is partially defined by diminished NO levels, and an impaired vascular relaxation, in the long term leading to high blood pressure, atherosclerosis, and overexertion of the heart muscle tissue [14, 118]. NO and superoxide, both being highly reactive, can form peroxonitrite, possibly mediated by all three NOS enzymes, in the absence of L-arginine [120]. The peroxonitrite anion is a more stable but equally reactive oxidizing and nitrating agent, and has been shown to react with aromatic moieties, carbon dioxide, aldehydes, thiol groups and catalytic metals in enzymes, thus modifying proteins and impairing metabolic pathways [121, 122].

The identification of antioxidant substances in our daily diet, and increased intake of those aliments, would clearly help to alleviate high levels of ROS and RNS and maintain a healthy redox balance, preventing a multitude of diseases.

1.6.3 Targets of Resveratrol in Redox Balance

Resveratrol interferes with the redox balance by a combination of direct radical scavenging [123], induction of detoxifying enzymes (SOD, CAT, GPTx, HO-1) and increasing their activities [124, 125], and inhibition of radical producing enzyme systems [126]. Although some human intervention studies with oral Resveratrol in high risk patients or patients with manifested diseases (diabetes, obesity, cancer) have shown promising effects on disease related factors and ROS [12, 127], a beneficial effect on ROS and detoxifying enzyme levels in healthy humans could not yet be determined.

Considering the high metabolism rate and extensive plasma protein binding of the parent compound, but a lower binding rate for its metabolites, it might be possible that metabolites are responsible for the beneficial effects in patients with manifested diseases.

Introduction

To gain insight into the ability of Resveratrol metabolites to influence ROS levels and detoxifying enzymes, the publication (5) *Impact of Trans-Resveratrol-Sulfates and -Glucuronides on Endothelial Nitric Oxide Synthase Activity, Nitric Oxide Release and Intracellular Reactive Oxygen Species* assesses the effects of Resveratrol and single metabolites on the nitric oxide system in endothelial cells *in vitro*, and publication (6) *Concentration dependent effects of resveratrol and metabolites on the redox status of human erythrocytes in single dose studies* addresses the issue of the differential influence of low and high oral bolus doses on the redox balance in plasma and erythrocytes in healthy humans, comparing plasma ROS and enzyme activities to results from a cell free antioxidative assay.

2 Objectives

The overall aim of this PhD thesis was the bioactivity profiling and the identification of cellular targets of physiologically formed Resveratrol metabolites in comparison to effects already known for the parent compound. Experiments were conducted over a broad range of concentrations, but preferably under consideration of bioavailability and metabolism of oral Resveratrol, with physiological concentrations of <50 μM .

The efficacy of these compounds could strongly correlate to intracellular drug concentrations. Therefore, one objective of this thesis was to assess the uptake of Resveratrol and its metabolites. The time-, concentration- and/or transporter-dependent uptake of Resveratrol and metabolites was established in various *in vitro* models of immune and cancer cells. With cancer being a worldwide health issue, another aim was the assessment of anti-cancer properties in the here used cancer cell lines, not only to monitor growth inhibition, but also including the impact on DNA damage via the chemotherapy target topoisomerase II.

Another objective of this thesis was to establish the alleviating effects of Resveratrol metabolites on selected cytokines and chemokines in activated macrophages, as macrophages play the key role in immunity and inflammation, and are strongly involved in chronic and acute diseases.

Balancing cellular reactive oxygen species (ROS) is also a way to interfere with inflammation, atherosclerosis, cancer, or aging. The objective in regard of atherosclerosis was to assess the impact of Resveratrol and its metabolites on eNOS and NO levels in endothelial cells. The expression of phase II detoxifying enzymes, and radical scavenging by antioxidants present in plasma are mechanisms to cope with elevated levels of ROS. Thus, this thesis also aimed at phase II detoxifying enzyme activities, as well as plasma ROS levels in human blood after oral intake of high and low single dose oral Resveratrol.

3 Results

3.1 Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells

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In this work, the OATP-mediated uptake of selected physiological Resveratrol sulfate and glucuronide conjugates was investigated. CHO cells were transfected with three subtypes of OATP transporters (OATP1B1, OATP1B3, and OATP2B1). Uptake was measured and uptake kinetics were calculated. Additionally, OATP1B1 transporter was knocked down in the breast cancer cell line ZR-75-1, to establish OATP1B1-selective uptake and cytotoxicity of Resveratrol and Resveratrol-disulfate.

I synthesized and purified the glucuronated metabolites used in this study. Additionally, I participated in discussions regarding method development and participated in writing the manuscript.

RESEARCH ARTICLE

Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): Impact on growth of ZR-75-1 breast cancer cells

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Scope: Resveratrol is a naturally occurring polyphenolic compound with various pharmacological activities. These effects are observed despite its low bioavailability, which is particularly caused by extensive phase II metabolism. It is unknown whether resveratrol and its metabolites can accumulate to bioactive levels in organs and tissues through protein-mediated transport mechanisms. Because organic anion transporting polypeptides (OATPs) mediate the uptake of many clinically important drugs, we investigated their role in the cellular transport of resveratrol and its major glucuronides and sulfates.

Methods and results: Uptake experiments were performed with resveratrol and its glucuronides and sulfates in OATP-expressing Chinese hamster ovary (CHO) and breast cancer (ZR-75-1) cells. The uptake rates for resveratrol in OATP1B1-, OATP1B3-, and OATP2B1-transfected Chinese hamster ovary cells were four- to sixfold higher compared to wild-type cells. Resveratrol-3-O-4'-O-disulfate was transported by OATP1B1 and OATP1B3, while resveratrol-3-O-sulfate was exclusively transported by OATP1B3. However, resveratrol-4'-O-sulfate, resveratrol-3-O-glucuronide, and resveratrol-4'-O-glucuronide did not show any affinity for these OATPs. OATP-dependent uptake of resveratrol was also confirmed in ZR-75-1 cells.

Conclusion: Our data revealed that OATPs act as cellular uptake transporters for resveratrol and its major sulfates, which must be considered in humans following oral uptake of dietary resveratrol.

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Abbreviations: BSP, bromosulphophthalein; CHO, Chinese hamster ovary; OATP, organic anion transporting polypeptides; WT, wild-type

1 Introduction

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a naturally occurring compound found at low concentrations in grapes, berries, peanuts, and red wine [1]. Over the last decade, it has been shown that resveratrol exhibits a wide variety of biological and pharmacological properties. In several in vitro, animal models and human studies, resveratrol has been found to be active in the prevention and treatment of cancer, cardiovascular diseases, inflammation, ischemic injuries, and

neurodegenerative diseases, and resveratrol may also act as an antiobesity and antiaging compound [2]. The observed pharmacological activities of resveratrol cannot be explained by the low blood and tissue concentrations of unchanged resveratrol based on extensive metabolism in the gut and liver, leading to five to tenfold higher circulating plasma concentrations of resveratrol glucuronides and resveratrol sulfates compared with unconjugated resveratrol [3,4]. Currently, limited information is available regarding the possible benefits of resveratrol metabolites. Based on in vitro studies, resveratrol sulfates have been found to have comparable or greater potency than resveratrol against specific molecular targets, namely, COX 1 and 2, quinone reductase 1, nuclear factor κ B as well as similar ability to scavenge free radicals [5–7]. Furthermore, sulfates were also very recently shown to attenuate the *Escherichia coli*-LPS-induced IL-6 and TNF- α release [8]. In contrast to resveratrol sulfates, the few published studies have shown that resveratrol glucuronides are ineffective in various human cell lines, macrophages, and HIV-1 infection [8–12]. However, the in vitro activity of resveratrol metabolites may not necessarily reflect their in vivo function given that intracellular sulfatases or β -glucuronidases could easily convert the conjugates back to resveratrol [13]. Recent data from our laboratory have indeed shown that intracellular resveratrol-3-O-sulfate is readily hydrolyzed in breast tissue samples by members of the sulfatase family to regenerate parent resveratrol [13]. Monosulfate metabolites were also converted back to the parent compound in human colorectal cells [9]. The observed induction of autophagy and senescence, however, was abrogated by inclusion of a sulfatase inhibitor, which reduced intracellular resveratrol. Interestingly, cellular uptake of resveratrol-3-O-sulfate and resveratrol-4'-O-sulfate into cytoplasm of human colon cancer cells was dependent on cell line (HCEC < HCA-7 < HT-29), strongly indicating uptake transport mechanisms rather than passive diffusion [9]. An active transport for resveratrol and its conjugates is also supported by several in vitro and in vivo studies showing that the multidrug resistance-associated proteins (MRPs) namely MRP2 and MRP3 are responsible for the efflux of resveratrol glucuronides whereas breast cancer-resistance protein has been found to be the major efflux pump for native resveratrol and resveratrol sulfates [14,15]. We therefore hypothesize that resveratrol and its conjugates may accumulate to bioactive levels in cells, organs, and tissues through active transport mechanisms.

One major cellular uptake mechanism is via members of the organic anion transporting polypeptide (OATP) family [16–18]. Among the 11 human OATPs, OATP1B1 and OATP1B3 are highly expressed in the liver and mediate the uptake of numerous drugs into hepatocytes. OATP2B1 shows substantial expression in the apical membrane of enterocytes, where it contributes to the intestinal absorption of many endogenous compounds and clinically important drugs, thereby affecting drug disposition [19,20]. Based on the structural similarity of resveratrol-3-O-sulfate with estrone-3-sulfate, for which OATP1B1-, OATP1B3-, and OATP2B1-mediated up-

take exists, we hypothesized that resveratrol and its conjugates might also be substrates of these OATPs. Any differences in the affinity of resveratrol glucuronides and resveratrol sulfates to these transporters might also explain the observed differences in their pharmacological activity.

Recent data from our laboratory have also shown high expression of various OATPs in human hormone receptor-positive (MCF-7 and ZR-75-1) and negative (MDA-MB-231) breast cancer cell lines [21,22]. As pronounced resveratrol uptake into the cytoplasm was also observed in these cell lines we hypothesized that members of the OATP family may mediate intracellular resveratrol concentrations, thereby affecting cell growth.

Therefore, in the present study, we investigated the transport of resveratrol and its major metabolites, namely resveratrol-3-O-glucuronide, resveratrol-4'-O-glucuronide, resveratrol-3-O-sulfate, resveratrol-4'-O-sulfate, and resveratrol-3-O-4'-disulfate, in OATP1B1-, OATP1B3-, and OATP2B1-transfected Chinese hamster ovary (CHO) cells. In addition, the OATP-mediated transport of resveratrol and its correlation with cytotoxicity were also investigated in the human breast cancer cell line ZR-75-1.

2 Materials and methods

2.1 Chemicals

trans-Resveratrol was purchased from Sigma-Aldrich (Munich, Germany), and *trans*-resveratrol-3-O-glucuronide was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). *trans*-Resveratrol-3-O-sulfate, *trans*-resveratrol-4'-O-sulfate, *trans*-resveratrol-3-O-4'-O-disulfate, and *trans*-resveratrol-4'-O-glucuronide were synthesized as described previously [23,24]. MeOH and water were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals and solvents were commercially available and of analytical grade and were used without further purification.

2.2 Cell culture

CHO cells that were stably transfected with OATP1B1, OATP1B3, and OATP2B1 and wild-type (WT) CHO cells were provided by the University of Zurich, Switzerland and have been extensively characterized previously [25,26]. The CHO cells were grown in DMEM supplemented with 10% FCS, 50 μ g/mL L-proline, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The selective medium for stably transfected CHO cells additionally contained 500 μ g/mL geneticin sulfate (G418) [27]. All of the media and supplements were obtained from Invitrogen (Karlsruhe, Germany). The mammalian ZR-75-1 breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was maintained in RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, 100 μ g/mL

streptomycin, and 1% GlutaMAX. The cells were grown in T-flasks with a 25 cm² growth area (BD Biosciences, Franklin Lakes, NJ, USA), maintained at 37°C under 5% CO₂ and 95% relative humidity. The cells were passaged once a week and were used up to passage 55 [22].

2.3 OATP1B1 knockdown in ZR-75-1 cells via lentiviral transduction with a short hairpin RNA

For transduction, ZR-75-1 cells were plated in 24-well tissue culture plates at a density of 40 000 cells/well in 0.5 mL of growth medium. After 24 h, 250 µL of medium supplemented with 8 µg/mL polybrene (Sigma, H9268) were added. Transductions were performed by the addition of 10 µL of shRNA (Mission® Transduction Particles NM_006446, Sigma, TRCN0000043203, coding sequence CCGGGCCTTCATCTAAGGCTAACATCTCGA-GATGTTAGCCTTAGATGAAGGCTTTTGT). Twenty-four hours after transduction, the cell culture medium was changed, and 1 mL of growth medium supplemented with 1 or 5 µg/mL of puromycin (Sigma, P9620) was added to select infected cells after an additional 24 h. The obtained silencing efficiency was evaluated after 3 weeks via real-time PCR and immunofluorescence.

2.4 Real-time RT-PCR

Total RNA was extracted from cell lines using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration, purity, and integrity of the RNA samples were determined through UV absorbance and electrophoresis. Two micrograms of total RNA were reverse transcribed to cDNA using random hexamer primers and the RevertAid™ H Minus M-MuLV Reverse Transcriptase system (Fermentas, St. Leon-Rot, Germany), as recommended by the manufacturer. TaqMan® Gene Expression Assays (Applied Biosystems, Warrington, UK) were purchased for human OATP1B1. The 18S gene was used as a reference gene as previously described [21]. Multiplex quantitative real-time RT-PCR was performed in an amplification mixture with a volume of 20 µL. The target gene amplification mixture contained 10 µL of 2X TaqMan® Universal PCR Master Mix, 1 µL of the appropriate Gene Expression Assay, 1 µL of the TaqMan® endogenous control (human β-actin or 18S), 10 ng of template cDNA diluted in 5 µL of nuclease-free water and 3 µL of nuclease-free water. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence generation due to TaqMan® probe cleavage via the 5'→3' exonuclease activity of DNA polymerase was measured with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All samples were amplified in triplicate. To cover the range of expected Ct values for the target mRNA, a standard curve of six serial dilutions from 50 ng to

500 pg of pooled cDNA was analyzed using Sequence Detection Software (SDS 1.9.1., Applied Biosystems). The results were imported into Microsoft Excel for further analysis. Comparable cDNA contents in the experimental samples were calculated according to the standard curve method. Relative gene expression data are given as the *n*-fold change in transcription of target genes normalized to the endogenous control. Real-time RT-PCR was performed with the following prefabricated TaqMan® Gene Expression Assays (Applied Biosystems) containing intron-spanning primer Hs00272374_m1 for OATP1B1.

2.5 Immunofluorescence

ZR-75-1 OATP1B1-knockdown cells and cells transfected with the empty vector were allowed to attach on culture slides overnight (8-Chamber Polystyrene Vessel Tissue Culture-Treated Glass Slides, BD Falcon). Formalin fixation was followed by a washing step and a blocking step (by 5% BSA). The primary antibody against OATP1B1 (OATP1B1/1B3 mMDQ mouse mAb; Acris Antibodies, Herford, Germany) was diluted 1:100, and incubation was performed for 2 h. Optimal antibody concentrations were determined by titrating serial antibody dilutions. The applied dilutions corresponded to the minimum concentration necessary to produce a positive signal. WT and OATP1B1-transfected CHO cells were used as controls. Following incubation with the secondary antibody (1:1000 dilution; Alexa Fluor® 488 Goat Anti-Mouse IgG; Invitrogen, Carlsbad, CA, USA) for 30 min, cell nuclei were stained with 0.5 µg/mL Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA). Thereafter, the slides were rinsed with distilled water before being mounted in Mowiol 4–88 (Carl Roth, Karlsruhe, Germany). Fluorescent staining was visualized with an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Images were captured using an AxioCam HRC2 Color CCD digital camera and Axiovision 4.8 software (Carl Zeiss Vision GmbH, Aalen, Germany). To minimize background signals and to make the signal intensity and extension in different samples comparable, the exposure times for the individual antibodies were evaluated and kept constant between the samples.

2.6 Western blotting

Western blotting to confirm OATP1B1-, OATP1B3-, and OATP2B1-overexpression in transfected CHO cells was performed with membrane extracts (Pierce, Rockford, IL, USA) from these cells. WT CHO cell membrane extracts were used as controls. Protein content was determined with an assay kit from Bio-Rad Laboratories (Hercules, CA, USA) with BSA as the standard. For immunoblotting, 25 µg of protein were separated by SDS polyacrylamide gel 12% electrophoresis and transferred onto Hybond PVDF membranes. To control equal sample loading, protein on the membranes were stained

with Ponceau S. In the immunoblot, 5% nonfat dry milk in TBS/0.1% Tween 20 was used for blocking. As primary antibodies an anti-OATP1B1/1B3 mouse mAb from Acris Antibodies (BM5542) and an anti-OATP2B1 rabbit mAb from Abcam Antibodies (ab83532, Cambridge, UK) diluted 1:500 in blocking solution were applied. Peroxidase-conjugated species-specific IgGs were used as secondary antibodies (dilution 1:2000). Detection was done with an ECL detection kit from Thermo Scientific (Portsmouth, NH, USA) and Amersham Hyperfilms (GE-Healthcare, Amersham, UK).

2.7 Uptake assays in CHO cells

Transport assays were performed on 12-well plates as described in detail elsewhere [25]. Briefly, CHO cells were seeded at a density of 350 000 cells per well on 12-well plates (BD Biosciences). Uptake assays were generally performed on day 3 after seeding, when the cells had grown to confluence. Twenty-four hours before starting the transport experiments, the cells were additionally treated with 5 mM sodium butyrate (Sigma-Aldrich) to induce nonspecific gene expression [28]. Resveratrol and its sulfates were dissolved in DMSO and diluted with uptake buffer pH 7.4 (final DMSO concentration of 0.5%, which was constant in all transport experiments) to 12.5–600 μ M. Control experiments contained DMSO in the medium in place of resveratrol. Prior to the transport experiment, the cells were rinsed twice with 2 mL of prewarmed (37°C) uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4 , 0.8 mM MgSO_4 , 5.5 mM D-glucose, and 20 mM Hepes; pH adjusted to 7.4). Uptake was initiated by adding 0.25 mL of uptake buffer containing the substrate. After the indicated time period at 37°C, uptake was stopped by removing the uptake solution and washing the cells five times with 2 mL of buffer (pH 7.4). The cells were then trypsinized by the addition of 100 μ L of trypsin and transferred into test tubes. Next, the cell membranes were disrupted via repeated (five times) shock freezing in liquid nitrogen and thawing. Following centrifugation at $13\,500 \times g$ for 5 min, 100 μ L of the supernatant was diluted with methanol/water (2:1; v/v), and aliquots (80 μ L) were analyzed via HPLC [22].

As most OATPs are stimulated by an acidic pH, uptake experiments were also performed at pH 6.5 under identical conditions as mentioned above except now using acidic uptake and washing buffers.

2.8 Uptake assays in ZR-75-1 cells

Transport assays were performed on 12-well plates as described above for CHO cells. Resveratrol and its disulfate were dissolved in DMSO and were diluted with uptake buffer (pH 6.5; final DMSO concentration of 0.5%) to 12.5–400 μ M. The experiments were performed in triplicate. Control experiments contained DMSO in the medium in place of resveratrol. Prior to the transport experiment, the cells were rinsed twice with 2 mL of prewarmed (37°C) uptake buffer. Uptake

was initiated by adding 0.25 mL of uptake buffer containing the substrate. After 1 min at 37°C, uptake was stopped by removing the uptake solution and washing the cells five times with 2 mL of buffer. After trypsinization, cell membranes were disrupted via repeated shock freezing in liquid nitrogen. Following centrifugation, the supernatant was diluted with methanol/water, and aliquots (80 μ L) were analyzed via HPLC as described above.

2.9 Inhibition analysis

For the inhibition experiments with rifampicin and bromosulphophthalein (BSP; Sigma-Aldrich), stock solutions of these compounds were prepared in DMSO containing the indicated concentrations. CHO cells grown on 12-well plates were first washed twice with prewarmed uptake buffer (pH 7.4) and incubated for 10 min at 37°C under 5% CO_2 with 1 μ M resveratrol or 10 μ M resveratrol sulfates (resveratrol-3-O-disulfate and resveratrol-3-O-4'-O-disulfate) in the presence of the inhibitors ranging from 0.0001 to 100 μ M. Control experiments were performed without BSP and rifampicin under identical conditions as mentioned above.

2.10 Metabolism studies in ZR-75-1 cells

Cells were plated on six-well plates and allowed to attach overnight. Resveratrol was dissolved in DMSO and diluted with medium (final DMSO concentration <0.1%) to a concentration of 5–100 μ M. The experiments were performed under each set of conditions in triplicate. Control experiments contained DMSO in the medium in place of resveratrol. After 72 h, the medium was aspirated via suction, and aliquots (100 μ L) were subsequently analyzed through HPLC. The cells were then trypsinized by the addition of 100 μ L of trypsin, washed three times with phosphate buffered saline, and lysed by repeated (five times) shock freezing in liquid nitrogen and thawing. Following centrifugation at $13\,500 \times g$ for 5 min, 80 μ L of the supernatant (cytoplasm) was subsequently analyzed by HPLC [22]. Additionally, the cell pellets containing the membranes were extracted with 200 μ L of methanol and analyzed by HPLC for their resveratrol content. The protein concentration in the cell pellets was determined using a bicinchoninic acid protein assay kit (Pierce Science, Rockford, IL, USA), with BSA as a standard.

2.11 Cytotoxicity assay

CellTiter-Blue (Promega, Southampton, UK) is a colorimetric and fluorescent assay used to measure cell viability via nonspecific redox enzyme activity (reduction from resazurin to resorufin by viable cells). ZR-75-1 cells (50 000 cells/mL) were seeded into 96-well flat-bottomed plates and incubated for 24 h at 37°C under 5% CO_2 . For cytotoxicity assays, the cells were incubated with various

concentrations of resveratrol (5–400 μM) for 72 h. The CellTiter-Blue (20 μL) reagent was added to the wells, and the plate was incubated for 2 h, protected from light. The absorbance was recorded for resazurin (605 nm) and resorufin (573 nm). The assay results were measured on a Tecan M200 multimode plate reader (Tecan Austria GmbH, Groedig, Austria). The absorbance was also measured in CellTiter-Blue assays in blank wells (without resveratrol) and deducted from the values from experimental wells. The viability of the treated cells was expressed as a percentage of the viability of the corresponding control cells. All experiments were repeated at least three times.

2.12 Determination of protein concentrations

Total protein was determined using the colorimetric bicin-choninic acid protein assay kit (Pierce Science) with BSA as a standard and quantification at a wavelength of 562 nm on a spectrophotometer (UV-1800, Shimadzu). Raw data were analyzed using UVProbe software (version 2.31, Shimadzu). The protein concentrations were consistent among the plates (0.150 ± 0.005 mg/well).

2.13 HPLC analysis

Resveratrol and its glucuronidated and sulfated biotransformation products were quantified by HPLC as described previously [22, 29] using a Dionex UltiMate 3000 system (Sunnyvale, CA, USA) equipped with an L-7250 injector, an L-7100 pump, an L-7300 column oven (set at 15°C), a D-7000 interface and an L-7400 UV detector (Thermo Fisher Scientific, Waltham, MA, USA) set at a wavelength of 307 nm. Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed by spiking drug-free cell culture medium with standard solutions of *trans*-resveratrol, *trans*-resveratrol-3-O-sulfate, *trans*-resveratrol-4'-O-sulfate, *trans*-resveratrol-3-O-4'-O-disulfate, *trans*-resveratrol-3-O-glucuronide, and *trans*-resveratrol-4'-O-glucuronide to give a concentration range from 0.00257 to 25.7 μM (average correlation coefficients: >0.999). For this method, the lower limit of quantification for resveratrol, resveratrol sulfates and resveratrol glucuronides was 11, 13, and 25 nM, respectively. Coefficients of accuracy and precision for these compounds were $<11\%$.

2.14 Data analysis

Kinetic analysis of the uptake of resveratrol and metabolites was performed over a substrate concentration range of 1–600 μM . Prior to these experiments, the linearity of cellular uptake over time (1, 3, and 10 min) was individually determined for WT and OATP-transfected CHO cells by using resveratrol (50 μM) as a substrate. Cellular uptake rates are presented

after normalization for the incubation time and total protein content. Net uptake rates were calculated as the difference in the uptake rate of the transfected and WT cells for each individual concentration. The data were fitted to the Michaelis–Menten model. Kinetic parameters were calculated using the Graph-Pad Prism Version 5.0 software program (GraphPad Software, San Diego, CA, USA) for Michaelis–Menten: $V = V_{\max} \times S / (K_m + S)$, where V is the rate of the reaction; $V_{\max}$ is the maximum velocity; K_m is the Michaelis constant, and S is the substrate concentration. The intrinsic clearance, which is defined as the ratio $V_{\max}/K_m$, quantifies the transport capacity. IC_{50} values were determined by plotting the log inhibitor concentration against the net uptake rate and nonlinear regression of the dataset using the equation:

$$\gamma = \frac{a}{1 + [I/(\text{IC}_{50})^s + b]}$$

in which γ is the net uptake rate (pmol/ μg protein/min), I is the inhibitor concentration (μM), s is the slope at the point of inversion, and a and b are the maximum and minimum values for cellular uptake. Net uptake was calculated for each inhibitor concentration as the difference in the uptake rates of the transporter-expressing and WT cell lines. Unless otherwise indicated, values are expressed as mean $\pm$ SD of three individual experiments. Significant differences from control values were determined using a Student's paired t -test at a significance level of $p < 0.05$.

3 Results

3.1 Accumulation of resveratrol and metabolites in transfected CHO cells

To investigate whether resveratrol and its major conjugates are substrates of OATPs, uptake analysis were performed in OATP1B1-, OATP1B3-, and OATP2B1-transfected CHO cells which highly expressed these transporters in the membranes (Supporting Information Fig. 1). Uptake of resveratrol (12.5–400 μM) at pH 7.4 for all three OATPs was only linear for up to 1 min (Fig. 1). We therefore finalized all experiments at 1 min (initial linear phase). As shown in Table 1 and Fig. 2A, the initial net OATP1B1-, OATP1B3-, and OATP2B1-mediated accumulation rates (transfected-WT) for resveratrol followed Michaelis–Menten kinetics, with approximately twofold higher $V_{\max}$ values for OATP1B1 compared to OATP1B3 and OATP2B1 ($V_{\max}$: 1100 versus 818 versus 640 pmol/mg protein/min at pH 7.4). K_m -values were the lowest for OATP1B3 (62.0 μM) and followed by OATP2B1- (88.1 μM) and OATP1B1-transfected cell lines (88.4 μM). The uptake of resveratrol-3-O-4'-O-disulfate (25–600 μM) in OATP1B1- and OATP1B3-transfected CHO cells, however, was less pronounced, showing $V_{\max}$ values of 100 and 107 pmol/mg protein/min, respectively (Fig. 2C). Interestingly, the affinity

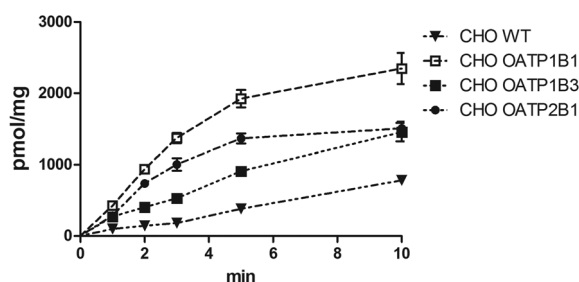


Figure 1. Time-dependent uptake of resveratrol in OATP-transfected CHO cells. The uptake of resveratrol (50 μM) after 1, 2, 3, 5, and 10 min was determined in wild-type, OATP1B1-, OATP1B3-, and OATP2B1-transfected CHO cells at pH 7.4, 37°C. The data represent the mean $\pm$ SD of three individual determinations.

of resveratrol-3-*O*-4'-*O*-disulfate for OATP1B1 and OATP1B3 was about twofold higher with K_m -values of 37.6 and 37.5 μM compared to resveratrol. Resveratrol-3-*O*-sulfate (25–600 μM) was only taken up by OATP1B3, again with low K_m (101 μM) and V_{max} (122 pmol/mg protein/min) values. The uptake of resveratrol-4'-*O*-sulfate, resveratrol-3-*O*-glucuronide, and resveratrol-4'-*O*-glucuronide by OATP1B1-, OATP1B3-, and OATP2B1-transfected and WT CHO cells was below the detection limit (13–25 nM). As it is well known that most OATPs are stimulated by an acidic extracellular pH [27], we also performed uptake experiments at pH 6.5 with WT and OATP-transfected CHO cells. Table 1 revealed that the K_m values for the uptake of resveratrol and its sulfated conjugates were in general lower at pH 6.5 compared to pH 7.4 but only reached a level of significance for resveratrol-3-*O*-4'-*O*-disulfate by OATP1B1 and OATP1B3. V_{max} values also showed a pH dependency which was, however, less

pronounced and only significant for resveratrol uptake by OATP1B3 and OATP2B1.

3.2 Effect of OATP inhibitors on the accumulation of resveratrol and major sulfates in transfected CHO cells

To investigate whether known OATP inhibitors impact OATP1B1-, OATP1B3-, and OATP2B1-mediated resveratrol accumulation, resveratrol was quantified after incubation of 1 μM in OATP1B1-, OATP1B3-, and OATP2B1-expressing CHO cells at pH 7.4 in the absence and presence of increasing concentrations of BSP and rifampicin. The inhibition of resveratrol uptake by BSP in OATP1B3-expressing CHO cells was more potent (IC_{50} : 1.09 μM) compared to OATP1B1- and OATP2B1-expressing CHO cells (IC_{50} values: 1.39 and 1.47 μM , respectively). Rifampicin was an even more potent inhibitor of resveratrol uptake in OATP2B1 (IC_{50} : 0.48 μM) but not in OATP1B1- and OATP1B3-transfected CHO cells (IC_{50} : 1.24 and 1.34 μM , respectively). Uptake inhibition of 10 μM resveratrol-3-*O*-sulfate in OATP1B3-transfected CHO cells by BSP and rifampicin was also pronounced with IC_{50} values of 3.52 and 0.33 μM , respectively. Inhibition of resveratrol-3-*O*-4'-*O*-disulfate uptake by BSP and rifampicin resulted in even lower IC_{50} values for OATP1B1 and OATP1B3 (0.27 and 0.29 μM versus 0.25 and 0.23 μM).

3.3 OATP1B1 knockdown in ZR-75-1 cells

The PCR data from various lentiviral-transfected clones revealed an up to tenfold reduction of OATP1B1 expression in ZR-75-1 cells. The cells exhibiting the lowest expression

Table 1. pH dependency of Michaelis–Menten parameters in stably transfected CHO cells

Substrate	pH 7.4		pH 6.5	
	K_m [μM]	V_{max} [pmol/mg/min]	K_m [μM]	V_{max} [pmol/mg/min]
OATP1B1				
Res	88.4 $\pm$ 17.3	1100 $\pm$ 67.9	79.8 $\pm$ 16.9	1149 $\pm$ 74.9
3OS	n.d.	n.d.	n.d.	n.d.
DiS	37.6 $\pm$ 5.23*	100.3 $\pm$ 3.12*	20.7 $\pm$ 7.38*	83.9 $\pm$ 4.13*
OATP1B3				
Res	62.0 $\pm$ 10.6*	818 $\pm$ 39.3*	42.9 $\pm$ 12.5*	653 $\pm$ 50.4*
3OS	101 $\pm$ 28.1*	122 $\pm$ 9.61	80.6 $\pm$ 29.1*	120.5 $\pm$ 13.0
DiS	37.5 $\pm$ 5.87*	107.6 $\pm$ 3.76*	13.5 $\pm$ 8.17*	99.6 $\pm$ 6.19*
OATP2B1				
Res	88.1 $\pm$ 17.8	640 $\pm$ 40.5*	77.0 $\pm$ 18.92	990.6 $\pm$ 73.7*
3OS	n.d.	n.d.	n.d.	n.d.
DiS	n.d.	n.d.	n.d.	n.d.

Values are means $\pm$ SD of three individual determinations. The net OATP-mediated uptake values were calculated by subtracting the values obtained with the wild-type CHO cells from those obtained with the stably transfected cells. Kinetic parameters were calculated by fitting the data to the Michaelis–Menten (K_m) equation with nonlinear regression. Res, resveratrol; 3OS, resveratrol-3-*O*-sulfate; DiS, resveratrol-3-*O*-4'-*O*-disulfate; n.d., not determined. K_m and V_{max} values in bold and marked with an asterisk are significantly different ($p < 0.05$) between pH 6.5 and pH 7.4.

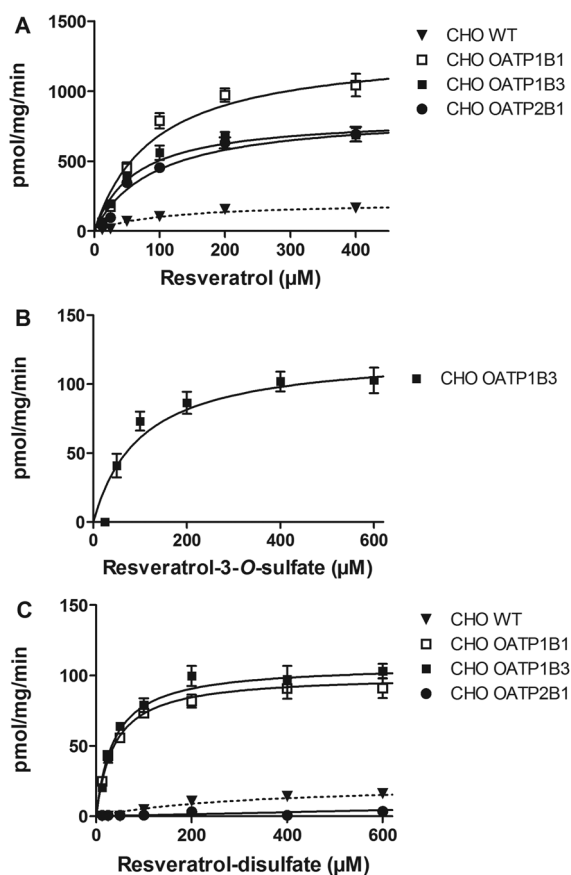


Figure 2. Uptake of resveratrol and its major sulfates in OATP-transfected and wild-type CHO cells. The uptake of resveratrol (12.5–400 μ M) (A), resveratrol-3-*O*-sulfate (25–600 μ M) (B), and resveratrol-3-*O*-4'-*O*-disulfate (25–600 μ M) (C) by OATP1B1-, OATP1B3- and OATP2B1-transfected CHO cells and wild-type CHO cells was determined after 1 min at pH 7.4, 37°C. The data represent the mean $\pm$ SD of three individual determinations.

of OATP1B1 (relative mRNA expression was reduced from 14.78 ± 0.26 to 1.19 ± 0.02 based on the change to the calibrator) were chosen for further experiments. Because ZR-75-1 cells do not express OATP1B3 and OATP2B1, but do express OATP1B1 [21], the expression of the OATP1B1 protein was confirmed via immunofluorescence using a specific OATP1B1/1B3 mouse mAb. Figure 3 clearly shows normal expression (bright green fluorescence) of OATP1B1 in ZR-75-1 empty vector cells (A) and extremely low expression of this transporter in ZR-75-1 OATP1B1-knockdown cells (B). OATP1B1-transfected CHO cells (C) were used as positive control, compared to WT CHO cells (D) without fluorescence.

3.4 Resveratrol accumulation in WT and ZR-75-1 OATP1B1-knockdown cells

Based on the much higher OATP1B1 mRNA level found in the WT ZR-75-1 breast cancer cell line compared to the

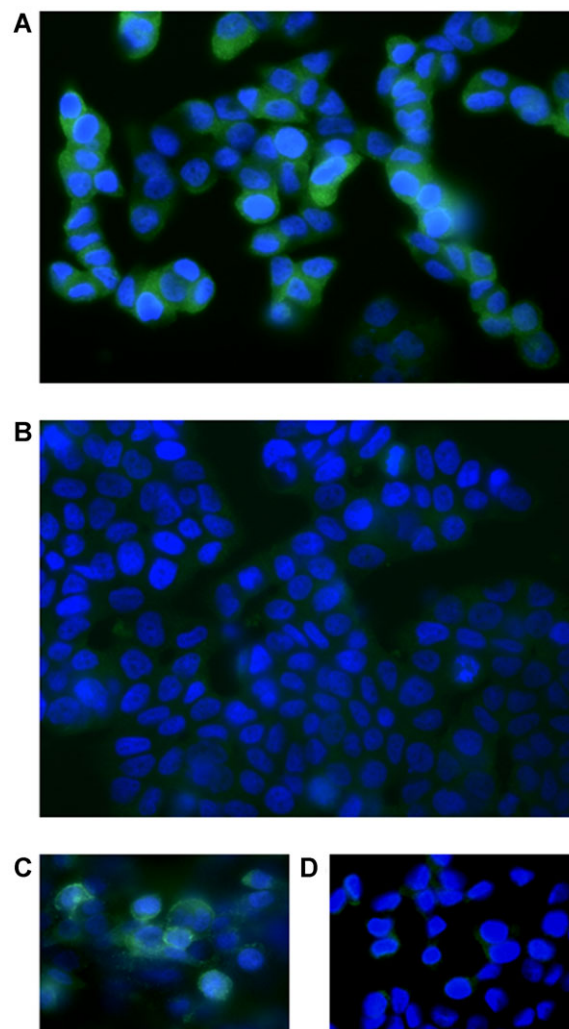


Figure 3. Immunofluorescent characterization of OATP1B1 in ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. Cells were grown on culture slides and stained with an antibody against OATP1B1 (green) while nuclei were visualized by Hoechst 33342 staining to give a bright blue color (see Section 2). Immunofluorescence was performed in ZR-75-1 empty vector-transfected cells (A), ZR-75-1 OATP1B1 knockdown cells (B), OATP1B1-transfected CHO cells (C), and CHO wild-type cells (D). Bright green fluorescence was seen in ZR-75-1 empty vector-transfected cells (A) and OATP1B1-transfected CHO cells (C).

OATP1B1-knockdown clone, we expected that OATP1B1 expression might be directly correlated with intracellular resveratrol concentrations. For kinetic analysis, an incubation time of 1 min was selected in order to prevent cellular uptake from interference with cellular efflux mechanisms such as MRPs and breast cancer-resistance protein. Figure 4A depicts representative Michaelis–Menten plots for resveratrol uptake by WT and ZR-75-1 OATP1B1-knockdown cells, where significantly higher uptake rates and approximately twofold lower K_m values were found in the OATP1B1-expressing control cells (V_{max} : 3360 versus 2412 pmol/mg protein/min; K_m : 144

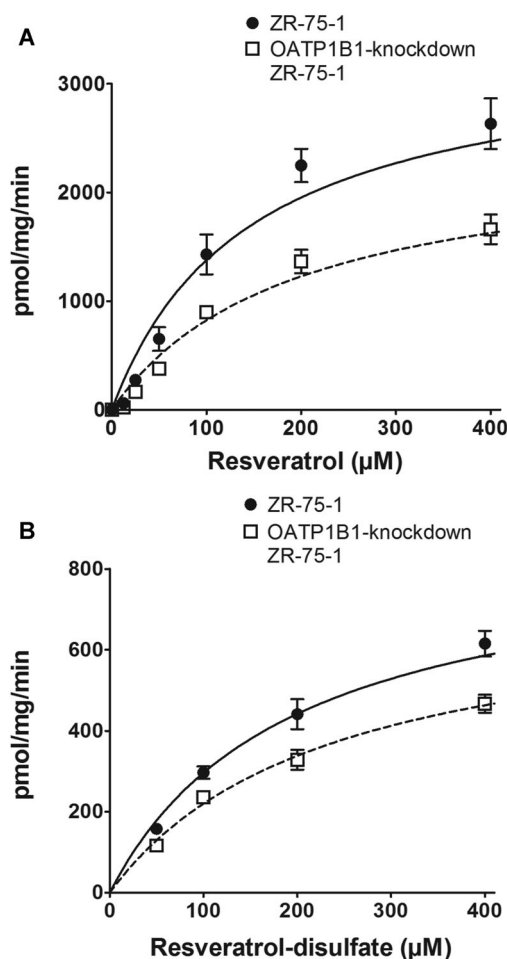


Figure 4. Uptake of resveratrol and resveratrol disulfate in ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. The uptake of resveratrol (12.5–400 μ M) (A) and resveratrol-3-*O*-4'-*O*-disulfate (12.5–400 μ M) (B) in ZR-75-1 empty vector-transfected and OATP1B1 knockdown ZR-75-1 cells was determined after 1 min at pH 7.4, 37°C. The data represent the mean $\pm$ SD of three individual determinations.

versus 193 μ M). Resveratrol-3-*O*-4'-*O*-disulfate, a substrate of OATP1B1, also showed higher uptake rates and a lower affinity for this transporter in ZR-75-1 cells transfected with the empty vector compared with the OATP1B1-knockdown clone ($V_{\max}$: 870 versus 734 pmol/mg protein/min; K_m : 194 versus 234 μ M). However, incubation with resveratrol-3-*O*-sulfate, which is a substrate of OATP1B3 but not of OATP1B1, did not show any differences in uptake in the two cell lines, thus strongly indicating the impact of OATP1B1 on resveratrol transport.

3.5 Cytotoxicity of resveratrol and resveratrol disulfate in ZR-75-1 OATP1B1-knockdown cells

The cytotoxicity of resveratrol in WT and OATP1B1-knockdown ZR-75-1 breast cancer cells was quantified using

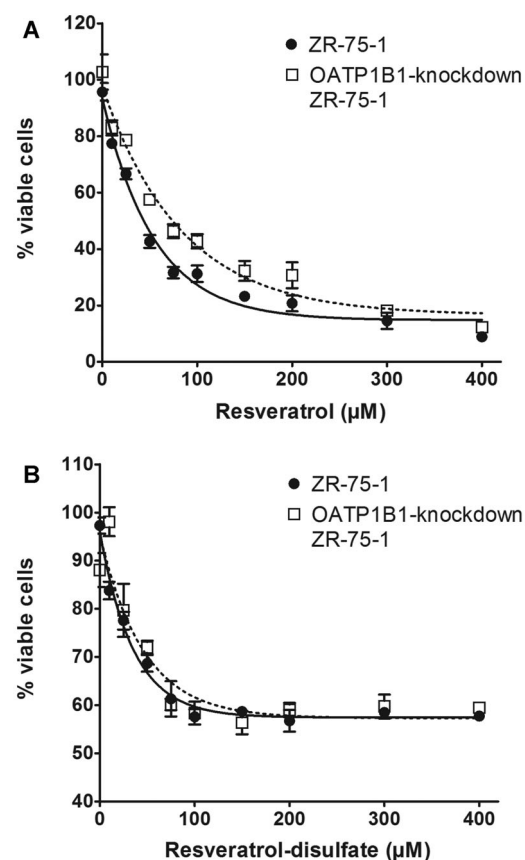


Figure 5. Cytotoxicity of resveratrol and resveratrol disulfate to ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. After incubation for 72 h with 10–400 μ M resveratrol (A) and resveratrol-3-*O*-4'-*O*-disulfate (B) at 37°C percent viable cells were determined (see Section 2). Dose response curves were obtained by nonlinear curve fitting using GraphPad Prism 5.0 program. The data represent the mean $\pm$ SD of three individual determinations.

the CellTiter-Blue test kit from Promega, as described above. As shown in Fig. 5, resveratrol exhibited an approximately twofold lower IC_{50} value in control ZR-75-1 cells (37.7 μ M) compared to the OATP1B1 knockdown clone (58.0 μ M), supporting the importance of OATP1B1-dependent resveratrol uptake. This trend was also true for resveratrol-3-*O*-4'-*O*-disulfate, for which the IC_{25} value was lower in WT cells (6.97 μ M) compared to OATP1B1-knockdown cells (9.15 μ M). Due to the overall low cytotoxicity of resveratrol-3-*O*-4'-*O*-disulfate, we could not determine IC_{50} values in either cell line.

3.6 Differences in the metabolism of resveratrol and resveratrol disulfate in ZR-75-1 cells

To further evaluate the differences in resveratrol biotransformation, WT and OATP1B1-knockdown ZR 75-1 breast cancer cells were incubated for 72 h with resveratrol and

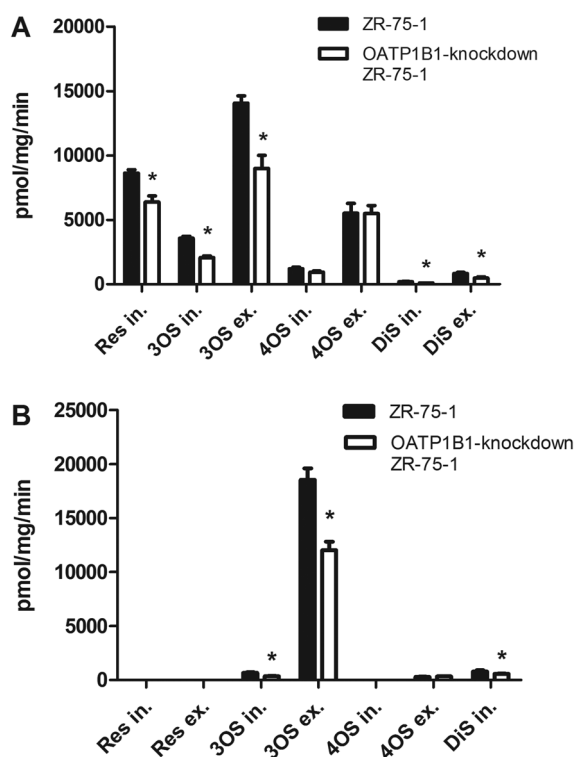


Figure 6. Metabolism of resveratrol and resveratrol disulfate in ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells. The concentrations of resveratrol, resveratrol-3-O-4'-O-disulfate as well as the formation of metabolites (DiS, resveratrol disulfate; 3OS, resveratrol-3-O-sulfate; and 4OS, resveratrol-4'-O-sulfate) were determined after incubation for 72 h with 50 μ M resveratrol (A) and 50 μ M resveratrol-3-O-4'-O-disulfate (B) in ZR-75-1 and ZR-75-1 OATP1B1-knockdown cells at 37°C. Concentrations were determined in the medium (ex., extracellular) and in the cytoplasm (in., intracellular). The data represent the mean $\pm$ SD of three individual determinations. Columns marked with an asterisk are significantly different ($p < 0.05$).

resveratrol-3-O-4'-O-sulfate (50 μ M), and the cytoplasm and cellular supernatant were assayed to quantify resveratrol, resveratrol-3-O-sulfate, resveratrol 4'-O-sulfate, and resveratrol-3-O-4'-O-disulfate via HPLC.

As shown in Fig. 6A, the intracellular concentrations of resveratrol after incubation with 50 μ M resveratrol for 72 h were significantly lower in the OATP1B1-knockdown cells compared to WT cells (6398 ± 457 versus 8620 ± 270 pmol/h/mg protein), leading to decreased formation of resveratrol-4'-O-sulfate (943 ± 102 versus 1215 ± 113 pmol/h/mg protein) and resveratrol-3-O-4'-O-disulfate (104 ± 12 versus 188 ± 36 pmol/h/mg protein).

In order to verify that most of resveratrol reached the cytoplasm did not remain in the membranes, cell pellets (containing the membranes) were extracted with methanol and analyzed by HPLC. Resveratrol concentrations in the cellular membranes were low (<5%) excluding any accumulation in the lipid bilayers.

As observed in the cytoplasm, resveratrol-3-O-sulfate was the major biotransformation product in the cellular supernatant ($14\,060 \pm 561$ pmol/h/mg protein in WT cells versus 8992 ± 1022 pmol/h/mg protein in ZR-75-1 OATP1B1-knockdown cells). Similarly, the concentrations of resveratrol-4'-O-sulfate and resveratrol-3-O-4'-O-disulfate were lower in ZR-75-1 OATP1B1-knockdown cells compared to WT cells (5532 ± 751 versus 5488 ± 627 pmol/h/mg protein; and 849 ± 79 versus 496 ± 83 pmol/h/mg protein).

Notably, the concentrations of resveratrol metabolites were approximately threefold higher in the medium compared to the cytosolic samples, thereby strongly indicating the occurrence of rapid cellular efflux after formation. As shown in Fig. 6B, the major metabolite following the uptake of 50 μ M resveratrol-3-O-4'-O-disulfate was the deconjugation product, resveratrol-3-O-sulfate, followed by resveratrol-4'-O-sulfate. Parent resveratrol was below the detection limit of 11 nM.

4 Discussion

To identify the relevance of uptake transporters to the in vivo activity and to identify the human OATP isoforms responsible for the hepatic uptake of resveratrol and its glucuronidated and sulfated conjugates, we employed cells that stably expressed these OATPs. As shown in Fig. 2 and Table 1, resveratrol exhibited saturable uptake kinetics at pH 7.4 for OATP1B1, OATP1B3, and OATP2B1. The highest affinity was observed for OATP1B3, with a K_m of 62 μ M, whereas the affinities for OATP1B1 and OATP2B1 were lower.

Interestingly, resveratrol-3-O-sulfate was only transported by OATP1B3 with a low affinity (K_m : 101 μ M), while resveratrol-3-O-4'-O-disulfate was transported with equally high affinities by OATP1B1 (K_m : 37.6 μ M) and OATP1B3 (K_m : 37.5 μ M). The K_m/V_{max} value for the OATP1B1-mediated uptake of resveratrol-3-O-4'-O-disulfate was similar to that for OATP1B3 (2.67 versus 2.87 μ L/min· μ g) (see Table 2). Resveratrol-4'-O-sulfate did not show any affinity for any of the three OATPs. Notably, there was also no uptake of resveratrol-3-O-glucuronide or resveratrol-4'-O-glucuronide, indicating that resveratrol sulfates, and not glucuronides, provide the intracellular pools for resveratrol generation [9].

Based on Michaelis–Menten parameters, however, the exact contribution of OATP2B1 in the gut and of OATP1B1, OATP1B3, and OATP2B1 in the liver to the overall uptake of resveratrol and its major sulfates in humans cannot be determined because of high interindividual variability (up to tenfold) in OATP levels as determined by quantitative proteomics or by Western blotting [30–32]. Our data also suggest that OATP1B1, OATP1B3, and OATP2B1 are low-affinity transporters. Because of the low bioavailability, the portal concentration of resveratrol is probably lower than the K_m values. Indeed, administration of resveratrol (5.0 g/day) for up to 28 days to healthy volunteers showed peak plasma concentrations of 4 μ M and 13 μ M [33] for unconjugated resveratrol

Table 2. pH dependency of Michaelis–Menten parameters in stably transfected CHO cells

Substrate	pH 7.4 $V_{\max}/K_m$ [$\mu\text{L}/\text{min}\cdot\mu\text{g}$]	pH 6.5 $V_{\max}/K_m$ [$\mu\text{L}/\text{min}\cdot\mu\text{g}$]
OATP1B1		
Res	12.4 $\pm$ 3.07	14.4 $\pm$ 3.88
3OS	n.d.	n.d.
DiS	2.67 $\pm$ 0.45	4.05 $\pm$ 1.93
OATP1B3		
Res	13.2 $\pm$ 2.79	15.2 $\pm$ 5.82
3OS	1.21 $\pm$ 0.44	1.5 $\pm$ 0.75
DiS	2.87 $\pm$ 0.55	7.41 $\pm$ 8.27
OATP2B1		
Res	7.26 $\pm$ 1.85*	12.9 $\pm$ 4.07*
3OS	n.d.	n.d.
DiS	n.d.	n.d.

Values are means $\pm$ SD of three individual determinations. The net OATP-mediated uptake values were calculated by subtracting the values obtained with the wild-type CHO cells from those obtained with the stably transfected cells. Kinetic parameters were calculated (see Table 1). Res, resveratrol; 3OS, resveratrol-3-O-sulfate; DiS, resveratrol-3-O-4'-O-disulfate; n.d., not determined. $V_{\max}/K_m$ values in bold and marked with an asterisk are significantly different ($p < 0.05$) between pH 6.5 and pH 7.4.

and resveratrol-3-O-sulfate, respectively. However, concentrations in the colon tissue after administration of resveratrol (1.0 g/day) for up to 8 days to patients with colorectal cancer were significantly higher with mean values of 674 μM for resveratrol and 67 μM for resveratrol-3-O-sulfate [34] and presumably also resveratrol-3-O-4'-O-disulfate, a metabolite recently shown to be present in human plasma at the same level as resveratrol-3-O-sulfate [35].

Interestingly, affinity of resveratrol and its sulfates to OATPs was overall higher at pH 6.5 compared to 7.4. This is in accordance with literature data [27] also showing pH differences in the uptake of OATP substrates. As OATP2B1 is expressed in the upper part of the gut, which also shows a slightly acidic microenvironment [36] and as several literature data have shown an acid pH in the tumor environment [37, 38], pH dependency of Michaelis–Menten parameters for OATPs have to be considered in vivo.

To further demonstrate the importance of OATP1B1 for the uptake of resveratrol and its sulfates, hormone-dependent

ZR-75-1 cells that were previously shown to express high levels of OATP1B1, but not OATP1B3 and OATP2B1 [21], were incubated for 1 min with increasing concentrations of resveratrol and resveratrol-3-O-4'-O-disulfate. Indeed, the uptake of resveratrol by the ZR-75-1 OATP1B1-knockdown cells was significantly reduced compared to control cells, as indicated by higher K_m and lower $V_{\max}$ values (Fig. 4 and Table 3). Concomitant with the decreased uptake detected in ZR-75-1 knockdown cells, we also observed decreased formation of the metabolites resveratrol-4'-O-sulfate and resveratrol-3-O-sulfate after 72 h, which led to a higher IC_{50} value in the cytotoxicity assay in OATP1B1-expressing WT cells (37.7 versus 58 μM ; Fig. 5). As expected, we also observed lower uptake of the OATP1B1 substrate resveratrol-3-O-4'-O-disulfate in the ZR-75-1 OATP1B1-knockdown cells, which was confirmed by decreased formation of resveratrol-3-O-sulfate. Due to its several-fold lower cytotoxicity in breast cancer cell lines [24], the IC_{50} values for resveratrol-3-O-4'-O-disulfate could not be determined in the ZR-75-1 control and OATP1B1-knockdown cells. However, the IC_{25} values were again lower in WT cells (6.97 μM) compared to the OATP1B1-knockdown cells (9.15 μM). As demonstrated in a previous study, metabolite concentrations in the cellular medium were up to 15-fold higher compared to the cytoplasm, supporting the important role of ABCG2 in resveratrol sulfate efflux [22].

The observed OATP-dependent uptake of resveratrol disulfate was in agreement with recent data from Patel and co-workers [9], who found that the uptake of sulfates in human colon cancer cell lines was correlated with the expression of OATP1B3, which is high in HT-29 and HCA-7 cells, but low in HCEC cells. Indirect involvement of OATP1B1 and OATP1B3 has been reported by Gui et al. [18], who observed inhibition of OATP1B1- and OATP1B3-mediated fluorescein-methotrexate uptake by resveratrol, with an approximately twofold lower IC_{50} value being found for OATP1B1 than for OATP1B3 (11 versus 23 μM).

Any variations in OATP expression may significantly alter the uptake of resveratrol and its sulfates into targeted cells and tissues, thereby strongly affecting the efficacy of treatment. Patients with low or no detectable expression of OATP1B1, OATP1B3, and OATP2B1 may therefore show decreased response rates or even no response to resveratrol. Concomitant administration of OATP inhibitors may also interfere with the uptake of resveratrol, leading to

Table 3. Michaelis–Menten parameters determined in ZR-75-1 cells

Substrate	ZR-75-1		OATP1B1 knockdown ZR-75-1	
	K_m [μM]	$V_{\max}$ [pmol/mg/min]	K_m [μM]	$V_{\max}$ [pmol/mg/min]
Resveratrol	144 $\pm$ 34.4*	3360 $\pm$ 281*	192.7 $\pm$ 38.7*	2412 $\pm$ 193*
Resveratrol-3-O-4'-O-disulfate	194 $\pm$ 40.7*	870 $\pm$ 68.7*	234 $\pm$ 40.3*	734 $\pm$ 50.2*

Values are means $\pm$ SD of three individual determinations. $V_{\max}/K_m$ values in bold and marked with an asterisk are significantly different ($p < 0.05$) between ZR-75-1 and OATP1B1 knockdown ZR-75-1 cells.

transporter-mediated drug/drug interactions. Our data demonstrated that BSP and rifampicin effectively inhibited resveratrol uptake in CHO cells, mediated by OATP1B1, OATP1B3, and OATP2B1 (IC_{50} values: 0.48–1.47 μ M). Additional potential inhibitors include clarithromycin, erythromycin, and roxithromycin, which inhibit the uptake of pravastatin in OATP1B1- and OATP1B3-transfected HEK293 cells (showing IC_{50} values of 32–37 μ M) [39]. Moreover, cyclosporin A significantly decreases the OATP1B1- and OATP1B3-mediated uptake of bosentan [26] and fexofenadine [40] in HEK293 and CHO cells. In addition to clinically applied drugs, naturally occurring flavonoids also interfere with the OATP uptake of dehydroepianstrosterone, thus indicating that they constitute a novel class of OATP1B1 modulators [12]. Ongoing studies are verifying the interactions of drugs and dietary supplements with the OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake of resveratrol and its sulfates.

Whether other transporters such as OATP2A1 and OATP4C1 which are expressed in ZR-75-1 WT cells [21] are also involved in the uptake of resveratrol and its conjugates is not known yet. Possible candidates may be organic anion transporters (OATs). Besides numerous clinically used drugs, OATs are also involved in the transport of polyphenol conjugates clearly showing substrate specificity. While OAT1-overexpressing human embryonic kidney 293H cells demonstrated enhanced uptake for sulfate and glucuronide conjugates, such as quercetin-3'-O-sulfate, daidzein-7-O-glucuronide, genistein-7-O-glucuronide, and quercetin-3'-O-glucuronide, OAT3 seems to have a higher affinity for sulfates such as quercetin-3'-O-sulfate but not for the isoflavone glucuronides. [41, 42] Another candidate protein might be the sodium-dependent glucose transporter SGLT1 which was shown to be responsible for the cellular uptake of resveratrol glycoside (*trans*-piceid), but not of *trans*-resveratrol in Caco-2 cells [15]. In vascular endothelial HUVEC cells, however, Chen et al. could very recently demonstrate that resveratrol uptake involves both passive diffusion and, at least partly, an SGLT1-mediated process [43]. Active mediated uptake of resveratrol was also supported by data from Maier-Salamon et al. [44] and from Delmas and Lin [45], clearly showing that in Caco-2 and hepatoblastoma cells, resveratrol uptake was significantly higher (around 50%) at 37°C than at 4°C when protein-mediated transport should be minimal.

Contrary to previous data in Caco-2 and HUVEC cells, we could not confirm any passive diffusion mechanism for the uptake of resveratrol as uptake kinetics in WT and OATP1B1-knockout ZR-75-1 cells were saturable, strongly indicating active transport. As passive diffusion and active transport work in concert it was not possible to discriminate each part from another in our breast cancer cell model.

In conclusion, our data revealed that OATPs act as transporters for resveratrol, resveratrol-3-O-sulfate, and resveratrol-3-O-4'-O-disulfate but not for resveratrol-4'-O-sulfate and resveratrol glucuronides. The OATP-dependent uptake of resveratrol sulfates in concert with intracellular sulfatases, which rapidly deconjugate sulfates to the pharma-

cologically active parent compound, represents a key factor explaining the observed pharmacological activity of resveratrol. Future in vivo studies should focus not only on the concentration of resveratrol and its conjugates in target tissues but also on the expression levels of OATPs.

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The authors have declared no conflict of interest.

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3.2 Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells

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This publication assesses the inhibitory potential of selected physiological Resveratrol sulfate and glucuronide conjugates on Topoisomerase II in a cell free assay. Additionally, cytotoxicity and apoptotic potential was determined in colon cancer cell lines CaCo-2 and HT-29. Additionally, uptake of Resveratrol and Resveratrol-3-sulfate, as well as any further metabolism in colon cancer cells was determined.

I synthesized and purified the glucuronated metabolites used in this study. Additionally, I participated in discussions about method development, preparation of figures and tables and revising the manuscript.

RESEARCH ARTICLE

Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells

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Scope: The cytotoxic and genotoxic potential of phase II metabolites of resveratrol (RSV) was investigated in human colon cells with special emphasis on human topoisomerase (TOP) II.

Methods and results: Cell-free screening of topoisomerase II (TOPII) inhibition by the decatenation assay showed inhibitory potential for RSV ($\geq 200 \mu\text{M}$) and for the first time for the three human phase II metabolites RSV-3-sulfate ($\geq 200 \mu\text{M}$), RSV-3-glucuronide ($\geq 100 \mu\text{M}$) and RSV-disulfate ($\geq 100 \mu\text{M}$). Conjugation at the C4'-position (RSV-4'-sulfate and RSV-4'-glucuronide) resulted in loss of the inhibitory potential in this assay. Cell-based experiments with RSV and the most abundant metabolite in humans, RSV-3-Sulf, revealed no TOP poisoning in HT29 and Caco-2 cells up to $250 \mu\text{M}$. Further, the phase II metabolite exhibited only minor effects in the comet assay and showed negligible cytotoxic effects and apoptotic potential after 1 and 24 h incubation. Fluorescence microscopy and HPLC-DAD analysis identified cellular uptake of RSV and of RSV-3-Sulf although to a lesser extent when compared to RSV. Furthermore, within the cells fractional deconjugation of RSV-3-Sulf to the parent compound was observed.

Conclusion: Sulfate- and glucuronide-phase II metabolites might contribute to the genotoxic potential of RSV by inhibition of TOPII activity. By deconjugation at the target site RSV-3-Sulf might serve as a pool of the parent compound.

Keywords:

Colon cancer cells / DNA damage / Resveratrol / Resveratrol metabolites / Topoisomerase

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1 Introduction

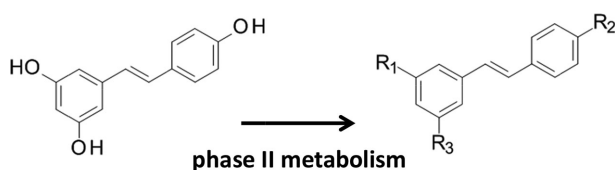
Resveratrol (3,5,4'-trihydroxystilbene, RSV) is a polyphenol produced by plants as a response to stress stimuli and is therefore classified as phytoalexin [1]. RSV can be found in a broad range of dietary sources, but is most abundant in grapes and red wine. To date, numerous studies have reported that RSV has, among others, chemopreventive, antioxidant, and anticancer potential in vitro and in vivo, pushing it into the role of a popular food supplement [2]. In contrast, RSV has

recently been reported to act as a topoisomerase II (TOPII) poison, as well as a TOPII catalytic inhibitor in human cell lines [3–5]. TOP enzymes regulate the topology of the DNA and are crucial for cellular processes including replication and transcription. To unwind or unknot DNA, TOPII introduces transient double-strand breaks, while forming covalently linked complexes with the 5' end of the double helix called cleavage complex. TOP poisons stabilize cleavage complexes, whereas catalytic inhibitors interrupt the catalytic cycle of the enzyme without an increase in cleavage complex formation. Both mechanisms lead to torsional stress, which may result in DNA strand breaks and subsequent cell death [6, 7]. Thus, on the one hand TOP inhibitors represent an intensively used concept in chemotherapy, but on the other hand may be associated with application-limiting toxicity regarding intake as e.g. food supplements when observed for bioactive food constituents.

However, after dietary intake RSV is rapidly metabolized in the liver [8–12]. Several studies have identified

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Abbreviations: ETO, etoposide; fpg, formamidopyrimidine-DNA glycosylase; Gluc, glucuronide; ICE assay, isolating in vivo complexes of enzyme to DNA assay; LDH, lactate dehydrogenase; RSV, resveratrol; Sulf, Sulfate; TOPII, topoisomerase II; WST-1, water-soluble (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate])



	R ₁	R ₂	R ₃
RSV-3-Sulf	- SO ₃ -Na ⁺	- OH	- OH
RSV-3-Gluc	- C ₆ H ₉ O ₆	- OH	- OH
RSV-4'-Sulf	- OH	- SO ₃ -Na ⁺	- OH
RSV-4'-Gluc	- OH	- C ₆ H ₉ O ₆	- OH
RSV-Disulf	- SO ₃ -Na ⁺	-SO ₃ -Na ⁺ / -OH	-SO ₃ -Na ⁺ / -OH

Figure 1. Structure of RSV and its physiological phase II metabolites in humans (*R*₁, *R*₂, *R*₃ indicate the substituents). *R* = rest.

RSV-3-sulfate (RSV-3-Sulf), RSV-4'-Sulf, RSV-3-glucuronide (RSV-3-Gluc), RSV-4'-Gluc, RSV-3,5-disulfate, and RSV-3,4'-disulfate (RSV-Disulf; Fig. 1) as phase II metabolites in animals and humans [13–17]. After oral intake of doses between 0.5 and 5 g RSV, the mean plasma levels of these phase II metabolites have been reported to far exceed the parent compound [10, 12, 13, 18]. Furthermore, it has been suggested that the conjugated metabolites contribute to the biological effects of RSV in vivo [8, 9]. Several studies hypothesize that these metabolites might possess bioactive properties by themselves. RSV-sulfates have been reported to exhibit an anti-inflammatory potential in human macrophages in plasma-representative concentrations quantified after dietary-scale loads [19]. Moreover, they were also found to inhibit the proliferation of human colon cancer cells as well as breast cancer cells [20] and to induce autophagy [18, 21]. RSV-Glucs appear to be less active, but have been shown to induce G1 arrest in human colon cells [22]. In addition to potential intrinsic bioactivity, these metabolites could also serve as a pool for the parent compound through hydrolysis of conjugates at the target site. Recently, Patel et al. [18] were the first to show that RSV can be regenerated from RSV-sulfates in human colon cancer cells and in human colon tissue. It is therefore of great interest to further elucidate the potential bioactivity of RSV metabolites, and to reveal their possible role as a reservoir of the parent compound.

Currently, there is no data available on the potential impact of RSV metabolites on human TOP, and as a consequence, on DNA integrity. In the present study, we are the first to address whether phase II metabolites of RSV possess TOP-targeting properties and whether respective effects are of relevance for the maintenance of DNA integrity. Considering the low systemic bioavailability of RSV, the study was carried out using the well characterized human colon cancer cell line HT29.

To further confirm the results obtained with HT29 cells, key experiments including TOP inhibition and strand-breaking potential were conducted in an additional cell line (Caco-2). Both cell lines originate from the intestinal tract, where high concentrations up to 640 μM can be locally detected after oral intake [18, 23, 24].

2 Materials and methods

2.1 Chemicals

All chemicals were purchased from Carl Roth GmbH+Co. KG (Karlsruhe, Germany) and Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or where indicated separately. RSV metabolites were synthesized as described in detail by Wenzel et al. [16]. The purity of RSV-3-Sulf, RSV-4'-Sulf, RSV-Disulf, RSV-3-Gluc, and RSV-4'-Gluc was determined by NMR and LCMS to be 95, 98, 98, 95, and 99%, respectively.

2.2 Cell culture and treatment

The human colon carcinoma cell lines HT29 and Caco-2 were obtained from the German Collection of Microorganisms and Cells Cultures (Braunschweig, Germany). Cells were kept under humidified conditions in incubators at 37°C and 5% CO₂ and routinely tested and found negative for mycoplasma contamination. HT29 cells were cultured in DMEM, supplemented with 1% v/v penicillin/streptomycin and 10% v/v heat-inactivated fetal calf serum (Invitrogen Life Technologies, Karlsruhe, Germany). Caco-2 cells were cultured in DMEM Glutamax, supplemented with 1% v/v penicillin/streptomycin and 10% v/v heat-inactivated fetal calf serum (Invitrogen™ Life Technologies).

Treatment of the cells was performed in serum-free media with a final solvent concentration of 1% v/v DMSO. Due to substance limitations only RSV-3-Sulf was included in the cell-based testing. Concentrations tested for RSV-3-Sulf were restricted to 50, 100, and 250 μM.

2.3 Decatenation assay

To investigate the inhibitory potential of the test substances on TOPII α the cell-free decatenation assay was performed as described previously [4]. Briefly, 200 ng kinetoplast DNA (TopoGEN, Port Orange, FL, USA) and 0.8 U of TOPII α (TopoGEN) were incubated with the test substances (final solvent control concentration of 1.7%) in a final volume of 30 μL containing 50 mM Tris (pH 7.9), 120 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, 1 mM adenosine triphosphate (ATP), 0.03 mg/mL BSA for 60 min at 37°C. The reaction was stopped by the addition of 1 mg/mL proteinase K (in 10% v/v SDS) followed by further incubation at 37°C for 30 min.

Samples were analyzed by agarose gel (1% w/v) electrophoresis (3 h at ~ 3 V/cm) in Tris-acetate/EDTA buffer (40 mM Tris, 1 mM EDTA, pH 8.5, and 20 mM acetic acid). Gels were stained with ethidium bromide (10 μ g/mL) and analyzed with the LAS-4000 system (Fujifilm, Düsseldorf, Germany).

2.4 Isolating in vivo complexes of enzyme to DNA (ICE) assay

In order to detect the effects of the test substances in cellular models, the ICE assay was performed in HT29 cells and Caco-2 cells with minor modifications [4]. Briefly, 5×10^6 HT29 cells or 3×10^6 Caco-2 cells were seeded in a 15 cm Petri dish and allowed to attach for 48 h. Cells were treated with the test compounds or the solvent control (1% v/v DMSO). Etoposide (ETO; 50 and 100 μ M) served as positive control. Following 1 h incubation, cells were washed twice with PBS and harvested by the addition of 5 mL of lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1% w/v *N*-lauroylsarcosine) with cellular and nuclear membranes disrupted by shearing the cell lysate through a 9-gauge needle. After laying the cell lysate on a cesium chloride gradient, samples were centrifuged for 24 h at $100\,000 \times g$ in order to separate free enzymes from those bound to the DNA. The gradient was divided into 20 fractions and the DNA-rich fractions, which contain the cleavage complexes, were transferred to a nitrocellulose membrane using the Minifold II Slot-Blot System (Whatman, Little Chalfont, UK) with TOPII proteins detected by specific TOPII α and TOPII β antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

2.5 Single-cell gel electrophoresis (comet assay)

The strand-breaking potential of the test compounds was evaluated by the comet assay. Briefly, 3×10^5 (for 1 h incubation) and 1.5×10^5 (for 24 h incubation) HT29 cells or 4×10^5 (for 1 h incubation) and 2.5×10^5 (for 24 h incubation) Caco-2 cells were seeded into a Petri dish ($d = 5$ cm) and allowed to grow for 48 h. Subsequently, cells were incubated with the test compounds for 1 or 24 h. UV-B irradiation ($\lambda = 312$ nm; dosage: 448 J/cm², 2 min) served as positive control. The assay was performed according to the method of Tice et al. [25] with minor modifications. After cell harvesting, cell number and viability was determined by Trypan blue staining. For examination, 3×10^4 (HT29) or 5×10^4 (Caco-2) cells from each sample were added to low melting agarose (0.8% w/v) and placed on agarose-coated slides. Cell-coated slides were incubated in lysis buffer containing 89 mL lysis buffer stock solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris pH, 1% w/v *N*-lauroylsarcosine, pH 8.0), 10 mL of DMSO, and 1 mL of Triton X-100 for 24 h at 4°C. To detect additional oxidative DNA damage, the slides were treated with formamidopyrimidine-DNA glycosylase (fpg; 1:3000 dilution; New England Biolabs Inc., Vienna, Austria) for 30 min at

37°C. Electrophoresis was performed after equilibration for 20 min in 300 mM NaOH, 1 mM EDTA (pH 13.5). DNA was stained with 10 μ g/mL ethidium bromide (10 μ g/mL) and tail intensity (amount of nuclear DNA found in the tail) was analyzed using the software Comet IV (Perceptive Instruments, Suffolk, UK).

2.6 Cytotoxicity

Two different approaches were used to determine cytotoxicity, the commercially available water-soluble WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate) test kit (Roche Applied Science, Mannheim, Germany) as well as the lactate dehydrogenase (LDH) kit (Roche Applied Science). The WST-1 assay allows the monitoring of the modulation of the activity of mitochondrial dehydrogenases, while the LDH assay provides information of the integrity of the cell membrane. The assays were performed according to the manufacturer's instructions and as described previously [26]. Briefly, 3×10^4 cells were seeded in each well of a 96-well plate and allowed to attach for 48 h and incubated for either 1 or 24 h. Triton X-100 (1% v/v) was used as positive control and DMSO (1% v/v) served as negative control. After incubation, 50 μ L of the supernatant was used to determine the amount of LDH kit. For the WST-1 assay, cells were washed and then incubated for 20 min with 10 μ L of the WST-1 reagent in 100 μ L of serum-free DMEM. Plates were measured in a plate reader (Victor³ V, PerkinElmer, Waltham, MA, USA) at $\lambda = 450$ nm. The percentage of either mitochondrial activity or LDH activity in relation to DMSO and Triton X-100 was analyzed.

2.7 Apoptosis (cell death detection ELISA^{Plus})

The impact of the test compounds on apoptosis was evaluated using the Cell Death Detection ELISA^{Plus} kit (Roche Applied Science) as described in the manufacturer's protocol. Briefly, 1×10^5 cells were seeded in each well of a 24-well plate. The cells were allowed to attach for 48 h and then treated for 24 h in serum-free medium with the solvent control DMSO or with rising concentrations of RSV or RSV-3-Sulf. The cells were then harvested and cell number and viability determined by flow cytometry (Guava EasyCyte plus, Millipore, Hayward, CA, USA). To do this, an aliquot of 1×10^4 cells of each sample was added into the wells of a streptavidin-coated microplate. The amount of histone-associated DNA fragments formed after induction of cell death was determined by mononuclear antihistone antibodies and peroxidase-linked antibodies. The amount of nucleosomes of each sample was quantified by photometrical measurement (Victor³ V, PerkinElmer) at $\lambda = 405$ nm. A reference wavelength of $\lambda = 490$ nm as well as a blank were subtracted and the values expressed as A405–A490 nm.

2.8 Cellular localization

For the localization of RSV and RSV-3-Sulf, 5×10^4 HT29 cells were seeded in multiwell microscopy slides (Falcon, Corning, USA) and allowed to attach for 72 h. Cells were then incubated with 250 μ M RSV, 250 μ M RSV-3-sulfate or 1% DMSO (solvent control) for 5 min or 1 h. At the end of the incubation, cells were fixed with prewarmed formaldehyde (3.7%; 15 min), rinsed with PBS, and incubated with 100 mM glycine to mask reactive sites. Slides were mounted with Roti-Mount FluorCare with DAPI (Roth, Graz, Austria) and sealed. Images of three independent experiments were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS.1 system using a Plan Apochromat 63X/1.4 oil objective, zoom 2. Within the same experiment, for a proper comparison of the different conditions, at least three optical fields were acquired for each treatment, while keeping acquisition parameters constant.

2.9 Cellular uptake

Cells (5×10^6) were seeded in Petri dishes ($d = 15$ cm) and allowed to grow for 48 h. Cells were then incubated for 1 h in serum-free media with either 250 μ M of RSV, 250 μ M of RSV-3-Sulf, or with the solvent control DMSO (1% v/v final concentration). After incubation, cells were washed twice with PBS and harvested by adding lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1% w/v *N*-lauroylsarcosine) and the cell lysate disrupted by shearing 20 times through a 9-gauge needle. Proteins were precipitated by the addition of methanol and separated by centrifugation. After resolving the vacuum-evaporated supernatant in methanol, the solution was filtered through a polyvinylidene fluoride (PVDF) filter (0.45 μ m) and 40 μ L of the filtrate used for HPLC analysis (Dionex Ultimate 3000RS, Thermo Scientific, Vienna, Austria). Separation of the compounds was performed at 25°C on a C18 column (Phenomenex Luna, 5 μ m, 250×3 mm²) with an ammonium formate (10 mM, pH 8.2) methanol gradient and a flow rate of 0.5 mL/min (UV detector 300 nm). Starting with 97% ammonium formate, the methanol content was raised to 75% within 45 min and returned to initial conditions within 15 min. RSV and its respective metabolites were identified by comparing retention times with the retention times of standards for each compound. For quantification an external calibration was used. The concentration of the compounds found in the cell lysate was referred to the respective protein concentration of the sample and is therefore expressed as microgram compound per milligram protein. The protein concentration was measured by Bradford assay. The recovery rate was $84.3 \pm 1.3\%$.

2.10 Statistics

The results are presented as the mean values of at least three independent experiments $\pm$ SD. Statistical analysis was

performed using the software OriginPro 9.0G (OriginLab Corporation, MA, USA). Test of normality was done by Shapiro–Wilk test ($p > 0.05$). Significance was calculated by one-way analysis of variance (ANOVA), followed by posthoc testing with Fisher's least significant difference least significant difference (LSD) test in case of normality. If no normality was given, Kruskal–Wallis ANOVA followed by the Mann–Whitney test was used. A level of $p < 0.05$ was considered as significant (indicated as letters in the graphs).

3 Results

3.1 Cell-free decatenation assay

The inhibitory potential of the test compounds on the catalytic activity of human TOPII was investigated under cell-free conditions in the decatenation assay using the TOPII α isoform. Active TOPII releases free minicircles from the high molecular weight kinetoplast DNA, thus enabling migration that is detectable in agarose gel electrophoresis (Fig. 2). RSV itself suppressed TOPII activity at a concentration of 200 μ M with a complete inhibition observed at 250 μ M. RSV-3-Sulf affected TOPII activity to a comparable extent with an onset of inhibition at 200 μ M and a complete block of activity at 250 μ M. RSV-3-Gluc showed TOPII-inhibitory properties at concentrations ≥ 100 μ M. The metabolites conjugated at the C4' position (RSV-4'-Sulf and RSV-4'-Gluc) did not appear to affect TOPII activity up to a concentration of 250 μ M. In contrast, concentrations higher than 100 μ M of RSV-Disulf, representing a mixture of RSV-3,5-Disulf and RSV-3,4'-Disulf, led to a complete block of TOPII α activity. The potency of the test compounds to inhibit TOPII activity under cell-free conditions can be summarized as RSV-3-Gluc $\approx$ RSV-Disulf $>$ RSV $\approx$ RSV-3-Sulf $>>$ RSV-4'-Sulf $\approx$ RSV-4'-Gluc (Table 1). However, the impact of phase II conjugation on solubility and therefore potentially on cellular kinetics, raises the question whether TOPII-targeting by RSV metabolites is of relevance within cells. For this reason, RSV-3-Sulf, one of the most abundant metabolites in human plasma and available in sufficient amounts for testing in cell-based assays, was selected for further mechanistic studies.

3.2 Cell-based ICE assay

The impact on the level of cleavage complexes (TOPII-DNA-intermediates) in HT29 and Caco-2 cells was determined using the ICE assay where poisoning by TOPII is indicated by an increased level of cleavage complexes in DNA-rich fractions. Figure 3A demonstrates the distribution of DNA in the different fractions. Following ultracentrifugation, the highest content of DNA was found in fractions from seven to ten. These fractions were examined by Western analysis using antibodies against TOPII α or TOPII β , respectively (Fig. 3B). The solvent DMSO showed no or very weak TOPII signals

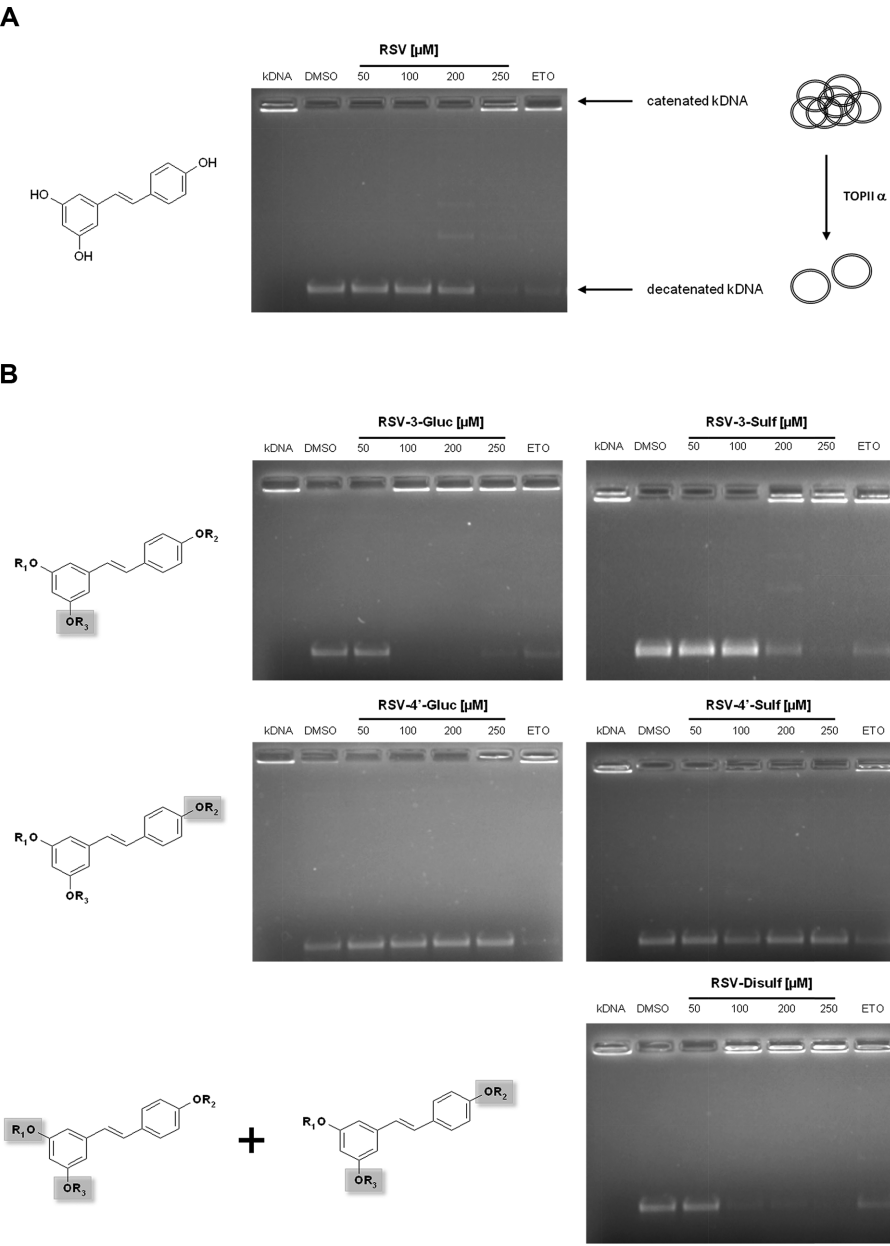


Figure 2. Inhibition of TOP2 α under cell-free conditions (decatenation assay). The activity of the enzyme was monitored by the release of decatenated minicircles, which migrate through the gel and were compared to the catenated form. One representative gel for each substance out of at least three gels with comparable outcome is shown for (A) RSV (B) RSV metabolites. Lane 1: catenated kinetoplast DNA (200 ng) without enzyme, lane 2–6: 0.8 U TOP2 α together with the negative control DMSO (1.7%; lane 2), rising concentrations of test substances (lane 3–6) or 50 μ M of ETO serving as positive control (lane 7).

in the DNA-rich fractions, whereas incubation with ETO, a well-known TOP2 α poison, clearly enhanced the level of cleavage complexes in these fractions reflected by strong chemiluminescence in both tested cell lines. However, after incubation with RSV and RSV-3-Sulf up to concentrations of 250 μ M no substantial increase in the level of cleavage complexes was detected when compared to DMSO controls, arguing against TOP2 α poisoning by these compounds in HT29 and Caco-2 cells, at least in the selected time and concentration range examined. Nevertheless, the maintenance of DNA integrity is not only influenced by the potential genotoxic mechanism of TOP2 α poisoning, but may also result from the persistent blocking of the catalytic activity causing torsional stress and subsequent DNA strand breakage.

Table 1. Concentration at which first inhibitory effects of RSV and phase II metabolites on TOP2 α were detected in the cell-free decatenation assay

Compound	Inhibitory concentration
RSV	≥ 200 μ M
RSV-3-Sulf	≥ 200 μ M
RSV-3-Gluc	≥ 100 μ M
RSV-4'-Sulf	No inhibition
RSV-4'-Gluc	No inhibition
RSV-Disulf	≥ 100 μ M

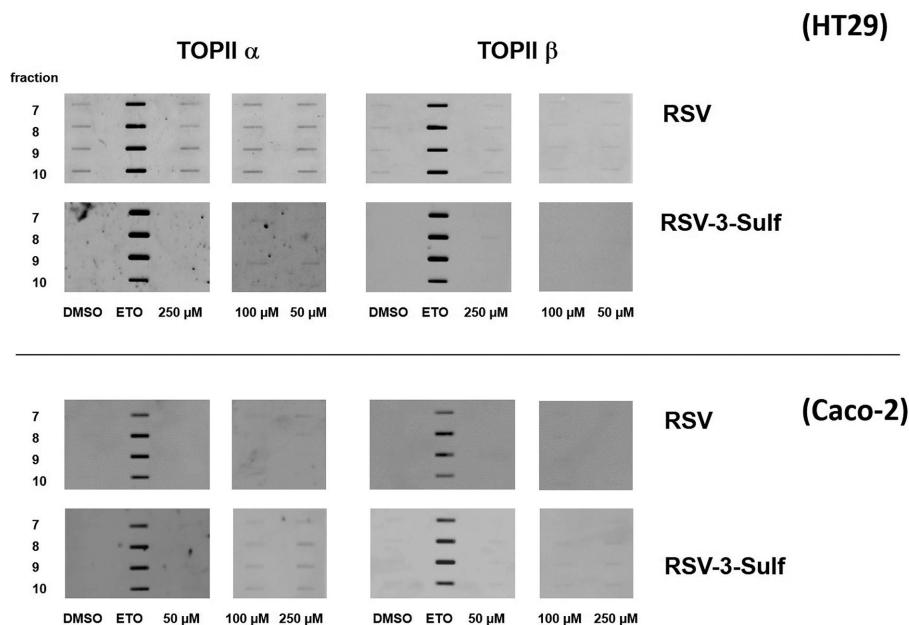


Figure 3. Potential poisoning effects of TOP1I in HT29 and Caco-2 cells after 1 h incubation with RSV or RSV-3-Sulf (ICE assay). Poisoning of the enzyme was determined by the amount of cleavage complexes found in the DNA-rich fractions. DMSO served as negative control, 50 μM (HT29 cells) and 100 μM (Caco-2 cells) ETO served as positive control. (A) Concentration of the DNA in the different fractions after ultracentrifugation of a representative sample. The DNA-rich fractions (fraction 7–10) were blotted on a nitrocellulose membrane and the amount of TOP1I in these fractions measured by the intensity of the chemiluminescence signal (B). Displayed are representative membranes from three experiments with comparable outcome after incubating HT29 or Caco-2 cells with RSV or RSV-3-Sulf.

3.3 Impact on DNA integrity

Potential effects of RSV and RSV-3-Sulf on DNA integrity in HT29 and Caco-2 cells were determined by single-cell gel electrophoresis (comet assay, Fig. 4). In order to detect the potential oxidative damage induced by the compounds, an additional treatment with the DNA repair enzyme fpg was

included in the assay. Incubation with RSV for 1 h resulted in a concentration-dependent increase in the level of DNA strand breaks in both colon cancer cell lines showing statistical significance at concentrations ≥ 50 μM in HT29 cells and ≥ 100 μM in Caco-2 cells. A maximum in tail intensity of $3.9 \pm 2.1\%$ in HT29 cells and $10.1 \pm 2.2\%$ in Caco-2 cells was observed. Additional treatment with fpg did not

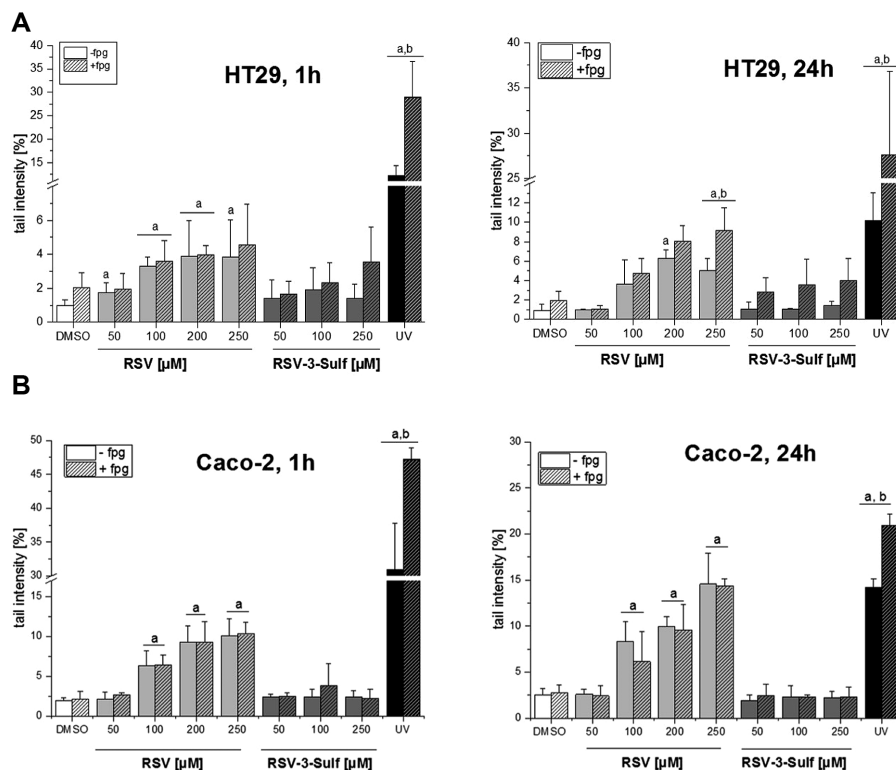


Figure 4. DNA strand-breaking potential in HT29 (A) and Caco-2 (B) cells after incubation with RSV or RSV-3-Sulf for 1 or 24 h investigated by comet assay. Additional treatment with fpg was performed to detect fpg-sensitive sites. DMSO served as negative control and 2 min of UV-B radiation was used as positive control. Data is presented as tail intensity (percentage of DNA found in the tail) and represents the mean $\pm$ SD of at least three independent experiments. Statistical analysis was performed by Kruskal–Wallis ANOVA and Mann–Whitney test. Significance refers to the negative control DMSO (a), or to differences between fpg-treated and nontreated samples (b).

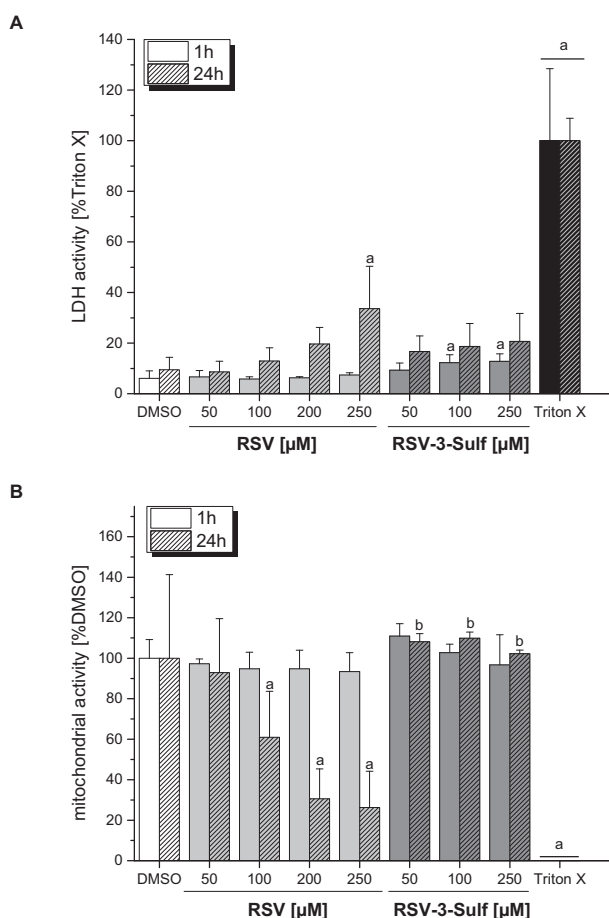


Figure 5. Cytotoxic effects in HT29 cells after 1 and 24 h incubation with RSV or RSV-3-Sulf at the indicated concentrations. DMSO was used as negative control while 1% v/v of Triton X-100 served as positive control. Cytotoxicity was monitored by two different approaches: the LDH assay (A) and the WST-1 assay (B). Data are expressed as mean $\pm$ SD of at least three independent experiments. The values are presented as percentage of the respective control (Triton X-100 for LDH and DMSO for WST-1). Statistical analysis was performed by one-way ANOVA with Fisher's LSD test. Significances refer to the negative control DMSO (a) or to differences between the same concentration of RSV and RSV-3-Sulf (b).

significantly enhance the amount of DNA damage. RSV-3-Sulf showed no strand-breaking potential up to the highest tested concentration of 250 μ M in both tested cell lines.

After 24 h incubation with the parent compound RSV significantly enhanced tail intensities were observed in concentrations ≥ 200 μ M peaking at $6.3 \pm 0.9\%$ in HT29 cells and at $14.5 \pm 3.4\%$ for 250 μ M in Caco-2 cells. However, a significant increase of fpg-sensitive sites was only observed in HT29 cells at the highest concentration of 250 μ M RSV ($9.1 \pm 2.4\%$). In contrast, RSV-3-Sulf seemed to have little impact on DNA integrity showing tail intensities of $1.4 \pm 0.4\%$ and $2.2 \pm 0.7\%$ after incubation with the highest tested concentration (250 μ M) in both cell lines.

3.4 Cytotoxicity

Potential cytotoxic effects of RSV and RSV-3-Sulf in HT29 cells were then monitored after 1 h and 24 h by assessing membrane integrity (LDH assay) and mitochondrial activity (WST-1 assay; Fig. 5). After 1 h incubation no cytotoxic effects were detected up to the highest tested concentration of 250 μ M RSV in the LDH assay (Fig. 5A). Incubation with RSV-3-Sulf resulted in a significant increase in LDH activity at 100 μ M ($12.3 \pm 3.2\%$) and 250 μ M ($12.8 \pm 2.9\%$) when compared to the solvent control, but this was not significantly different from the parent compound. After 24 h incubation 250 μ M RSV significantly enhanced LDH activity up to $33.6 \pm 16.8\%$. Short-term incubation (1 h) with the test compounds did not affect mitochondrial activity as verified by the WST-1 assay. In contrast, a concentration-dependent decrease of mitochondrial activity was observed after 24 h incubation with RSV to $30.6 \pm 14.8\%$ (200 μ M) and $26.3 \pm 17.9\%$ (250 μ M), respectively (Fig. 5B). In contrast, RSV-3-Sulf had no significant influence on the mitochondrial activity in HT29 cells up to a concentration of 250 μ M.

3.5 Induction of apoptosis

The process of apoptosis might influence the level of DNA damage observed in the comet assay after 24 h. Thus, the induction of apoptosis was determined by ELISA measuring the amount of mono- and oligo-nucleosomes in the cytoplasm indicative of early apoptotic events. After 24 h incubation, 200 and 250 μ M RSV significantly increased the amount of nucleosomes approximately 1.6- and 1.5-fold, respectively (Fig. 6), with no effect observed for RSV-3-Sulf over the tested concentration range.

3.6 Cellular localization

The localization of 250 μ M RSV and RSV-3-Sulf was investigated by fluorescence microscopy taking advantage of the intrinsic fluorescence of RSV species as previously described [27,28] (Fig. 7). A cellular uptake of RSV was observed already after 5 min incubation with enhanced fluorescence present in the cytoplasm and in the region of the nucleoli. Fluorescence intensity seemed lower after 1 h incubation but was still clearly visible with comparable cellular distribution of the compound. However, after 5 min and 1 h incubation with RSV-3-Sulf the intracellular fluorescence signal detected was comparable to that of the controls.

3.7 Cellular uptake

The uptake of RSV and RSV-3-Sulf was investigated by HPLC analysis after 1 h incubation in HT29 cells (Fig. 8). The intracellular concentrations of the two compounds are summarized in Table 2. After 1 h incubation with RSV or RSV-3-Sulf,

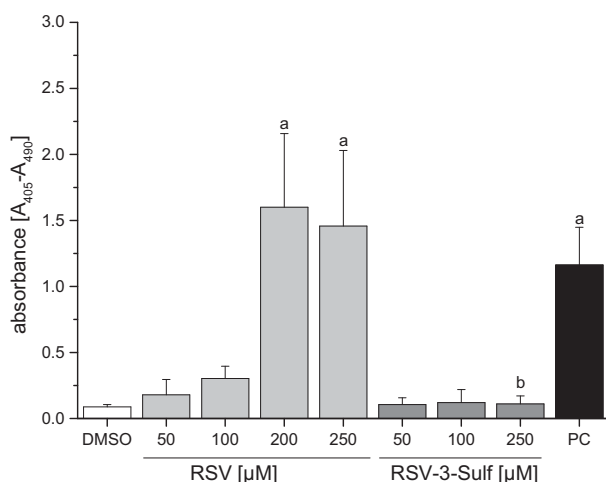


Figure 6. Apoptosis induction potential of RSV and RSV-3-Sulf after 24 h incubation in HT29 cells. Cells were incubated with the solvent control DMSO or with rising concentrations of RSV or RSV-3-Sulf. A DNA–histone complex served as positive control (PC). Data are expressed as difference between photometric measurements at $\lambda = 405$ nm and the reference wavelength of $\lambda = 490$ nm ($A_{405} - A_{490}$). Statistical significances were calculated by one-way ANOVA followed by Fischer's LSD test. Significance refers to the negative control DMSO (a) or to differences between the same concentration of RSV and RSV-3-Sulf (b).

RSV-related species were detected in the cell lysate; in parallel no RSV or respective phase II metabolites were detected after incubation with the solvent control DMSO (for LODs, see Table 2). The peak of RSV eluted at 32.5 min (1) and RSV-3-Sulf at 27.6 min (2). The metabolites formed during incubation were identified as RSV-4'-Gluc (20.2 min; 6), RSV-Disulf (21.2 min; 4), RSV-3-Gluc (22.6 min; 3), and RSV-4'-Sulf (25.8 min; 5). Phase II metabolites were detected and determined after incubation with both RSV and RSV-3-Sulf. After incubation with the parent compound RSV-monoconjugated sulfate and Gluc metabolites were found in the cells with RSV-3-Sulf representing the predominant metabolite (2.8 $\mu\text{g}/\text{mg}$ protein, $\sim 6.1\%$). The second most abundant metabolite was RSV-4'-Gluc (approximately 5%, 2.3 $\mu\text{g}/\text{mg}$ protein). RSV itself was detected in a concentration of 40.4 $\mu\text{g}/\text{mg}$ protein, representing approximately 86.6% of all identified RSV-related species. However, after incubation with RSV-3-Sulf only 9.7 $\mu\text{g}/\text{mg}$ of RSV-3-Sulf was found in the cells equating to 75.7%. Interestingly, the second most abundant RSV-related compound after incubation with RSV-3-Sulf was free RSV (2.2 $\mu\text{g}/\text{mg}$ protein), representing approximately 16.9%. Additionally, minor amounts of mono- and de-conjugated metabolites were observed.

4 Discussion

In this study, we addressed whether phase II metabolites of RSV contribute to the genotoxic and cytotoxic effects of the

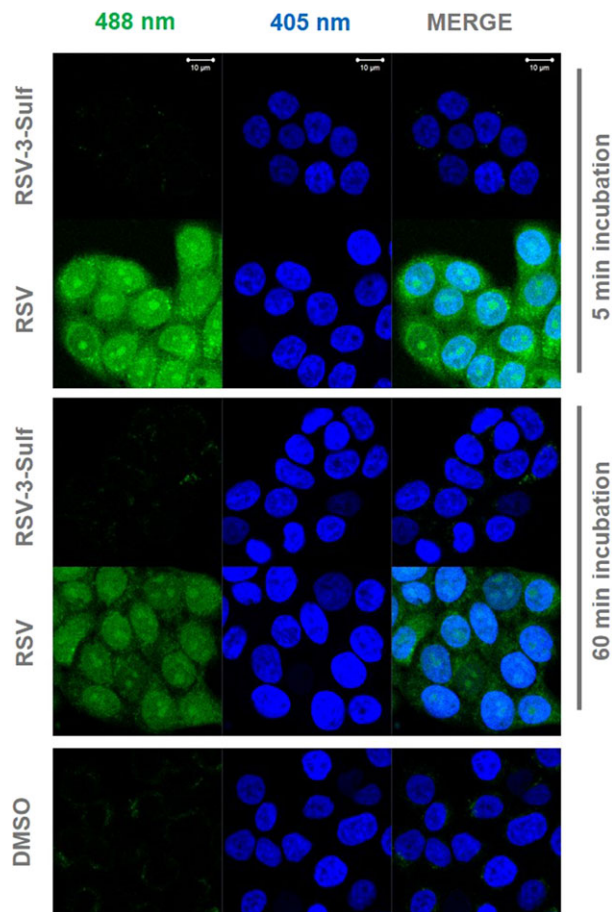


Figure 7. Cellular localization of 250 μM RSV and RSV-3-Sulf in HT29 cells analyzed by fluorescence microscopy. Representative images were selected after the performance of three independent experiments incubating the compounds for 5 min or 1 h. On the left column is displayed the intrinsic fluorescence of the test compounds (488 nm), in the middle column of the nuclear staining with DAPI (405 nm), and in the right column merge of the two images (63 $\times$ magnification, zoom 2).

parent compound with special emphasis on the interference with human TOP2. Several studies have shown that RSV possesses TOP2-targeting properties in cell-free test systems [4, 29, 30], but, to the best of our knowledge, this is the first report demonstrating that phase II metabolites can also affect the activity of the enzyme. In this study, metabolites conjugated in the C3-position were identified as potential TOP2 inhibitors (with RSV-3-Gluc and RSV-Disulf exceeding the potential of the parent compound), whereas conjugation at the C4'-position was found to be associated with a loss of inhibitory properties. The results indicate that the position of conjugation is critical for the bioactivity and targeting of TOP2. In silico modeling suggested that the C4', C3-, and C5-positions are necessary for the binding of RSV to DNA and to the enzyme. In particular, the C4'-position seems to be critical for the binding of RSV to the enzyme [3]. Conjugation at this position may hamper the binding of RSV to

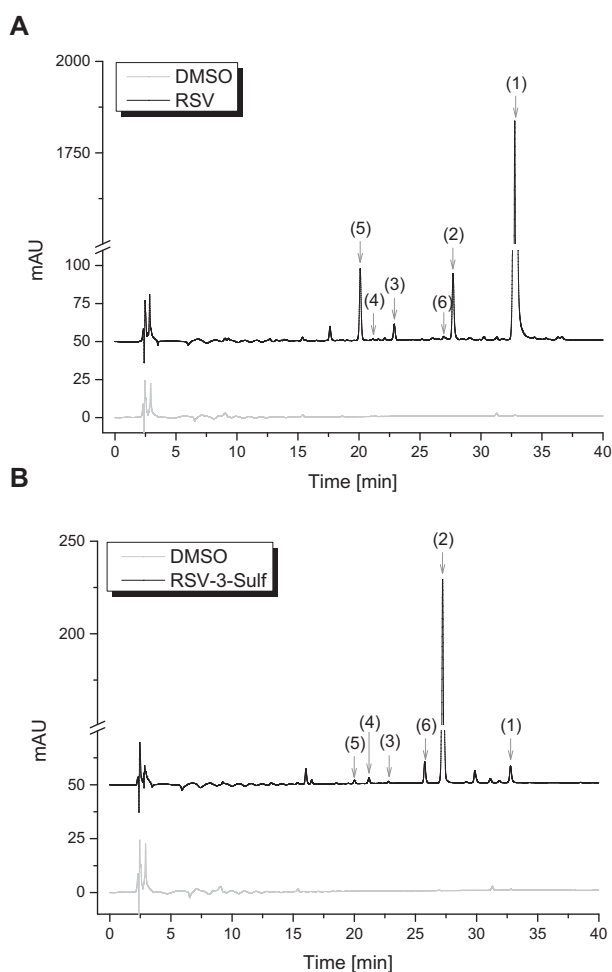


Figure 8. Cellular uptake and metabolism of either 250 μ M RSV (A) or 250 μ M RSV-3-Sulf (B) in HT29 cells after 1 h incubation analyzed by HPLC-DAD. Displayed are two representative HPLC chromatograms out of at least three independent experiments. Numbers of the peaks refer to RSV (1), RSV-3-Sulf (2), RSV-3-Gluc (3), RSV-Disulf (4), RSV-4'-Sulf (5), RSV-4'-Gluc (6).

TOPII leading to a reversion of its inhibitory potential. Due to substance limitations, only RSV-3-Sulf, one of the most abundant metabolites in humans [11, 18, 31], could be included in mechanistic studies. In contrast to the results in the cell-free decatenation assay, neither RSV nor RSV-3-Sulf showed a poisoning effect on TOPII in both tested colon cell lines (ICE assay). This is in contrast to Leone et al. [3], who previously reported the poisoning potential of RSV in cells (U87 human glioma cells) at 120 μ M. However, more recently RSV has been shown to not poison TOPII in HT29 cells in a comparable concentration range. The above-mentioned results were obtained using a competitive incubation with the well-known TOPII poison doxorubicin suggesting a potential catalytic inhibition of the enzyme by RSV [5]. In contrast, Baechler et al. [4] showed a weak poisoning by 100 μ M RSV in human epidermoid A431 cells. Overall, there is evidence that RSV shows a cell-type specific inhibition of TOP. In addition, RSV-3-sulf might act as a catalytic inhibitor in HT29 cells and Caco-2 cells, explaining the absence of TOPII cleavage complex formation.

Potential effects of RSV and RSV-3-Sulf on DNA integrity were monitored in the comet assay. Several studies have previously reported the cell-type and concentration-dependent DNA strand-breaking potential of RSV [29, 32, 33]. However, to date, data on the potential influence of RSV-3-Sulf on DNA integrity are limited. In the present study a concentration-dependent, though not significant increase in tail intensity was observed, after 1 h as well as 24 h incubation in HT29 cells, but not in Caco-2 cells. However, the parent compound exceeded the effects observed for the metabolite by far in both cell lines. This is in concordance with Aires et al. [21], who observed an increase in the DNA double-strand break marker γ H2AX by Western blot analysis after 48 h incubation with RSV-3-Sulf in metastatic colon cancer cells, although at a lower level than for RSV itself. The phosphorylation of H2AX is a very sensitive marker of DNA strand breaks and may lead to more precise results when compared with the comet assay [34]. The potential genotoxicity of RSV observed after 24 h incubation is most likely a secondary effect caused by

Table 2. Overview to the concentrations, percentages, and LODs of RSV and the metabolites found in the cell lysate of HT29 cells after 1 h incubation with either 250 μ M RSV or 250 μ M RSV-3-Sulf analyzed by HPLC

RSV species identified	LOD (μ g/mg protein)	RSV		RSV-3-Sulf	
		Concentration (μ g/mg protein)	Percentage (%)	Concentration (μ g/mg protein)	Percentage (%)
RSV (1)	0.006	40.4 $\pm$ 9.41	86.6	2.2 $\pm$ 1.09	16.9
RSV-3-Sulf (2)	0.020	2.8 $\pm$ 0.06	6.1	9.7 $\pm$ 3.87	75.7
RSV-3-Gluc (3)	0.013	0.7 $\pm$ 0.21	1.4	<LOD	—
RSV-4'-Sulf (5)	0.020	0.2 $\pm$ 0.00	0.5	0.3 $\pm$ 0.04	2.8
RSV-4'-Gluc (6)	0.013	2.3 $\pm$ 0.70	5.0	0.3 $\pm$ 0.05	2.7
RSV-Disulf (4)	0.020	0.2 $\pm$ 0.06	0.4	0.2 $\pm$ 2.23	1.9

Intracellular concentrations are calculated by referring to the respective protein concentration in the cell lysate determined by Bradford assay. Columns 4 and 6 provide information about the proportion (calculated as percentage of the overall amount of RSV species found) of each substance in the cell lysate after incubation with RSV or RSV-3-Sulf, respectively. Data represent the mean $\pm$ SD of three independent experiments. Numbers in brackets refer to the respective peak in the HPLC chromatograms shown in Fig. 8.

cytotoxicity and apoptosis, both well known to increase DNA damage [35]. Cytotoxic effects, monitored by WST-1 and LDH assay, were observed after 24 h incubation with 100 μ M of RSV. Following this, significant concentrations of mono- and oligo-nucleosomes as marker for apoptosis were observed after incubation with 200 and 250 μ M of RSV, supporting DNA strand breaks observed in the comet assay. These results are comparable to previous findings demonstrating the cytotoxic and apoptotic potential of RSV in HT29 cells [36–38]. In contrast to the parent compound RSV, RSV-3-Sulf had only a minor impact on cell viability showing significant effects exclusively in the LDH assay after 1 h incubation. Studies investigating the effects of RSV-3-Sulf in human colon cancer cells have previously reported first significant effects between 10 and 50 μ M after 48 h incubation indicating differences in sensitivity between cell lines originating from the same tissue [18, 21, 22]. In addition, significant effects have been shown exclusively for treatments lasting at least 48 h. Patel et al. [18] incubated a mixture of RSV-sulfates in HT29 cells for 7 days and found a significant decrease in cell number starting at 50 μ M. The rather low effects observed in this study might therefore be explained by the relatively short incubation times applied. However, published data regarding RSV and its main phase II metabolite, RSV-3-Sulf, indicate minor effects of the metabolite in comparison to the parent compound, which was also observed in this study [18, 21, 22]. These differential effects may result from differences in the cellular localization of the compounds. RSV has been shown to effectively enter the nucleus [28], while the cellular localization of the metabolites remains to be clarified. Fluorescence microscopy conducted within this study revealed that RSV is indeed localized in the cytoplasm and in the area of the nucleolus of the cells, which is in line with the findings of Lancon et al. [28]. However, RSV-3-Sulf was not detected in HT29 cells by this method, which might be explained by several reasons. First of all, the sulfate conjugation of RSV may cause a shift in the intrinsic fluorescence of RSV, which has already been described for RSV triacetate [27]. Moreover, the absence of an intracellular fluorescence signal after incubation with RSV-3-Sulf might as well be caused by a reduced cellular uptake. To address this question, we additionally investigated the uptake and subsequently the metabolism of RSV and RSV-3-Sulf in HT29 cells by HPLC analysis, which revealed that the uptake of RSV-3-Sulf was indeed around four times lower when compared to RSV. The low intracellular concentration might explain the limited biological effects observed in comparison to the parent compound and the discrepancy of the TOP-inhibiting potential between cell-free and cell-based experiments. However, it was shown that RSV and its phase II metabolites were taken up and further processed by phase II enzymes generating, among others, the parent compound RSV. This was also reported by Patel et al. [18], thereby strongly supporting the idea that metabolites could serve as a pool for the parent compound. Interestingly, this group also reported that the uptake and metabolism of RSV and RSV monosulfates are comparably low in the non-

cancer colon cell line human colonic epithelial cells (HCEC) [18] indicating a potential-specific sensitivity of colon cancer cells to RSV species.

Of note, concentrations used in the study are rather high and, at least in case of the parent compound RSV, very unlikely to be achieved systemically after dietary intake. However, RSV represents a popular food supplement, proposed not only as a cancer therapy, but also as a stand-alone product or in combination with classical chemotherapeutics [2, 15, 39, 40]. As a therapeutic, it is likely that the plasma as well as tissue concentrations exceed those obtained by the consumption of RSV-containing food. In addition, the concentrations of the sulfate metabolites after repeated oral intake of 1 g of RSV, a dose also provided by food supplements, are found to average around 50 μ M and can reach up to ~640 μ M in colonic tissue [18], which is far higher than the concentrations tested in this study. Toxicological effects can therefore not be excluded in this concentration range.

In conclusion, the results of the present study indicate that the phase II metabolites of RSV can indeed contribute to the effects of the parent compound either by directly affecting targets such as TOPII or by serving as a pool for the parent compound. Although RSV-3-Sulf had only minor effects in HT29 and Caco-2 cells further studies should consider the potential effects of mixtures of RSV and its metabolites, representative for the in vivo situation [15, 23]. Subsequently, in vivo studies should clarify the impact of the phase II metabolites on the genotoxic and cytotoxic potential of RSV and investigate, if the metabolites indeed influence targets in the colonic tissue relevant for the maintenance of DNA integrity such as TOPII. Furthermore, these studies could provide data, which might help evaluate if RSV species locally protect colonic tissue against cooccurring TOP poisons or whether there are any associated potential health risks for the consumer or patient to be considered.

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The authors have declared no conflict of interest.

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3.3 Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 Expression in U-937 Macrophages

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This publication uses a model of pgLPS activated human macrophages to establish the impact of selected Resveratrol conjugates on gene and protein expression of selected chemokines (IL-8, MCP-1, MIP-1 β), chemokine receptors (CCR1, CCR2, CXCR2) and sirtuin-1 gene expression. Additionally, the uptake of Resveratrol, Resveratrol-3-sulfate and Resveratrol-4'-glucuronide was measured.

I synthesized and purified the glucuronated metabolites used in this study, and set up the experimental designs. I conducted all measurements and did statistical data interpretation, as well as preparation of figures and tables, and wrote the manuscript.

Sulfated and Glucuronated *trans*-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 Expression in U-937 MacrophagesKatharina Schueller,^{†,‡} Marc Pignitter,[†] and Veronika Somoza^{*,†,‡}[†]Department of Nutritional and Physiological Chemistry, Faculty of Chemistry, and [‡]Christian Doppler Laboratory for Bioactive Aroma Compounds, University of Vienna, Althanstraße 14, 1090 Vienna, Austria

S Supporting Information

ABSTRACT: The natural anti-inflammatory compound resveratrol (RES) is metabolized upon ingestion. After dietary-scale doses, plasma concentrations of sulfated and glucuronated metabolites in humans exceed those of RES. The aim of this in vitro study was to assess the effect of physiological concentrations (1 μ M) of the most abundant RES metabolites (RES-3-O-sulfate, R3S; RES-disulfates, RdS; RES-3-O-glucuronide, R3G; RES-4'-O-glucuronide, R4G) on genes and proteins involved in immune cell chemotaxis and inflammation (IL-8, MIP-1b, MCP-1, CCR1, CCR2, CXCR2, SIRT1) in a cell model of lipopolysaccharide (LPS)-activated U-937 macrophages. Levels of MCP-1 mRNA were comparably decreased after 3 h of treatment with R3S and RdS by -24.7 ± 5.51 and $-28.7 \pm 19.2\%$, respectively. LPS-induced MCP-1 protein release was reduced after 3 h of treatment by R3S ($-20.8 \pm 13.9\%$) and RdS ($-25.7 \pm 8.29\%$). After a 9 h treatment, RdS also inhibited IL-8 and MIP-1b protein release by -22.9 ± 3.57 and $-20.1 \pm 7.00\%$, respectively. Glucuronides showed differential effects after 6 h of treatment, with R4G up-regulating mRNA of MIP-1b ($24.5 \pm 14.8\%$) and R3G and R4G down-regulating CXCR2 surface protein compared to cells treated with LPS alone, by -5.33 ± 4.18 and $-15.2 \pm 5.99\%$, respectively. On the contrary, R3G and R4G up-regulated SIRT1 mRNA by 22.7 ± 17.9 and $22.8 \pm 16.9\%$, respectively, in LPS-stimulated U-937 macrophages, showing anti-inflammatory properties. In conclusion, sulfated RES metabolites show an interesting beneficial potential for attenuating inflammatory immune processes.

KEYWORDS: resveratrol metabolites, immune cells, macrophages, chemokines, receptors, MCP-1

INTRODUCTION

The ameliorating effect of the polyphenol *trans*-resveratrol (RES) in inflammatory diseases has been studied intensively over the past decades.¹ RES has been shown to influence signaling molecules, enzymes, proteins, and transcription factors by modulating the arachidonic acid cascade and prostaglandin synthesis,^{2,3} among other mechanisms, thus counteracting inflammatory processes in vitro.⁴ However, the beneficial effects of RES in vitro must be seen in context with its low bioavailability in vivo. Walle et al.⁵ measured a concentration of $<0.02 \mu$ M of free RES in plasma after a dietary-relevant oral dose of 25 mg (110 μ M). RES was found to be widely distributed and extensively metabolized, mainly in the kidney and liver, forming predominantly *trans*-RES-3-O-sulfate (R3S), *trans*-RES-3,5-O-disulfate, *trans*-RES-3,4'-O-disulfate (RdS), *trans*-RES-3-O-glucuronide (R3G), and *trans*-RES-4'-O-glucuronide (R4G) (Figure 1).^{6,7} In comparison to RES, the concentrations of RES metabolites in humans can be up to 20-fold higher.⁸ In a pharmacokinetic study conducted by Burkon and Somoza,⁹ maximum human plasma metabolite concentrations of 0.95, 1.27, 0.16, and 0.19 μ M for R3S, RdS, R3G, and R4G, respectively, were reached following a single oral dose of 85.5 mg/70 kg body weight piceid, corresponding to 50 mg/70 kg body weight RES (220 μ mol) administered to healthy volunteers. Neither piceid nor free RES was detected in plasma or urine. Taken together, the parent compound RES is largely metabolized upon absorption and is, therefore, barely bioavailable, in contrast to its metabolites, leading to the

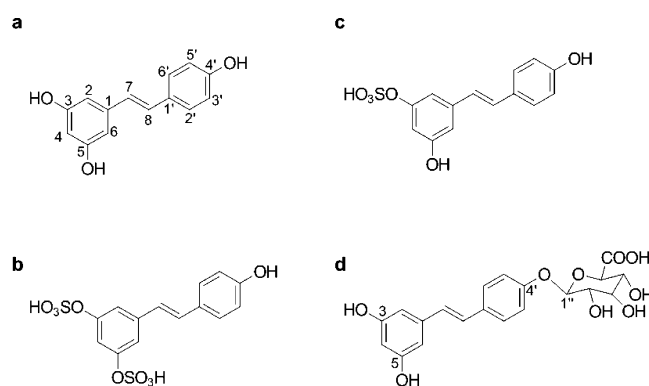


Figure 1. Structures of (a) *trans*-RES, (b) *trans*-RES-3,5-O-disulfate, (c) *trans*-RES-3-O-sulfate, and (d) *trans*-RES-4'-O-glucuronide. Numbering of structure a also applies to metabolites b–d.

hypothesis that the biological activity of RES in humans is elicited by metabolites.¹⁰

Recent in vitro studies indicate that the metabolites of RES may indeed mediate or contribute to anti-inflammatory health benefits, originally attributed to the parent compound. The glucuronated metabolites were found to suppress the cytokine-elicited induction of secreted eotaxin-1, a CC motif chemokine

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involved in the development of coronary heart disease, by 10% at 25 μM .¹¹ R3S, R3G, and R4G could be shown to reduce colon cancer cell growth at 10–50 μM .¹⁰ Also, as recently shown by Walker et al.,¹² R3S, at a concentration as low as 1 μM , lowered IL-6 expression by 57.8% in an in vitro model of stimulated human macrophages after 6 h of co-incubation with *Escherichia coli* lipopolysaccharides (1 $\mu\text{g/mL}$). Lipopolysaccharide (LPS) from the outer membrane of bacteria is widely used for in vitro studies on inflammation because it activates the NF- κB signaling cascade in the host tissue.¹³ Down-stream of NF- κB activation, the CC motif chemokines monocyte chemoattractant protein-1 (MCP-1 = CCL2) and macrophage inflammatory protein 1 beta (MIP-1b = CCL4) and the CXC motif chemokine interleukin 8 (IL-8 = CXCL8) and chemokine receptors CXCR2 (transcript variants 1 and 2, CXCR2var1/2), CCR1, and CCR2 are important factors in triggering immune cell trafficking and infiltration from the bloodstream into tissues via chemotaxis, but also enhance local cell activation and proliferation of inflamed tissue and immune competent cells, leading to disease progression.^{14,15} Another factor involved in inflammatory processes is the deacetylase Sirtuin-1 (SIRT1), a member of the silent mating type information regulation 2 enzyme family. SIRT1 inhibits NF- κB transcription and is down-regulated in response to LPS.¹⁶ Thus, an up-regulation of SIRT1 results in decreased levels of pro-inflammatory cytokines.¹⁶

The ability of phenolic dietary compounds to inhibit immune cell migration has been shown in vivo, in a model of allergic airway inflammation in mice, where intraperitoneal epigallocatechin-3-gallate (10 mg/kg body weight for 4 days) potentially reduced infiltration of immune cells and was able to inhibit migration of Th1 cells in vitro by 30–50% at a concentration of 1 μM via direct interaction with CXC motif chemokines.¹⁷ RES has also been demonstrated to inhibit monocyte recruitment into endothelium via inhibition of adhesion over a concentration range 0.3–10 μM in vitro.¹⁸ Furthermore, RES inhibited mRNA expression of CCR2 in THP-1 monocytes by 12% after 6 h of treatment and blunted monocyte recruitment on vascular endothelia at a concentration of 10 μM .¹⁹ As for RES metabolites, in an enzymatic study, SIRT1 was activated to the same extent by RES, R3S, and RES-4'-O-sulfate over a concentration range from 10 to 500 μM , resulting in attenuated cytokine levels in vitro.²⁰ In a mouse model of colitis, in which a dose of 100 mg RES/kg body weight was administered for 7 days, the induction of SIRT1 has been linked with attenuated cytokine levels in vivo.²¹ Considering the low bioavailability of free RES in vivo, those effects could very likely be caused by present metabolites. Therefore, this in vitro study investigates the effect of RES and its main physiological metabolites on immune chemotactic genes and proteins, as well as SIRT1, in differentiated human U-937 macrophages (U-937 M ϕ). Macrophages were activated with LPS from *Porphyromonas gingivalis* (pgLPS), an oral pathogen responsible for disease progression in periodontitis.²² Macrophages are exposed to dietary substances either directly during ingestion or via the bloodstream. As the parent compound RES is barely bioavailable after dietary intake, we aimed to identify the potential of RES metabolites, each of which may have the same or higher benefit than the parent compound. Considering plasma metabolite concentrations after intake of dietary-scale doses, in this study, we deliberately used compounds at only 1 μM . The glucocorticoid dexamethasone (DEX), which is known to suppress inflammation via the NF- κB pathway, was

used as anti-inflammatory control at 100 nM.²³ Here, we show for the first time the attenuating impact of main physiological RES metabolites on genes involved in macrophage activation and recruitment (chemokines IL-8, MIP-1b, MCP-1, and chemokine receptors CXCR2var2, CCR1, and CCR2) on mRNA and protein levels, as well as their effects on SIRT1 gene expression. Our findings may contribute to the development of more potent nutraceuticals and adjuvants in local and systemic inflammatory diseases containing RES and/or conjugates thereof.

MATERIALS AND METHODS

Chemicals. Methanol (MeOH) of HPLC and LC-MS grade was purchased from Carl Roth (Karlsruhe, Germany). FBS was obtained from Invitrogen (Karlsruhe, Germany) and pgLPS from InvivoGen (San Diego, CA, USA); peqGOLD total RNA isolation kit, peqGOLD Ultra Low Range DNA-Ladder II, and qPCR plates were purchased from Peqlab (Erlangen, Germany); a high-capacity cDNA reverse transcription kit and fast SYBR green Mastermix for PCR experiments were from Life Technologies (Carlsbad, CA, USA). ELISA kits for IL-8, MIP-1b, and MCP-1 were obtained from Merck (Vienna, Austria), RayBiotech (Norcross, GA, USA), and Life Technologies, respectively. For flow cytometry, fluorescein-labeled CXCR2 and labeled isotype control antibodies (both R&D Systems, Minneapolis, MN, USA) and rabbit anti-human CCR-1 pAb (PromoCell, Heidelberg, Germany) and isotype control rabbit mAb IgG (New England Biolabs, Ipswich, MA, USA) were used. CCR1 and isotype control were labeled with AlexaFluor488 (Abcam, Cambridge, UK). All other chemicals were from Sigma-Aldrich (Vienna, Austria).

Synthesis and Purification of RES Metabolites. The method for synthesis of RES glucuronides was adapted from an already published method,²⁴ with the following modifications: 1 equiv of RES-phenolate was conjugated with 2 equiv of acetobromo- α -D-glucuronic acid methyl ester, and after 1 h of stirring at 40 °C, the reaction flask was put on ice and adjusted to pH 8–9 with 16.7 mM sodium carbonate solution. The mixture of RES conjugates was evaporated to dryness and kept at –80 °C prior to purification. Metabolites were purified on a Dionex Ultimate 3000 semipreparative LC system (Thermo Fisher Scientific, Vienna, Austria) with the UV detector set to 300 nm. After dissolution of the synthesis product in MeOH/water (1:1, v/v), the first purification step was done on an RP18 column (Synergi Fusion, 250 $\times$ 10 mm, 4 μm , Phenomenex, Aschaffenburg, Germany) at a flow rate of 1.5 mL/min, by gradient elution with 10 mM ammonium formate buffer at pH 8.2 and MeOH. LC gradient (t (min), % MeOH) was (0, 20), (20, 70), (25, 100), (30, 20), and the column was equilibrated for 10 min after each run. The major peak (retention time of 20.5 min) was collected and subjected to a second purification on an RP18 column (XTerra Prep MS C18, 100 $\times$ 50 mm, 5 μm , Waters, Eschborn, Germany). The flow rate was set at 11 mL/min, gradient elution was done with the eluents already mentioned, and the following gradient (t (min), % MeOH) was used: (0, 25), (23, 60), (26, 25). Again, the column was equilibrated for 10 min after each run. Five peaks were collected and subjected to LC-MS (LCMS-2020, Shimadzu, Columbia, MD, USA, with LabSolutions software) analysis with an MS detector with ESI source and quadrupole analyzer recording positive and negative total ion current (TIC($\pm$)) and negative single ion monitoring (SIM(–)), with the following MS interface settings: heatblock temperature, 300 °C; desolvation line temperature, 200 °C; nebulizing gas flow, 1.5 L/min; drying gas flow, 15 L/min; gas flows were obtained from a nitrogen generator, scanning speed, 682 u/s. After evaluation of TIC(–) and SIM(–) for m/z 227 (RES), m/z 403 (RES glucuronides), and m/z 589 (RES diglucuronides), structure identification for two fractions of RES monoglucuronides was done by 1D and 2D NMR on a Bruker Avance III 500 MHz instrument (^1H , $^1\text{H}^1\text{H}$, $^1\text{H}^{13}\text{C}$, ^{13}C) at the NMR center of the Faculty of Chemistry, Vienna, Austria. NMR spectra were analyzed with SpinWorks 3.1.8.1 software, and chemical shifts and coupling constants were compared to published data sets.²⁵

Table 1. Forward and Reverse Primer Pair Sequences and Number of Cycles per Run^a

target	direction	sequence (5'–3')	cycles	C _T
IL-8	forward	ACCGGAAGGAACCATCTCAC	40	15.4 ± 0.4
	reverse	GGCAAACTGCACCTTCACAC		
MIP-1b	forward	CATGAAGCTCTGCGTGACTG	45	19.0 ± 1.2
	reverse	CCACAAAGTTGCGAGGAAGC		
MCP-1	forward	CCGAGAGGCTGAGACTAACC	40	15.9 ± 0.8
	reverse	CTTTCATGCTGGAGGCGAGA		
CCR1	forward	AACCAGAGAGAAGCCGGGATG	45	21.3 ± 0.7
	reverse	ACGGAGTTGCATCCCCATAG		
CCR2	forward	GAGCGGTGAAGAAGTCACCA	55	31.0 ± 1.2
	reverse	GCAGTTTATTAAGATGAGGACGAC		
CXCR2var1	forward	TGTTCTCTGTGGGAATACCTCCCC	55	32.0 ± 1.7
	reverse	GCTGTGACCTGCTGTTATTGGAGT		
CXCR2var2	forward	TCTGCCTAGAGCTCTGACTACCA	55	30.0 ± 0.3
	reverse	TGGGCTTTTCACCTGTAGGAC		
SIRT1	forward	AGTAGGCGGCTTGATGGTAA	45	24.6 ± 0.8
	reverse	CCTCAGCGCCATGGAATAATG		

^aC_T is the cycle when amplification of a target is first detected. C_T values represent the mean ± SD for 6 h incubations with pgLPS (*n* = 3–4).

Compounds were dissolved in DMSO at 10 mM and stored in aliquots at –80 °C.

Cell Culture. U-937 cell line was obtained from the American Type Culture Collection and cultured in Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM glutamine in a humidified incubator with 5% CO₂ at 37 °C and were split every 2–3 days. Differentiation was achieved by incubation with PMA (10 ng/mL) over 48 h, as already published by our group.¹² Incubations were done with complete medium, and DMSO concentration was 0.1% in all experiments.

Cellular Uptake. Uptake of RES and R4G into U-937 Mφ was tested at 1 μM and, subsequently, 10 μM. Time points chosen were 10 and 30 min and 1, 1.5, 2, 2.5, and 3 h. For R3S, uptake was only measured after 3 h of treatment. Cells were seeded in 24-well plates at a density of 0.2 × 10⁶ cells/well and, after differentiation, incubated with compounds at 37 °C. Cell culture plates were then immediately put on ice, and supernatants were removed. For protein precipitation, MeOH (–20 °C) was added to the supernatant (3:1) and centrifuged for 10 min at 4 °C and 16000g. Immediately after removal of the supernatant, cells were washed once with ice-cold PBS and taken up in 300 μL of distilled water, and two freeze–thaw cycles were done by immersing the plates in liquid N₂ and thawing them at ambient temperature. Lysed cells were then centrifuged under the conditions already mentioned. Both supernatants of precipitated incubation medium and lysed cells were filtered through a 0.45 μM PVDF filter and quantified by LC-MS. Limit of detection (LOD, S/N ratio 3.3:1) and limit of quantification (LOQ, S/N ratio 10:1) were calculated by LabSolutions software to be 3.15 and 10.5 nM for RES, 21.3 and 64.5 nM for R3S, and 15.1 and 50.2 nM for R4G, as determined by calibration curve from 0.039 to 10 μM at 300 nm UV detector wavelength. Recoveries for supernatant were 79, 76, and 73% for RES, R3S, and R4G, respectively. Recoveries for cell lysate were 84, 90, and 66% for RES, R3S, and R4G, respectively.

RT-qPCR. For gene expression analysis, cells were seeded in the same manner as for uptake experiments. Compounds were co-incubated in triplicate for 3 and 6 h with 1 μg/mL pgLPS and either DEX (100 nM) or compounds at 1 μM. mRNA of three wells was pooled and isolated and, subsequently, 2 μg, as measured by

spectrophotometry at 260/280 nm (TecanInfinite200, Groedig, Austria), was reverse transcribed, following the instructions provided by the manufacturer. RT-qPCR experiments were carried out in triplicate on a StepOnePlus device (AppliedBiosystems, Carlsbad, CA, USA). Primers were designed using PrimerBlast, and data were normalized to PPIA (primer sequences already published).¹² Primers were used at 100 nM, and qPCR cycles were adapted to the number of cycles when amplification of a target is first detected (C_T values) to reach fluorescent plateau phases, with 40 cycles for IL-8 and MCP-1, 45 cycles for MIP-1b, CCR1, and SIRT1, and 55 cycles for CCR2, CXCR2var1, and CXCR2var2 (Table 1). For data analysis, LinRegPCR2012.3 was used, and baseline-corrected starting concentrations (N₀ values) were used for normalization over PPIA. Four independent experiments with three technical replicates were conducted. To confirm PCR product lengths and purities, gene copies were run on a 3.5% agarose gel, with a DNA ladder ranging from 25 to 700 bp. All PCR products showed single bands, and product lengths corresponded to DNA ladder within 10 ± 3 bp.

ELISA. Incubations for the measurement of chemokines in cell culture supernatant were done as described for qPCR experiments. The supernatants of three wells were pooled and centrifuged for 10 min at 4 °C and 16000g, and aliquots were stored at –80 °C. Samples were diluted with complete medium, if necessary. ELISA measurements for IL-8 (Merck, Vienna, Austria), MIP-1b (RayBiotech, Norcross, GA, USA), and MCP-1 (LifeTechnologies, Carlsbad, CA, USA) were completed following the manufacturers' protocols. Four independent experiments were conducted.

Flow Cytometry (FC). For FC measurements, cells were seeded in 6-well plates 48 h prior to experiments at 1 × 10⁶ cells/well. After 6 h of incubation, the medium was removed, cells were scraped and taken up in ice-cold FC buffer, consisting of PBS (170 mM NaCl, 3.4 mM KCl, 10.0 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), 10% FBS, and 0.5% sodium azide. All staining and washing steps were done in Eppendorf tubes, centrifugation conditions were 5 min at 4 °C and 400g. The use of sodium azide and working at 4 °C are common strategies to impede further metabolism and prevent membrane proteins from being internalized.²⁶ Aliquots of 0.2 × 10⁶ cells were incubated with antibodies in 200 μL of FC buffer, 30 min for CXCR2 antibody (1:100) and 15 min for CCR1 pAb (1:400), followed by 15

min AlexaFluor488 (1:2000). Subsequently, samples were washed twice with 1 mL of FC buffer and resuspended in Hank's balanced salt solution (Sigma-Aldrich). Samples were transferred to a 96-well plate in duplicate and measured with a Guava easyCyte 8HT flow cytometer (Millipore, Darmstadt, Germany). Geometric mean fluorescence intensity (MFI) was used for effect calculation (treated over pgLPS (%)). Four independent experiments with two technical replicates were conducted.

Statistical Analysis. Data represent means $\pm$ SD and were analyzed using SigmaPlot 11.0 (SystatSoftware, USA). Prior to comparative testing, all sets were normality tested (Shapiro–Wilk). If normality was passed, values were *t* tested (*, $p < 0.05$) against pgLPS-control; if equal variance testing failed, a Wilcoxon–Mann–Whitney rank sum test was performed. For comparison of compounds to DEX, ANOVA was used with a post hoc Holm–Sidak test; if prior normality testing failed, a Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn's test for multiple comparisons was performed.

RESULTS AND DISCUSSION

Purification and Structure Elucidation of RES-Metabolites. Compounds in Figure 1 were drawn with ChemBio-Draw Ultra 12.0 (Cambridgesoft, Cambridge, MA, USA). R3S and a mixture of RES-3,5-O- and -3,4'-O-disulfate (59:41, RdS) were synthesized and purified as reported previously.²⁷ The synthesis of RES-glucuronides resulted in a collection of five peaks after the second purification step, with retention times (Rt, min) of 10.9 ± 0.38 (1), 17.4 ± 0.77 (2), 20.9 ± 1.28 (3), 27.3 ± 1.62 (4), and 30.9 ± 0.27 (5) (averages of 12 measurements; for a representative HPLC chromatogram, see Supplemental Figure A). As TIC(–) and SIM(–) chromatograms for *m/z* 403 of peaks 2 and 3 showed only slight impurities (see Supplemental Figure B for MS chromatograms), NMR spectra of those two fractions were taken.

NMR Data. NMR shifts and coupling constants corresponded to data already published for R3G and R4G.²⁵ Peak 2 (Rt = 17.4 ± 0.77 min) was therefore identified as R4G with a purity of 87.8%, and peak 3 (Rt = 20.9 ± 1.28 min) corresponded to NMR and MS data for R3G, with a purity of 95.0%. Impurities consisted mainly of the other isomer and traces of solvent.

Cellular Uptake. A previously published study on the uptake kinetics of 3–100 μ M labeled RES by human hepatic cells showed that RES is taken up in a concentration-dependent manner through a combination of passive diffusion and carrier-mediated transport.²⁸ In this study, we measured the uptake of RES and R4G into U-937 M ϕ time-dependently over 3 h (Table 2) and of R3S after 3 h of incubation at 37 °C to determine the amount of compound available for biological effects. At the concentration of 1 μ M none of the substances could be detected in the cells. To exclude limitations due to LOD (3.15, 21.3, and 15.1 nM for RES, R3S, and R4G, respectively), uptake was repeated at a concentration of 10 μ M. At the 10-fold concentration, an uptake at all measured time points could be observed for RES with a maximum of 0.12 ± 0.001 μ M ($1.37 \pm 0.31\%$ of the applied concentration), after 3 h. Following an incubation with 10 μ M R3S, this compound could not be detected in the cells; however, after 3 h, its concentration in the supernatant showed a decrease of -1.22 ± 0.82 μ M compared to the concentration in the incubation medium ($p = 0.017$), indicating an uptake. Table 2 shows an uptake of R4G at 10 μ M into cells only at time points of 10 min (0.023 ± 0.001 μ M) and 30 min (0.028 ± 0.003 μ M), and the supernatant at 1 and 10 μ M showed no differences from the

Table 2. Time-Dependent Uptake of (A) RES and (B) R4G into U-937 M ϕ ^a

(A) RES				
time (min)	1 μ M RES (mean $\pm$ SD)		10 μ M RES (mean $\pm$ SD)	
	supernatant (μ M)	cells (μ M)	supernatant (μ M)	cells (μ M)
10	1.05 ± 0.13	nd	9.79 ± 1.04	0.08 ± 0.02
30	1.03 ± 0.14	nd	9.98 ± 1.21	0.10 ± 0.01
60	0.79 ± 0.07 a	nd	8.80 ± 1.24 a	0.11 ± 0.03
90	0.65 ± 0.10 a	nd	9.18 ± 1.16	0.10 ± 0.01
120	0.78 ± 0.46 a	nd	9.07 ± 1.02	0.120 ± 0.001 b
150	0.43 ± 0.09 a	nd	7.73 ± 1.16 a	0.120 ± 0.001 b
180	0.41 ± 0.09 a	nd	8.86 ± 1.25	0.120 ± 0.001 b

(B) R4G				
time (min)	1 μ M R4G (mean $\pm$ SD)		10 μ M R4G (mean $\pm$ SD)	
	supernatant (μ M)	cells (μ M)	supernatant (μ M)	cells (μ M)
10	1.05 ± 0.30	nd	9.24 ± 0.46	0.023 ± 0.001
30	1.18 ± 0.12	nd	9.31 ± 0.70	0.028 ± 0.003
60	1.05 ± 0.19	nd	8.87 ± 0.71	nd
90	0.84 ± 0.23	nd	8.62 ± 1.30	nd
120	1.07 ± 0.06	nd	8.95 ± 0.89	nd
150	0.90 ± 0.21	nd	9.12 ± 1.03	nd
180	0.98 ± 0.09	nd	8.64 ± 1.21	nd

^aData are depicted as the mean $\pm$ SD ($n = 3$, $tr = 2$). Data for supernatant were compared to the incubation medium by ANOVA; differences from incubation medium are marked with "a" ($p < 0.05$). Data for cells were compared by all-pairwise ANOVA, and according to ANOVA results, calculated differences to 10 min measurements are marked with "b" ($p < 0.05$).

incubation medium at any measured time point, indicating no further uptake into cells. Riha et al.²⁹ identified RES and R3S as substrates for organic anion transporting polypeptides in Chinese hamster ovary cells, whereas R4G was not taken up at concentrations between 12.5 and 400 μ M, partially confirming our results obtained in U-937 cells. The uptake patterns of RES, R3S, and R4G in macrophages could be due to efflux transporters, such as breast cancer resistance protein 1 (BCRP1) and multidrug resistance protein 3 (MRP3), both of which can be found on macrophages.³⁰ As shown in a study by van de Wetering et al.,³¹ RES, R3S, and R3G are substrates for those two ABC transporters. In said study, it was shown that the transport kinetics of RES and R3S for the efflux transporters BCRP1 and MRP3 were similar; therefore, studies for R3S were limited to 3 h of uptake. The glucuronated metabolite, on the other hand, had a much higher affinity to MRP3, leading to a higher efflux rate, as compared to RES and R3S, further confirming our results for RES and R4G.³¹ Another point to be considered is the high plasma protein binding of RES (nearly 100%) and metabolites (approximately 50%), reducing the amount of free compound susceptible to transport.⁹ In this uptake study in U-937 M ϕ , no further metabolism of substances such as degradation, dehydrogenation, or dimer formation could be observed.

Despite the very limited uptake, our data show effects on gene and protein expression (see following chapters). It is possible that the amounts of the here tested substances in the cells suffice for our observed effects via the already reported interaction with the NF- κ B pathway.¹ Another possibility for the observed effects is the interaction of RES with the estrogen receptor α . RES has been shown to be an agonist for the

estrogen receptor α ,³² albeit at concentrations of 3–10 μM , which are higher than the concentration used in this study. The activation of this receptor has been associated with the suppression of the inflammatory response in a model of arthritis in rats.³³

Activation of U-937 M ϕ with pgLPS. As a control study for the activation of U-937 M ϕ , the effects on gene and protein expression of cells treated with or without 1 $\mu\text{g/mL}$ pgLPS were compared at various time points. As shown in Figure 2, pgLPS significantly up-regulated mRNA levels of IL-8, MIP-1b, and MCP-1 at 3 and 6 h (Figure 2B), the largest effect was measured for MIP-1b at 3 h, with a (59.4 ± 6.98)-fold increase. The corresponding proteins (with the exception of MCP-1 at 3

h) also showed elevated levels, with the highest protein expression for MIP-1b after treatment for 6 h (53.6 ± 12.1)-fold, Figure 2C). Gene expression of chemokine receptors and SIRT1 (Figure 2A) was down-regulated by pgLPS with maximum decreases for CXCR2var2 (6 h) and CCR1 (3 h) of -74.9 ± 8.20 and $-64.6 \pm 5.05\%$, respectively, and $-22.4 \pm 4.16\%$ for SIRT1 (3 h). A similar decrease in mRNA expression for CXC and CC receptors was published for activated human leucocytes ex vivo and for rats in vivo, as well as down-regulation of SIRT1 deacetylase after incubation with *Escherichia coli* LPS in vitro in RAW264.7 macrophages.^{34–36} In U-937 M ϕ , in contrast to decreased receptor mRNA expression (Figure 2A), flow cytometric measurements of CXCR2 and CCR1 after 6 h of treatment with pgLPS showed an increase in surface protein compared to control of $7.70 \pm 0.13\%$ for CXCR2 ($p = 0.03$) and $0.7 \pm 0.01\%$ for CCR1 ($p = 0.02$). Keller et al.³⁷ reported time-dependent changes in cytokine gene and protein expression in cultured LPS-activated mouse macrophages. In their study, the gene and protein expression of transcription factors of inflammatory pathways underwent repeated oscillating changes over 48 h. Those changes were attributed to an “intrinsic clockwork” in macrophages. Thus, we hypothesize that this oscillation might have contributed to our differential findings for mRNA and protein levels. Also, as shown by Daigneault et al.,³⁸ the PMA-induced differentiation process of monocytes into macrophages might have caused our here observed elevated levels of membrane receptor proteins.

Gene Expression and Protein Release of Chemokines.

Glucocorticoids inhibit inflammation by direct interaction with the NF- κB -pathway.²³ Accordingly, in our experiments, the anti-inflammatory control DEX inhibited mRNA expression and protein expression of all chemokines at 100 nM (Figure 3). As recently reviewed by Joven et al.,³⁹ especially CC motif chemokines present a promising target for polyphenols in the prevention and treatment of a multitude of inflammatory diseases. In our study, qPCR analysis of MIP-1b and MCP-1 confirmed these results only partially, by showing that mean mRNA levels of MCP-1 (CCL2, Figure 3E), but not MIP-1b (CCL4, Figure 3C), after 3 h of incubation are comparably decreased after treatment with pgLPS and RES, R3S, and RdS by -24.3 ± 11.1 , -24.7 ± 5.51 , and $-28.7 \pm 19.2\%$, respectively. For RES, a similar effect has been shown in adipocytes at a concentration of 10 μM .⁴⁰ Yang et al.⁴¹ obtained similar results for human pulmonary artery endothelial cells and reported an attenuating effect on cytokine-induced eotaxin (CCL11) for RES, but not for R3G and R4G at 25 μM . Although IL-8 and MCP-1 are regulated by the same transcription factor in U-937 cells, NF- κB ,⁴² the here tested compounds had no apparent effect on IL-8 gene expression at the chosen time points (Figure 3A). Interestingly, though, in our experiments, RES, RdS, and R3G did show an inhibiting effect on IL-8 protein release after 9 h (-12.7 ± 7.65 , -22.9 ± 3.57 , and $-16.5 \pm 6.04\%$, Figure 3B). RES, R3S, RdS, and R3G also inhibited protein release for MIP-1b, with the highest inhibiting value of $-20.1 \pm 7.00\%$ for RdS followed by R3S with $-15.4 \pm 8.68\%$ (both 9 h of treatment, Figure 3D). An inhibition of MCP-1 protein was measured for RdS after 3 and 9 h treatments (-25.7 ± 8.29 and $-23.0 \pm 10.1\%$), RES (9 h, $-21.8 \pm 16.2\%$), and R3S (3 h, $-20.8 \pm 13.9\%$), as shown in Figure 3F. In contrast to sulfated metabolites and R3G, R4G showed a differential effect on MIP-1b, with an mRNA increase of $24.5 \pm 14.8\%$ after 6 h. In our test system, sulfated

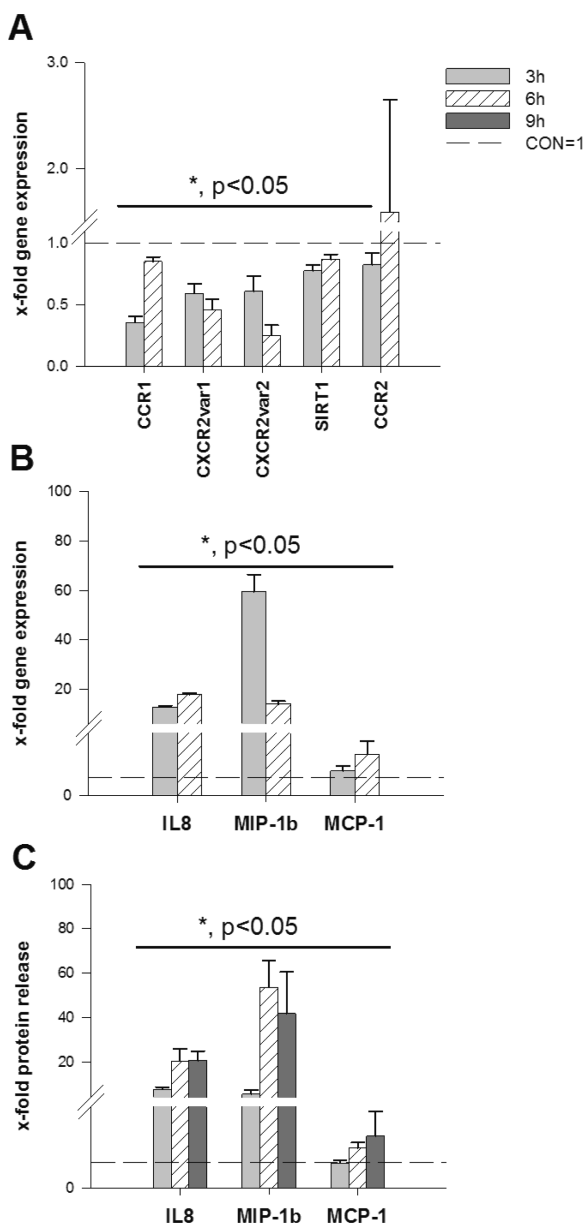


Figure 2. Control data for (A, B) mRNA and (C) protein expression of CCR1, CXCR2var1/2, SIRT1, CCR2, IL8, MIP-1b, and MCP-1 in U-937 M ϕ after treatment with 1 $\mu\text{g/mL}$ pgLPS for indicated time points (see legend). x -Fold changes in pgLPS treated cells were compared to DMSO-control (dashed line = 1-fold). Data represent means $\pm$ SD ($n = 3$ –4, $tr = 2$ –3). (*) $p < 0.05$ compared to untreated control.

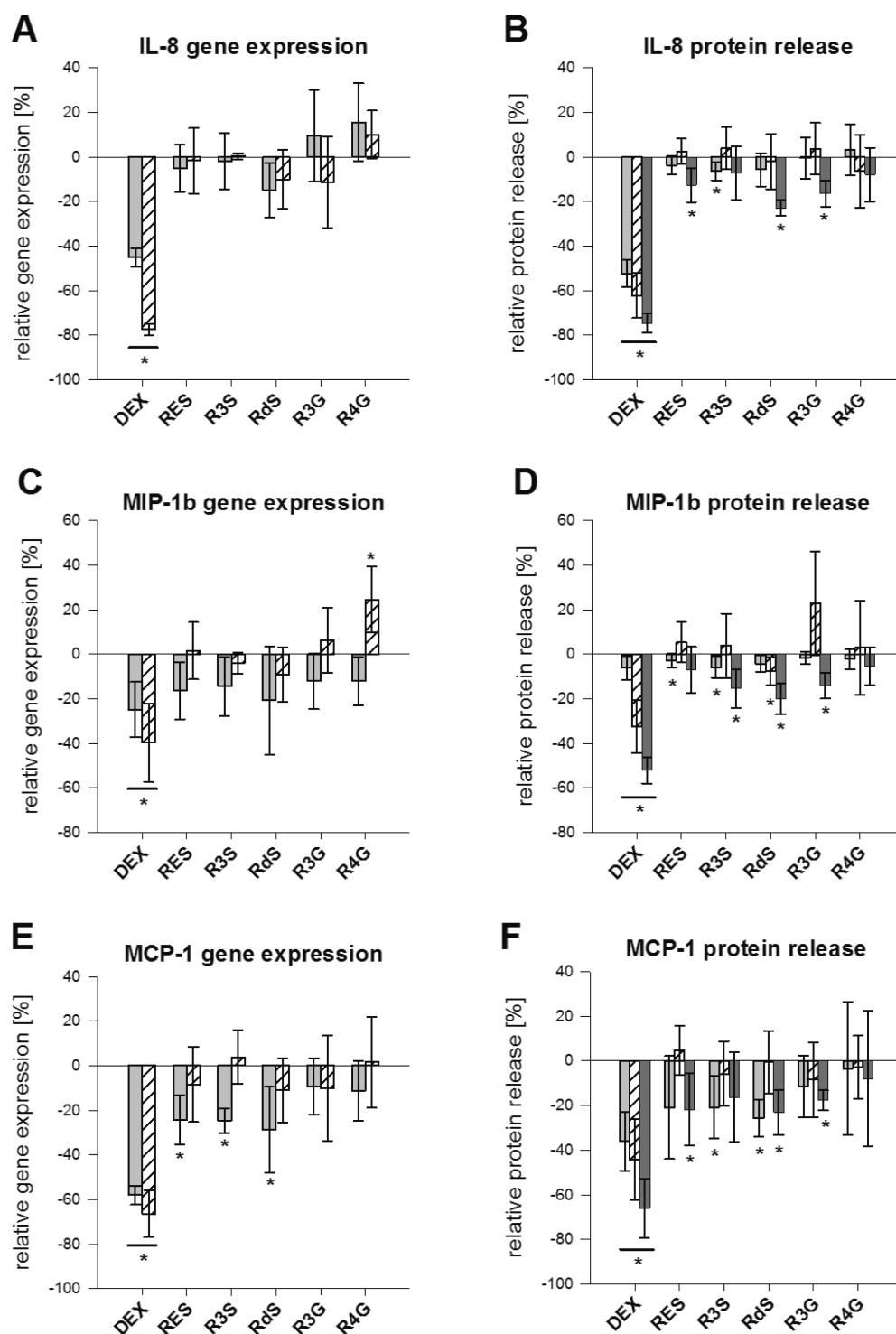


Figure 3. Changes in (A, C, E) mRNA expression and (B, D, F) protein release after 3 (light gray bars), 6 (slashed bars), and 9 h (dark gray bars) of co-incubation with pgLPS (1 μ g/mL) and substances at 1 μ M and 100 nM (DEX), respectively. pgLPS-control was set at 0% change; values are differences from pgLPS-control in percent. Data represent means $\pm$ SD ($n = 3-4$, $tr = 2-3$). (*) $p < 0.05$ compared to pgLPS-control.

metabolites were more anti-inflammatory active than glucuronated metabolites, which is in accordance with previously published work by our group, studying concentrations of 1–10 μ M RES, R3S, RdS, R3G, and R4G, where RES and sulfated, but not glucuronated, conjugates inhibited IL-6 and TNF- α protein release.¹² Similarly, in an in vitro study on colon cancer cell progression by Aires et al.,⁴³ R3S showed inhibiting effects at 30 μ M in contrast to R3G and R4G. Considering the results

of our uptake experiments, RES and R3S did indeed have effects on gene and protein expression, whereas R4G showed an uptake only at early time points at 10 μ M and no changes indicating any further uptake in the concentration of the supernatant at 1 or 10 μ M. The pro-inflammatory effect of R4G on MIP-1b could be an indication of the inflammatory properties of glucuronated phenolic metabolites, as reported by Liao and Lin for quercetin-3-glucuronide in isolated mouse

Table 3. Gene Expression Levels of CCR2 and CXCR2var1 at Various Incubation Times after Co-incubation with Compounds^a

CCR2	3 h	6 h	CXCR2var1	3 h	6 h
DEX	97 (63)	170 (87)	DEX	111 (101)	100 (61)
RES	81 (105)	142 (74)	RES	106 (137)	121 (117)
R3S	103 (100)	132 (61)	R3S	111 (129)	122 (91)
RdS	98 (196)	101 (13)	RdS	109 (167)	102 (72)
R3G	85 (110)	131 (94)	R3G	110 (95)	102 (113)
R4G	108 (89)	224 (49)*	R4G	104 (127)	175 (110)

^aData are median and percent range (%); (*) $p < 0.05$ compared to pgLPS-treated control set at 100%.

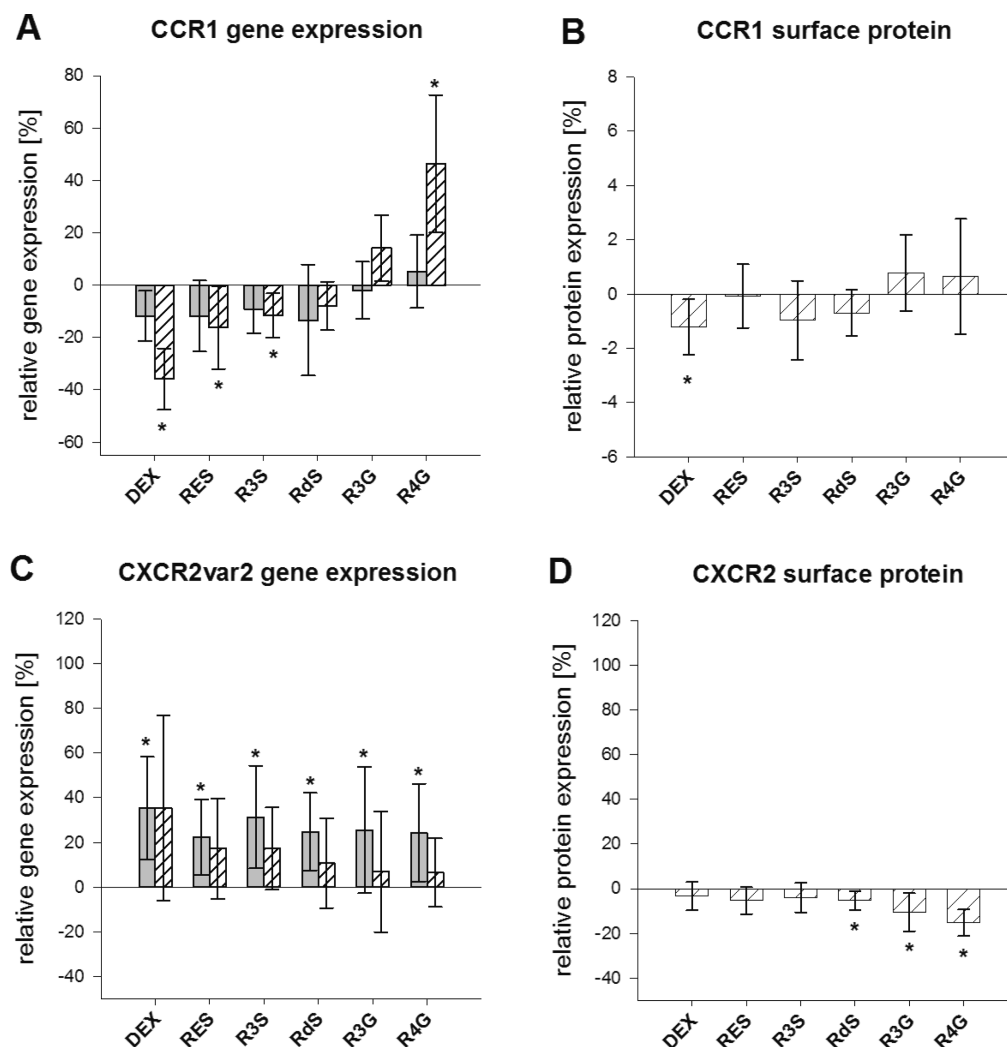


Figure 4. Changes in (A, C) mRNA and (B, D) surface protein expression after 3 (gray bars) and 6 h (slashed bars) of co-incubation with pgLPS (1 $\mu\text{g/mL}$) and substances at 1 μM and 100 nM (DEX), respectively. pgLPS-control was set at 0% change; values are differences from pgLPS-control in percent. Data represent means $\pm$ SD ($n = 3-4$, $tr = 2-3$). (*) $p < 0.05$ compared to pgLPS-control.

macrophages.⁴⁴ In our study, treatment with sulfated metabolites showed no significant difference (ANOVA) to treatment with the parent compound, as also found for COX-1 and iNOS inhibition at a compound concentration of 34 μM .⁴⁵ Considering the aggravated inflammatory profile in patients with rheumatoid arthritis, after application of a monoclonal MCP-1 antibody,⁴⁶ our results also support the hypothesis that sulfated RES metabolites could prove beneficial in inflammatory diseases, especially targeting MCP-1 and MIP-1b. Additionally, our results show that the impact of compounds should preferably be assessed on both gene and protein levels. Our data provide an interesting insight into the functional

impact of minute amounts of RES and the metabolites formed upon ingestion on inflammatory processes. The long-lasting and pronounced effects of sulfated metabolites in macrophages, especially on MCP-1, may be worth further investigation in animal or human studies.

Gene and Surface Protein Expression of Chemokine Receptors. Treatment with pgLPS led to down-regulation of chemokine receptor mRNA (Figure 2A); co-incubation with pgLPS and compounds/DEX for 3 and 6 h did not influence mRNA levels of CXCR2var1 (Table 3). Interestingly, only R4G showed an up-regulation of CCR2 by 224% with a percent range of 48.5% after 6 h (Table 3), suggesting an anti-

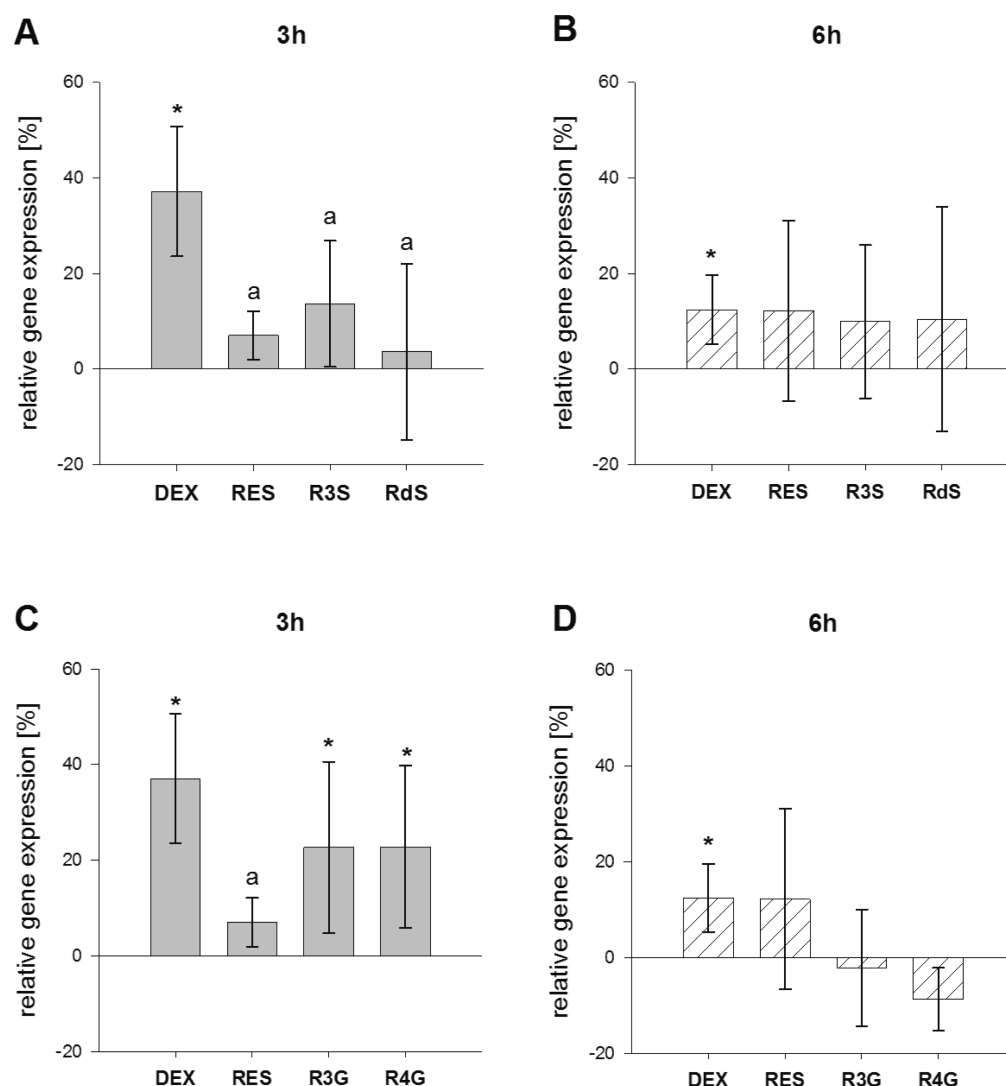


Figure 5. Impact of compounds at 1 μ M or 100 nM (DEX) in co-incubation with pgLPS (1 μ g/mL) on SIRT1 mRNA expression for (A, B) DEX, RES, R3S, and RdS after 3 and 6 h and for (C, D) DEX, RES, R3G, and R4G after 3 and 6 h. pgLPS-control was set at 0% change; values are differences from pgLPS-control in percent. Data represent means $\pm$ SD ($n = 3-4$, $tr = 2-3$). The asterisk (*) indicates statistically significant differences compared to pgLPS-treated control ($p < 0.05$). Letter "a" indicates results for one-way ANOVA versus anti-inflammatory control DEX.

inflammatory activity of this compound. It should be noted that our PCR results show high standard deviations, likely due to low abundances of mRNA in U-937 *M ϕ* for both genes, which resulted in very high CT values. Hence, we cannot exclude that the here tested compounds could elicit effects on CXCR2var1 mRNA expression in other immune cells. The same applies for CCR2 mRNA expression, as, for example, RES at 10 μ M has been shown to inhibit CCR2 expression in THP-1 monocytes.¹⁹

mRNA levels of chemokine receptors CCR1 and CXCR2var2 were differentially affected by RES metabolites (Figure 4). After 6 h, RES and R3S decreased CCR1 gene expression to the same extent as DEX, whereas R4G ($46.3 \pm 26.2\%$) showed the opposite effect (Figure 4A), as also reported for MIP-1b and CCR2 mRNA in this study (Figure 3C; Table 3). CCR1 receptor surface expression after 6 h of co-incubation with pgLPS was not affected by treatments, except for DEX, which down-regulated surface protein levels (Figure 4B). mRNA of CXCR2var2 was up-regulated to the same extent by all treatments, after 3 h of incubation, as tested by ANOVA (Figure 4C), showing that R4G affected the CXC

motif receptor differently from the CC motif receptors. After 6 h, no effect for RES and its metabolites on CXCR2var2 mRNA expression could be observed (Figure 4C). An up-regulation of chemokine receptors previously down-regulated by an inflammatory stimulus (Figure 2A) was also demonstrated in cytokine-induced rat synovial fibroblasts treated with green tea extract containing polyphenols (2.5–40 μ g/mL catechins).³⁴ However, it should be noted, that, in our study, the mRNA levels for two different transcript variants of CXCR2 (var1 and var2) were measured separately, with the CXCR2 antibody not being specific for a certain transcript variant, which could account for the differential effects on mRNA and surface protein levels.

In the here presented study, despite the up-regulation of mRNA compared to LPS, CXCR2 surface protein was down-regulated by RdS, R3G, and R4G by -5.33 ± 4.18 , -10.6 ± 8.49 , and $-15.2 \pm 5.99\%$, respectively (Figure 4D). This effect could be due to internalization of CXCR2 receptor proteins upon exposure to inflammatory stimuli.^{15,47} Another possibility for the differential effects on CXC-motif gene and protein expression would be the post-transcriptional influence of

microRNAs on CXC-motif receptors, as shown for microRNA146a and the CXC motif receptor 4 (CXCR4).⁴⁸ Considering our results on mRNA expression of chemokine receptors CCR1, CCR2, and CXCR2var2, we find it difficult to definitely attribute effects on receptors to be pro- or anti-inflammatory (Figure 4A). Further studies are needed to elucidate the complex mechanisms underlying chemokine receptor regulation.

Sirtuin-1 Gene Expression. High levels of SIRT1 have been demonstrated to exert protective effects in numerous inflammatory diseases.^{16,36} SIRT1 is an active inhibitor of NF- κ B, leading to reduced levels of chemokines and cytokines.²¹ As our data in Figure 5 show, after 3 h of treatment (Figure 5A,C), at 1 μ M, up-regulated SIRT1 gene expression for R3G and R4G was comparable to DEX (ANOVA versus DEX, $p = 0.392$), but not for RES or the sulfated metabolites (ANOVA versus DEX, $p = 0.017$). R3G and R4G even overcompensated the down-regulation of SIRT1 by pgLPS (Figure 2A) by 22.7 ± 17.9 and $22.8 \pm 16.9\%$, respectively. A similar up-regulation could be shown in HEPG2 cells, after treatment with 10 μ M RES and glucuronated conjugates.⁴⁹ Further confirming our in vitro results, in an in vivo study in mice, after ingestion of high doses of RES (440 μ M/kg bw per day for 2 weeks), an increase of SIRT1 expression of about 25% has been shown in lymphocytes from inflamed colon lamina propria.²¹ RES glucuronides are mainly formed and absorbed in the small intestine,^{25,50} but as glucuronides are transported via the bloodstream and are substrates for BCRP1 and MRP1,³¹ they could affect SIRT1 expression in colon mucosa and other tissues. In our study, in contrast to glucuronated metabolites, at 1 μ M, RES and the sulfated conjugates did not show effects on SIRT1 expression after 3 and 6 h of co-incubation with pgLPS (Figure 5A,B) and for glucuronated metabolites, no up-regulation could be measured after 6 h (Figure 5D).

The mRNA of SIRT1 and CXCR2var2, both being important factors in the development and progression of inflammatory diseases, was regulated comparably to the anti-inflammatory control DEX. The effects of sulfated and glucuronated metabolites, as well as DEX, on CC and CXC motif receptors on mRNA and protein levels are ambiguous and require further studies, as mentioned above. With regard to CC and CXC motif chemokines (IL-8, MCP-1, MIP-1b), the sulfated RES metabolites R3S and RdS were more potent than the tested RES glucuronates in inhibiting LPS-induced gene expression and protein expression in immune-competent macrophages. In summary, resveratrol metabolites formed in vivo, at concentrations reached after dietary-scale doses, were demonstrated to influence genes and proteins involved in inflammatory immune processes in immune-competent macrophages.

■ ASSOCIATED CONTENT

■ Supporting Information

Supplemental Figures A and B. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01830.

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Notes

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■ ABBREVIATIONS USED

BCRP1, breast cancer resistance protein 1; CCR, CC motif chemokine receptor; COX, cyclo-oxygenase; C_T , number of qPCR cycles when amplification of a target is first detected; CXCR, CXC motif chemokine receptor; DEX, dexamethasone; FC, flow cytometry; IL, interleukin; iNOS, inducible nitric oxide synthase; LOD, limit of detection; LOQ, limit of quantification; MCP-1, monocyte chemotactic protein-1; MeOH, methanol; MIP-1b, macrophage inflammatory protein 1 beta; MRP3, multidrug resistance protein 3; pgLPS, *Porphyromonas gingivalis* LPS; R3G, *trans*-RES-3-*O*-glucuronide; R4G, *trans*-RES-4'-*O*-glucuronide; R3S, *trans*-RES-3-*O*-sulfate; RdS, *trans*-RES-disulfates; RES, *trans*-resveratrol; Rt, retention time; SIM, single ion monitoring; SIRT1, Sirtuin-1; TIC, total ion current; TNF- α , tumor necrosis factor alpha; U-937 M ϕ , U-937 macrophages

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3.4 Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations

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This paper presents the establishment of a cell culture model of PMA differentiated macrophages, characterized by surface marker CD11b, as well as an inflammation model using ecLPS, characterized by gene transcription of IL-1 α , IL-1 β and IL-6, as well as release of MMP-2, MMP-9, IL-8 and IL-6 over time. Using this inflammation model, Resveratrol metabolites were tested in ecLPS activated human macrophages and their effect on cytokine gene transcription and release was established (IL-1 α , IL-1 β , IL-6, TNF- α).

I synthesized and purified the glucuronated metabolites used in this study, and participated in cell culture as well as RT-qPCR experiments. I also participated in data analysis and writing of the manuscript.

Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations

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Resveratrol has been shown to exploit various biological activities, including an anti-inflammatory activity. However, resveratrol is metabolized by phase II enzymes post-absorption to predominantly form glucuronides and sulfates. To investigate the anti-inflammatory effects of resveratrol and its dominating sulfated and glucuronated metabolites formed *in vivo*, U-937 macrophages were chosen as an immune-competent model system, known to release cytokines upon lipopolysaccharide stimulation. U-937 cells were stimulated with lipopolysaccharides from *Escherichia coli* (*E. coli*-LPS) to evoke an inflammatory reaction, and pre- or co-incubated with 1 or 10 μM of resveratrol (RES), resveratrol-3-sulfate (R3S), resveratrol-disulfates (RDS), resveratrol-3-glucuronide or resveratrol-4'-glucuronide. Time dependent gene expression of IL-6, IL-1 α/β and IL-1R by qPCR was studied at 1 h, 3 h, 6 h, 9 h, and 24 h of incubation, and the release of IL-6 and TNF- α , after 6 h was analysed by means of non-magnetic or magnetic bead analysis. As a result, 10 μM resveratrol completely inhibited the *E. coli*-LPS-induced release of IL-6, while resveratrol-3-sulfate and resveratrol-disulfates decreased it by respective $84.2 \pm 29.4\%$ and $52.3 \pm 39.5\%$. Whereas TNF- α release was reduced by $48.1 \pm 15.4\%$, $33.0 \pm 10.0\%$ and $46.7 \pm 8.7\%$ by RES, R3S and RDS, respectively. These results show that not only resveratrol but also resveratrol-3-sulfate and resveratrol-disulfates exhibit an anti-inflammatory potential by counteracting an inflammatory challenge in U-937 macrophages at plasma representative concentrations.

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Introduction

Resveratrol, a stilbenoid, is commonly found in peanuts, grapes and red wine.^{1,2} It has been identified to possess anti-oxidant,³ chemopreventive^{4,5} and anti-inflammatory activities.⁶ However, upon digestion and absorption, resveratrol is metabolized by phase II enzymes, resulting mainly in the formation of sulfated and glucuronated metabolites.^{7–10} In human intervention studies,^{8,10,11} the following metabolites have been identified in the human plasma and urine: resveratrol-3-sulfate (Res-3-sulf, R3S), resveratrol-3,5-disulfate, resveratrol-3,4'-disulfate (Res-disulfates, RDS), resveratrol-4'-sulfate, resveratrol-3-glucuronide (Res-3-gluc, R3G) and resveratrol-4'-glucuronide (Res-4'-gluc, R4'G). After intake of a bolus dose of 85.5 mg per 70 kg body weight of piceid, a resveratrol glucoside, the in 3-position monosulfated metabolite and the disulfated metabolites were the predominant metabolites detected after 6 h in the plasma of the volunteers,

reaching a maximum concentration of 1 μM .⁸ In addition, after consumption of a high bolus of 5 g resveratrol, resveratrol-3-sulfate was detected as the most abundant metabolite with a concentration of up to 14 μM in the plasma. In the same study, resveratrol sulfates and glucuronides were analyzed in the plasma with half-lives between 3.2 and 11.5 h, and 2.9 to 10.6 h, respectively.¹¹ Moreover, an uptake of repeated doses of up to 5 g resveratrol per day for 21 days resulted in a maximal plasma concentration of about 13 μM resveratrol-3-sulfate.¹² Incubation times and the concentrations used in *in vitro* studies focusing on the biological effects of these metabolites should, therefore, not exceed 14 μM .

The effects of resveratrol on the release of cytokines after stimulation with lipopolysaccharides (LPS) have been studied in various cell models. In a study using Caco-2 cells, a human colon cancer cell-line, it has been shown that resveratrol, up to a concentration of 50 μM , inhibits the secretion of the pro-inflammatory mediators prostaglandin E2 and cyclooxygenase-2, when treated with the compound for 1 h prior to a 48 h challenge with LPS. Furthermore, resveratrol has been shown to elicit an anti-inflammatory activity in macrophages by reducing the LPS-stimulated release of interleukin-1 β (IL-1 β)

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and tumor necrosis factor alpha (TNF- α), gene expression of IL-1 β in concentrations between 1 and 10 μ M after 6 h of incubation.¹³

Gram-negative bacteria, like *Escherichia coli* (*E. coli*), express the endotoxins LPS on their surface, which evoke an inflammatory reaction of the host when offered to the immune system.^{14,15} Rittig and co-workers¹⁶ showed by measurement of different cytokines that the immune response to LPS varies depending on the LPS phenotype. A treatment of human peripheral blood mononuclear cells with media from platelets which were stimulated with LPS from *E. coli* led to a release of the interleukins-6 (IL-6) and -8 (IL-8), as well as the matrix metalloproteases-2 (MMP-2) and -9 (MMP-9), with a different efficacy than a treatment of the platelets with LPS from *Salmonella minnesota*.¹⁷ However, *E. coli* are ubiquitous bacteria that can cause food poisoning as well as sepsis. Therefore, LPS from *E. coli* should be used analyzing a pro-inflammatory response in blood, monocytes or macrophages. Taken into account that different types of LPS may affect the release of cytokines from one cell model to another with different efficacies, prior to our investigation of the influence of resveratrol on the immune response, the optimal marker for the respective test system, considering the type of LPS and the cell-line used, needed to be identified. Of particular interest for the identification of immune-modulatory effects are immune-competent cells, like monocytes or macrophages. For *in vitro* studies, U-937 macrophages are widely studied since these cells grow as monocytes in suspension and have been demonstrated to release IL-6 after stimulation with IL-1 β ,¹⁸ and MMP-2 and MMP-9 after stimulation with *E. coli*-LPS.¹⁹ The U-937 monocytes can be differentiated with phorbol-12-myristate-13-acetate (PMA) into U-937 macrophages that may serve as a model system to identify an anti-inflammatory activity of natural compounds by means of a reduction of TNF- α , IL-6 and IL-1 β gene expression.²⁰ In our study, we first aimed to determine the optimal cell culture conditions to study the anti-inflammatory potential of a target compound after incubation of U-937 macrophages with LPS from *E. coli*. Afterwards, we chose *E. coli*-LPS-stimulated U-937 macrophages as an *in vitro* system to elucidate the anti-inflammatory effects of plasma representative

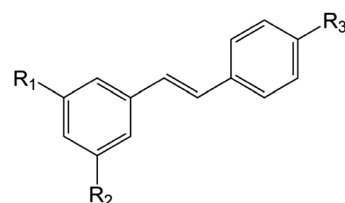
concentrations of resveratrol sulfates and glucuronides in comparison to their parent compound, since data on the anti-inflammatory potential of the resveratrol plasma metabolites are limited to the use of concentrations ≥ 30 μ M and incubation times of up to 48 h.^{21,22}

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich (Vienna, Austria), unless indicated otherwise. Lipopolysaccharide from *E. coli* (055:B5) was reconstituted to a concentration of 10 mg mL⁻¹ and aliquots were stored at -20 °C. The primers designed (primer3) were verified with primer BLAST (NCBI) and synthesized by VBC-Biotech (Vienna, Austria) (Table 1).

Trans-resveratrol metabolites were synthesized as described in previous publications of our group.^{8,9,23} The structure and purity of the synthesized compounds are shown in Fig. 1.



	R ₁	R ₂	R ₃	Purity
RES	-OH	-OH	-OH	99 %
R-3-S	-O-SO ₃ Na ⁺	-OH	-OH	95%
R-D-S	-O-SO ₃ Na ⁺ -O-SO ₃ Na ⁺	-O-SO ₃ Na ⁺ -OH	-OH -O-SO ₃ Na ⁺	98 %
R-3-G	-O-C ₆ H ₄ O ₆	-OH	-OH	95 %
R-4'-G	-OH	-OH	-O-C ₆ H ₄ O ₆	99 %

Fig. 1 Structure of *t*-resveratrol (RES), *t*-resveratrol-3-sulfate (R-3-S), *t*-resveratrol-disulfate (R-D-S), *t*-resveratrol-3-glucuronide (R-3-G) and *t*-resveratrol-4'-glucuronide (R-4'-G) and purity^a of synthesized compounds.

Table 1 Primer pairs used for the gene expression analyses by qPCR

Primer	Direction	Sequence	T _m [°C]
PPIA	Sense	5'-CCA CCA GAT CAT TCC TTC TGT AGC-3'	55.8
	Anti-sense	5'-CTG CAA TCC AGC TAG GCA TGG-3'	57.1
IL-6	Sense	5'-AAA CAA CCT GAA CCT TCC AAA GA -3'	57.1
	Anti-sense	5'-GCA AGT CTC CTC ATT GAA TCC A -3'	58.4
IL-6R	Sense	5'-CCA CCC CCA TGC AGG CAC TTA-3'	61.4
	Anti-sense	5'-CCA GGC TCC CTC CAG CAA CCA-3'	63.0
IL-1 α	Sense	5'-CCA GTG CTG CTG AAG GAG ATG-3'	61.9
	Anti-sense	5'-AGT GCC GTG AGT TTC CCA GAA-3'	58.8
IL-1 β	Sense	5'-GGA GAA CAC CAC TTG TTG CAC CAT-3'	60.2
	Anti-sense	5'-ACA GAG CCT CGC CTT TGC CG-3'	58.1
IL-1R	Sense	5'-CCC TCC TGA GAA GCT GGA CCC C-3'	61.9
	Anti-sense	5'-CGT TCC TTG CAT TCA GCC TCC A-3'	62.0
TNF- α	Sense	5'-CCC ATG TTG TAG CAA ACC CTC AA-3'	68.7
	Anti-sense	5'-TAT CTC TCA GCT CCA CGC CAT T-3'	67.0

Resveratrol and the resveratrol metabolites were dissolved in 100% DMSO to a concentration of 10 mM. These stock solutions were prepared freshly prior to the experiments and kept in the dark during handling procedures.

U-937 cell culture and differentiation procedure

The U-937 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.), cultured in Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Vienna, Austria), 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C, and split every 2–3 days. To differentiate the U-937 monocytes into macrophages, the cells were seeded in the respective volume and plate or dish in RPMI media containing 10% FBS, 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 2 mM glutamine and 10 or 50 ng ml⁻¹ phorbol-12-myristate-13-acetate (PMA). After 48 h, the differentiated cells were used for the experiments. The differentiation was verified by detection of CD11b using flow cytometry.²⁴ The cells were incubated with anti-CD11b antibody (sc-1186, Integrin α M, Santa Cruz Biotechnologies, Heidelberg, Germany) for 1 h and a fluorescein isothiocyanate (FITC)-coupled secondary antibody (sc-2078, goat anti-mouse, IgG₁-FITC, Santa Cruz Biotechnologies) for 30 min or a control antibody (sc-2855, normal mouse IgG₁-FITC, Santa Cruz Biotechnologies) for 1 h. The FITC signal was measured using flow cytometry (guava, Merck-Millipore, Vienna, Austria) and the data was analyzed with the InCyte software (v 2.2.2, Merck-Millipore).

U-937 macrophage cell viability and incubation media sampling

The viability of the U-937 macrophages was tested by trypan blue staining. The cells were seeded in 24-well plates with 200 000 cells per well and differentiated as described above. The differentiation media was exchanged with media containing 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 2 mM glutamine, 10% FBS, and the test compounds and incubated for 3, 6, 9 or 24 h, respectively. To test a preventive effect of the compounds the cells were incubated for 1 h with the respective compound, followed by 6 h stimulation with *E. coli*-LPS in absence of the compound. For resveratrol experiments, 1% DMSO was used as solvent control and 100 nM dexamethasone was used as an anti-inflammatory active positive control compound.^{25,26} After incubation, the incubation media was collected for cytokine analyses and stored at –80 °C. The cells were detached with 0.2% EDTA in PBS. The cell suspension was diluted 1 : 10 with trypan blue solution (0.4%) and the cell viability was determined using a hemocytometer with $n = 3$ and four technical replicates.

MMP-2 ELISA

The cell culture media of the 24 h incubation were thawed and used undiluted in the MMP-2 ELISA (Calbiochem/Millipore, Vienna, Austria), following the manufacture's protocol. The absorbance was measured at 450 nm against a reference of 595 nm using a multimode plate reader (Tecan infinite M2000,

Tecan, Groeding, Austria). Three independent experiments were carried out and duplicates of each sample were measured with the ELISA kit. The concentration of MMP-2 in the cell culture media was determined using a standard curve. All samples were compared to the *E. coli*-LPS treated positive control (100%) and are presented as T/LPS (%).

Cytokine measurement via bead-kit

The cytokines IL-6, IL-8 and MMP-9 were determined in the thawed incubation media using non-magnetic or magnetic bead kits (both ebioscience, Vienna, Austria).²⁷ For the measurement of MMP-9 the media had to be diluted to stay within the standard range depending on the incubation time since concentrations of MMP-9 increased over time, for 3 h 1 : 3, for 6 h 1 : 5, for 9 h 1 : 10 and for 24 h 1 : 50. Three to four independent experiments were carried out and duplicates of each sample were analyzed within the bead kit measurement following the manufacture's protocols. The detection of the non-magnetic beads was carried out on a flow cytometer (Guava, Merck-Millipore), with a bead emission at 700 nm and the phycoerythrin emission at 575 nm. Data were analyzed using the FlowCytoMix Pro software (ebioscience). For the detection and analysis using magnetic beads the MagPix system and Milliplex analyst software (Merck-Millipore) were used, respectively. The bead regions were gated and based on standard curves the results were calculated as pg ml⁻¹. The results were normalized to the stimulated control, 1 μ g ml⁻¹ *E. coli*-LPS (100%), as T/LPS in %.

Gene expression analysis of cytokines and cytokine receptors

For gene expression analysis of IL-1 α , IL-1 β , IL-1R, IL-6, IL-6R and TNF- α , PPIA (Table 1) was used as a reference gene.^{28,29} Five million U-937 cells were seeded in cell culture dishes (10 cm diameter) and differentiated for 48 h. Thereafter, the cells were incubated for 3, 6, 9 or 24 h with the respective compounds in combination with 1 μ g ml⁻¹ *E. coli*-LPS. After washing the cells twice with ice-cold PBS, the RNA was isolated using the SV RNA total isolation kit (Promega) and RNA concentration and purity were analyzed spectrophotometrically using a NanoQuant plate® (Tecan). The purities of all samples were measured with ratios at 260/280 nm between 1.8 and 2.3. Of each sample, 2 μ g of RNA were transcribed into cDNA using the high capacity reverse transcription kit with RNase inhibitor (Life Technologies). The cDNA concentration and purity was determined with a NanoQuant plate® (Tecan). The qPCR was carried out on a StepOnePlus instrument (ABI) using 50 ng of cDNA and 200 nM of the specific primer pairs (Table 1). For data analysis, the PCR efficiencies and corrected N_0 -values were determined using LinRegPCR.²⁸ The N_0 -values, the starting concentrations, of each sample were normalized to the reference gene, PPIA. The fold changes of the N_0 -values from *E. coli*-LPS-treated versus the untreated control cells were calculated and displayed for the analysis of time-dependent effects of *E. coli*-LPS stimulation on gene expression. The influence of resveratrol and resveratrol metabolites were compared to the *E. coli*-LPS treatment (100%) and presented as T/LPS control (%).

Statistical analysis

Three to four independent experiments were carried out for all analyses. In all experiments, outliers were excluded using a Nalimov outlier test with $p < 0.05$. The effects of PMA differentiation, *E. coli*-LPS stimulation and resveratrol and its metabolites on the LPS stimulation were tested using a two sided Student's *t*-test. Significant differences are displayed with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For the determination of the optimal incubation time and the effects of the different substances, a one-way ANOVA with a Holm-Sidak post-hoc test was performed between the different incubation times for each gene and each concentration. Significant differences ($p < 0.05$) are indicated with letters.

Results

Differentiation of U-937 monocytes into macrophages

The differentiation of U-937 monocytes into macrophages was initiated with phorbol-12-myristate-13-acetate (PMA).³⁰ The optimal PMA concentration was determined using integrin alpha M (cluster of differentiation 11b, CD11b) presentation capacity on the cell surface by flow cytometry and, additionally, the cell morphology was checked by microscopy (Fig. 2A). CD11b, one subunit of the heterodimeric integrin alpha M beta 2 receptor, has been established to determine the differentiation of macrophages.³¹ Treatment of U-937 monocytes with 10 ng ml⁻¹ PMA for 48 h led to differentiation of 78.6 ± 7.1%, whereas a treatment with 50 ng ml⁻¹ PMA for the same incubation time did not result in a higher proportion of differentiated cells (Fig. 2B). Thus, for all further experiments, the monocytes were differentiated into macrophages by treatment with 10 ng ml⁻¹ PMA for 48 h. For the experiments, the remaining monocytes in suspension were removed by washing with PBS and changing of media to incubation media, thus, only macrophages were used in the experimental setting.

Determination of the optimal incubation time for cytokine gene expression and cytokine release after stimulation of the U-937 macrophages with *E. coli*-LPS

The gene expression of IL-6 as well as IL-1α, IL-1β, IL-1R and IL-6R was measured after incubation with and without 1 μg ml⁻¹ *E. coli*-LPS for 1, 3, 6, 9 and 24 h. At all times, the incubation of the differentiated U-937 cells with 1 μg ml⁻¹ *E. coli*-LPS had no cytotoxic effect compared to the untreated control (data not shown). A strong increase in the expression of IL-1α, IL-1β and IL-6 to a fold change of 742 ± 130, 230 ± 72.5 and 1462 ± 235, respectively, was determined after 6 h of stimulation (Fig. 3). At the later time points, the increase was not as pronounced. The IL-1 receptor gene was up-regulated by *E. coli*-LPS stimulation for 6 h to a fold change of 4.92 ± 1.27, while IL-6R was down-regulated to 0.86 ± 0.08 (data not shown). Taken together, we

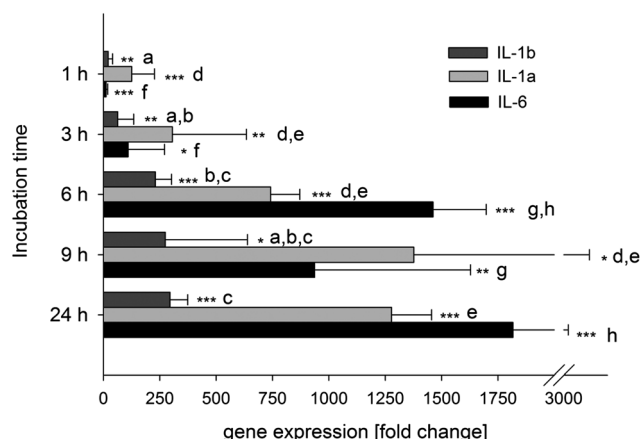


Fig. 3 Identification of the optimal incubation time of pro-inflammatory genes stimulated by incubation with 1 μg ml⁻¹ *E. coli*-LPS for 1, 3, 6, 9 and 24 h. Shown are the fold changes (mean ± S.D.) and of four independent experiments with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. untreated control (= 1) and significant differences over time are indicated with letters for each gene (a–c IL-6, d,e IL-1α, f–h IL-1β).

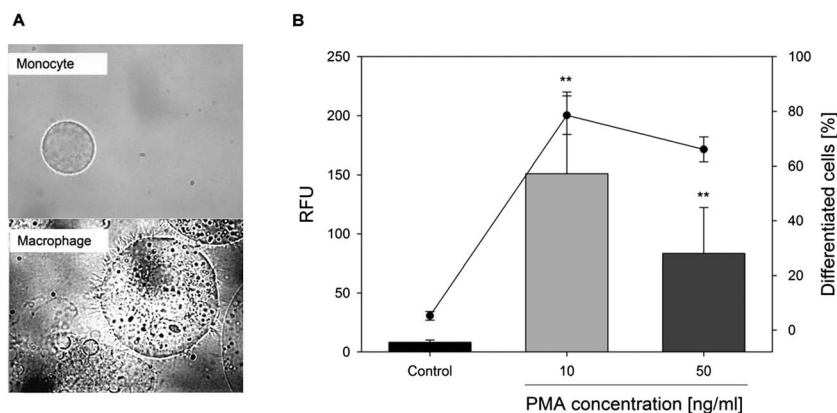


Fig. 2 Differentiation of monocytes into macrophages with 10 or 50 ng ml⁻¹ of phorbol-12-myristate-13-acetate (PMA) for 48 h by (A) microscopy (10 ng ml⁻¹ PMA) and (B) flow cytometry using a fluorescence labeled CD11b antibody. Shown are the mean relative fluorescence intensity units (RFU) ± S.D. (bars) and the percentage of differentiated cells (line) of five independent experiments, with ** $p < 0.01$ vs. the untreated control.

chose the 6 h incubation time to measure the influence of resveratrol and its metabolites on the gene expression in further experiments.

To determine the optimal incubation time for the cytokine and matrix metalloprotease (MMP) release by stimulation of U-937 macrophages with $1 \mu\text{g ml}^{-1}$ *E. coli*-LPS,³² the time dependent release of IL-6, IL-8, and MMP-9 was measured at 3, 6, 9 and 24 h. IL-6, IL-8 and MMP-9 secretion after 3, 6, 9 and 24 h incubation were measured in the cell culture media and the data is represented in Table 2 as fold change of the *E. coli*-LPS treatment vs. untreated control cells. The higher this value the more effective was the *E. coli*-LPS stimulation. After 3 h, only IL-8 was stimulated to a fold change of 1.74 ± 0.43 . However, this stimulation was not detectable after 6, 9 or 24 h. IL-6 release increased over time and was stimulated after exposure times of 6 h and longer. In contrast to the MMP-2 release, the MMP-9 release was stimulated after 24 h. After 6 h, IL-6 gene expression was stimulated to a maximum and also IL-6 release was significantly increased compared to the control. Finally, IL-6 was chosen as a biomarker in the *E. coli*-LPS-stimulated U-937 macrophages to determine the influence of resveratrol and its metabolites in the further experiments. In addition, the release of TNF- α , known to be up-regulated on a transcriptional level³³ and released from U-937 cells after LPS stimulation,^{34,35} was studied. An increased mRNA expression and release of this marker after 6 h of stimulation with $1 \mu\text{g ml}^{-1}$ *E. coli*-LPS of 14.3 ± 9.0 fold and 314.9 ± 54.1 fold, respectively, compared to the non-stimulated control was determined (data not shown).

Inhibition of the expression of pro-inflammatory genes by resveratrol and its metabolites

The impact of resveratrol, resveratrol sulfates and glucuronides on the expression of IL-6, TNF- α , IL-1 α , IL-1 β and the IL-1R in U-937 macrophages was determined after a 6 h co-incubation with *E. coli*-LPS (Table 3 and Fig. 4). Dexamethasone in a concentration of 100 nM was used as an anti-inflammatory control, up-regulating IL-1R mRNA expression to $344 \pm 16\%$ and down-regulating the gene expression of IL-1 α , IL-1 β (data not shown), IL-6 and TNF- α (Figure 4) to $5.6 \pm 0.7\%$, $27.9 \pm 2.7\%$, $9.9 \pm 1.6\%$ and $22.4 \pm 3.1\%$, respectively, compared to the *E. coli*-LPS treatment solely (100%).

Next, the impact of resveratrol and resveratrol metabolites on these target genes was investigated. Prior to the gene expression experiments using resveratrol or resveratrol metabolites in combination with LPS, false positive results due to cytotoxic effects had to be excluded. Co-incubation of $1 \mu\text{g ml}^{-1}$ *E. coli*-LPS and 10 μM resveratrol, resveratrol-3-sulfate, resveratrol disulfates, resveratrol-3-glucuronide or resveratrol-4'-glucuronide did not show cytotoxic effects compared to the solvent control (data not shown).

Resveratrol, at a concentration of 10 μM , suppressed the LPS-stimulated expression of IL-1 α and IL-1 β to $48.0 \pm 4.9\%$ and $79.1 \pm 15.0\%$, respectively (Table 3). The effects of the tested resveratrol-3-sulfate and resveratrol disulfates concentrations on the gene expression of IL-1 α and IL-1 β did not differ from the inhibitory effect of resveratrol, and reduced the expression of these genes to a maximum of $45.5 \pm 15.5\%$ and $58.4 \pm 16.7\%$, respectively. In contrast to the sulfated

Table 2 Determination of the optimal marker by the release of cytokines and matrix metalloproteases to detect an inflammatory response initiated by $1 \mu\text{g ml}^{-1}$ *E. coli*-LPS after 3, 6, 9 and 24 h. Presented are the fold changes compared to the control $\pm$ S.D. of at least three individual experiments with $**p < 0.01$ and $***p < 0.001$; n.d., not determined

	IL-6		IL-8		MMP-9		MMP-2	
3 h	1.80 ± 2.07		1.74 ± 0.43	**	0.90 ± 0.54		n.d.	
6 h	13.0 ± 1.87	***	0.85 ± 0.13		0.94 ± 0.34		n.d.	
9 h	58.6 ± 22.0	***	0.49 ± 0.02	***	1.63 ± 0.32		n.d.	
24 h	207 ± 45.0	***	0.32 ± 0.03	**	3.17 ± 0.28	***	0.55 ± 0.07	***

Table 3 Effect of resveratrol and resveratrol metabolites (1 μM and 10 μM) on the *E. coli*-LPS-stimulated mRNA expression of IL-1 α and IL-1 β . Dexamethasone (100 nM) was used as an anti-inflammatory control compound. Presented are the fold changes compared to the *E. coli*-LPS treatment $\pm$ S.D. of four individual experiments with $*p < 0.05$ (Student's *t*-test), letters indicate significant differences among the compounds for each marker and concentration (one-way ANOVA, $p < 0.05$)

Conc.	IL-1 α		IL-1 β	
	1 μM	10 μM	1 μM	10 μM
RES	$49.7 \pm 10.0^{*a}$	$48.0 \pm 4.9^{*a}$	$77.0 \pm 40.0^{a,b}$	$79.1 \pm 15.1^{*a,c}$
R3S	$61.2 \pm 17.0^{*a}$	$45.5 \pm 15.5^{*a}$	76.6 ± 29.5^a	$58.4 \pm 16.7^{*a}$
RDS	$55.3 \pm 25.2^{*a}$	$79.9 \pm 41.2^{*a,b}$	$59.8 \pm 33.6^{*a}$	$76.0 \pm 34.2^{*a,c}$
R3G	$86.5 \pm 10.6^{*b}$	$111 \pm 31.2^{*b}$	$102 \pm 14.1^{b,c}$	$127 \pm 12.0^{*b}$
R4'G	111 ± 14.1^c	$119 \pm 52.3^{*b}$	$128 \pm 32.6^{*c}$	$127 \pm 20.4^{*b}$
COM	$89.4 \pm 3.95^{*b}$	$117 \pm 13.0^{*b}$	$90.2 \pm 6.14^{*b}$	$94.8 \pm 7.60^{*c}$
DEX 100 nM	$5.56 \pm 0.74^*$		$27.9 \pm 2.67^*$	

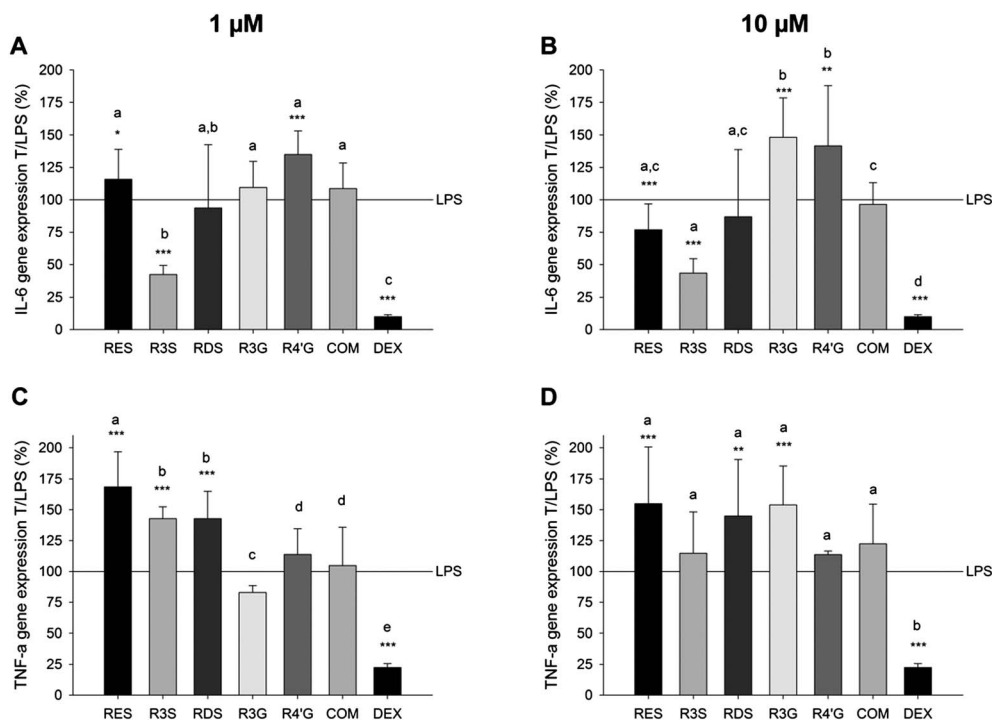


Fig. 4 Detection of the impact of resveratrol, resveratrol sulfates and resveratrol glucuronides in concentrations of 1 (A and C) or 10 μM (B and D) on the gene expression of IL-6 (A and B) and TNF- α (C and D) in U-937 macrophages stimulated with 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS after 6 h. Positive control: 100 nM dexamethasone (DEX). Presented are the means of a treatment with substance and *E. coli*-LPS in relation to the *E. coli*-LPS treatment only (T/LPS, %; LPS = 100%) $\pm$ S.D. of 3 to 5 individual experiments with * p < 0.05, ** p < 0.01 and *** p < 0.001. Letters indicate the different effects of the compounds for each gene and each concentration (p < 0.05).

metabolites, the tested resveratrol glucuronides mostly stimulated the gene expression of the tested genes by additional $8.6 \pm 15.6\%$ to $48.2 \pm 30.1\%$ compared to the LPS treatment solely. Only 1 μM resveratrol-3-glucuronide slightly inhibited the expression of IL-1 α and IL-1R to $86.5 \pm 10.6\%$ and $80.0 \pm 9.7\%$, respectively.

The gene expression of IL-6 was most effectively reduced to $42.2 \pm 11.2\%$ in the presence of 1 μM resveratrol-3-sulfate compared to resveratrol and the other metabolites (Fig. 4). However, at a concentration of 10 μM , resveratrol and the sulfated metabolites exploited similar anti-inflammatory activity by reducing the *E. coli*-LPS-stimulated IL-6 expression to a maximum of $43.4 \pm 11.1\%$ (R3S). In contrast, the TNF- α mRNA expression was up-regulated after co-incubation with 1 or 10 μM resveratrol and 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS for 6 h. Similar effects were found for the sulfated metabolites, while the glucuronated metabolites did not influence the gene expression of TNF- α in comparison to a treatment with LPS solely.

Taken together, with regard to the expression of the majority of tested pro-inflammatory genes, the resveratrol sulfates exhibited a strong anti-inflammatory potential, while resveratrol monoglucuronides did not. Thus, mixtures of either 1 or 10 μM of each of the metabolites were tested as well to prove the combined effect of resveratrol metabolites. This mixture (COM) did not impact the IL-6 or TNF- α mRNA expression (Fig. 4), but reduced the pro-inflammatory effect of *E. coli*-LPS by means of IL-1 α and IL-1 β mRNA expression levels (Table 3).

Resveratrol and resveratrol metabolites inhibit the IL-6 and TNF- α release of from U-937 macrophages after 6 h of incubation

On a functional level, it was analyzed whether resveratrol and its metabolites could inhibit the *E. coli*-LPS stimulated IL-6 and TNF- α release from U-937 macrophages. The anti-inflammatory control compound dexamethasone decreased the release of IL-6 and TNF- α , when co-incubated at a concentration of 100 nM together with 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS for 6 h, by $93.7 \pm 1.52\%$ and $57.2 \pm 7.69\%$, respectively, compared to the treatment with LPS solely (Fig. 5). Resveratrol strongly inhibited the pro-inflammatory response of *E. coli*-LPS by means of IL-6 release already at a concentration of 1 μM ($17.8 \pm 19.8\%$, p < 0.001, Fig. 5A) and completely at a concentration of 10 μM (Fig. 5B). This anti-inflammatory activity of resveratrol was also shown with regards to the LPS-stimulated TNF- α release. Here, 1 μM resveratrol inhibited the endotoxin-mediated cytokine release to $44.7 \pm 11.0\%$ (Fig. 5C). In addition, the sulfated metabolites showed an inhibition of the LPS-stimulated IL-6 and TNF- α release after 6 h of incubation. Here, the monosulfated metabolite tested suppressed the IL-6 release to a minimum of $15.8 \pm 29.4\%$ at 10 μM and the TNF- α release to a minimum of $66.4 \pm 8.7\%$ at 1 μM , while the disulfated metabolites reduced the IL-6 release to $27.5 \pm 23.0\%$ at 1 μM and the TNF- α release to $53.3 \pm 8.7\%$ at 10 μM . In contrast, the glucuronated metabolites did not inhibit the *E. coli*-LPS induced IL-6 or TNF- α release from U-937 macrophages. A concomitant incubation of 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS and a

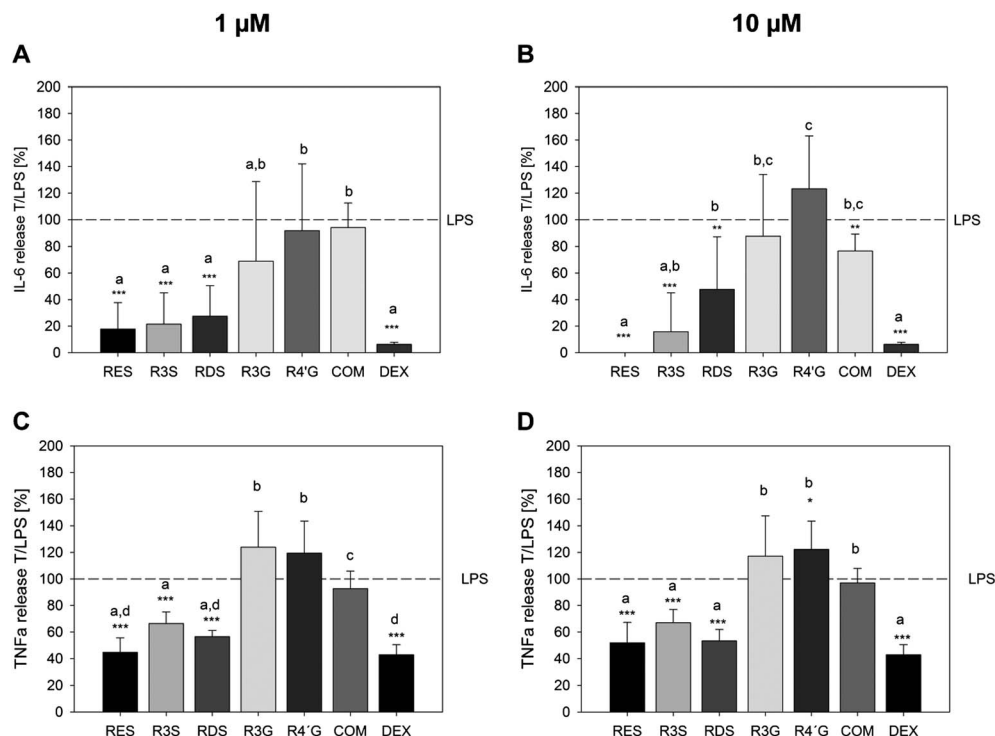


Fig. 5 Detection of IL-6 release from U-937 macrophages stimulated with $1 \mu\text{g ml}^{-1}$ *E. coli*-LPS after 6 h. Positive control: 100 nM dexamethasone (DEX). Presented are the means of the combined *E. coli*-LPS and compound treatment in relation to the *E. coli*-LPS treatment only (T/LPS, %, LPS = 100%) $\pm$ S.D. of 3 to 5 individual experiments with ** $p < 0.01$ and *** $p < 0.001$.

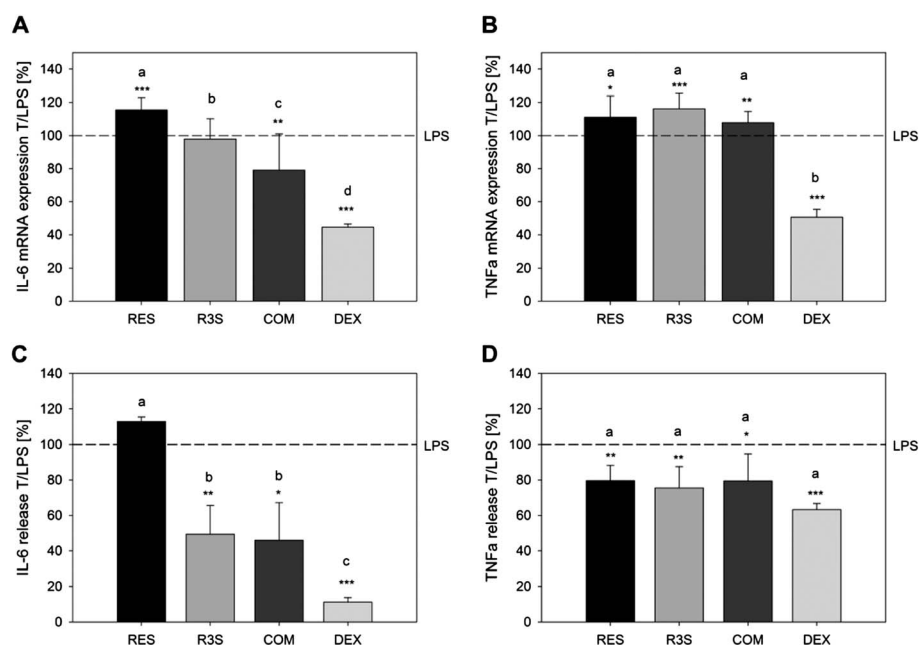


Fig. 6 Detection of the impact of a 1 h pre-incubation with resveratrol, resveratrol-3-sulfate and a combination of the sulfated and glucuronated metabolites in concentrations of $10 \mu\text{M}$ on the gene expression (A and B) and release (C and D) of IL-6 (A and C) and TNF- α (B and D) of U-937 macrophages stimulated with $1 \mu\text{g ml}^{-1}$ *E. coli*-LPS for 6 h. Positive control: 100 nM dexamethasone (DEX). Presented are the means of a treatment with substance followed by *E. coli*-LPS stimulation in relation to the *E. coli*-LPS treatment only (T/LPS, %, LPS = 100%) $\pm$ S.D. of 3 to 5 individual experiments with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Letters indicate the different effects of the compounds for each gene and each concentration ($p < 0.05$).

combination 10 μM of each of the metabolites resulted in a reduction of the LPS-stimulated IL-6 release (Fig. 5B), but did not influence the TNF- α release (Fig. 5D).

Resveratrol metabolites exhibit a preventive effect against a challenge of U-937 macrophages stimulated with *E. coli*-LPS

In addition to the effect of resveratrol and its metabolites concomitantly incubated with LPS, a preventive effect of the most active substances and the metabolite mixture against the pro-inflammatory activity of *E. coli*-LPS was investigated by incubation of U-937 cells with 10 μM resveratrol, resveratrol-3-sulfate or the combination of 10 μM of the resveratrol metabolites for 1 h, followed by a six hour stimulation with 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS. Dexamethasone served as an anti-inflammatory control, up-regulating the LPS-stimulated IL-1R mRNA expression and down-regulating the gene expression of IL-1 α , IL-1 β , IL-6 and TNF- α (Fig. 6A and B), as well as reducing the LPS-mediated release (Fig. 6C and D) of the pro-inflammatory cytokines IL-6 by $88.8 \pm 2.4\%$ and TNF- α by $46.8 \pm 3.4\%$ compared to the control (1 h 1% DMSO + 6 h 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS). Resveratrol did not inhibit the up-regulation of pro-inflammatory cytokine mRNA expression by LPS. In contrast, a pre-incubation with a mixture of 10 μM of each of the metabolites resulted in a decreased IL-6 mRNA expression (79.1 ± 22.0 , Fig. 6A) compared to the DMSO pre-treated control (100%). On a functional level, resveratrol also showed no inhibition of the *E. coli*-LPS-stimulated IL-6 release (Fig. 6C) but reduced the TNF- α release by $20.4 \pm 8.65\%$ (Fig. 6D). The tested sulfated metabolite and the metabolite mixture showed comparable effects on the LPS-mediated TNF- α release. However, a 1 h pre-treatment of the macrophages with resveratrol-3-sulfate or the metabolite mixture followed by a 6 h LPS-stimulation resulted in an inhibition of the IL-6 release to a minimum of $49.3 \pm 16.2\%$ and $46.0 \pm 21.2\%$, respectively. Thus, resveratrol-3-sulfate and a mixture of tested resveratrol metabolites showed a greater preventive potential against the pro-inflammatory endotoxin *E. coli*-LPS than the parent compound.

Discussion

Resveratrol is a known anti-inflammatory compound, but is metabolized upon absorption. We hypothesized that the resveratrol metabolites might also possess anti-inflammatory activities with regards to cytokine release in plasma representative concentrations. Since the release of cytokines depend on the type of LPS and cells used,^{16,17} prior to the analysis of an impact of resveratrol and its metabolites on the LPS-stimulated release of interleukins and matrix metalloproteases, the optimal marker needed to be identified. We determined IL-6 as well as TNF- α to be the ideal markers for our study by means of gene expression and interleukin secretion after 6 h of stimulation with 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS. In previous studies on U-937 monocytes, we measured the release of matrix metalloproteases (MMP-2 and MMP-9) after 24 h of incubation.¹⁹ In the here presented study, the release of MMP-9 could only be stimulated

with 1 $\mu\text{g ml}^{-1}$ *E. coli*-LPS after 24 h. This result demonstrates that the release of matrix metalloproteases is a late event in the immune response, while the release of interleukins is an early one. The release of IL-6 was already significantly stimulated after 6 h. Furthermore, it is known that macrophages release TNF- α upon stimulation with LPS.^{34,35} Since resveratrol and its metabolites have been detected in the plasma with half lives of 2.9 to 11.5 h^{7,8} the treatment time with the compounds of interest should be kept within this time frame. With respect to the here presented study, the incubation time of six hours applied represent biologically relevant conditions and optimal conditions with regards to IL-6 and TNF- α release from U-937 macrophages.

Resveratrol and resveratrol-3-sulfate have been shown to act as anti-inflammatory agents, affecting the expression of pro-inflammatory genes or inhibiting the release of pro-inflammatory cytokines. Furthermore, in U-937 macrophages, resveratrol was shown to down-regulate the gene expression of MMP-9.³⁶ Our results on the inhibition of the *E. coli*-LPS induced IL-6 and TNF- α release and gene expression of U-937 macrophages are in accordance with a study from Qureshi *et al.*,³⁷ showing that 0.1 to 10 μM resveratrol inhibit the LPS-stimulated release of TNF- α and gene expression of TNF- α , IL-1 β , IL-6 and iNOS from RAW 264.7 macrophages.

Here, we could also show that not only resveratrol but also its sulfated metabolites exhibit anti-inflammatory activities by inhibiting the *E. coli*-LPS stimulated mRNA expression of pro-inflammatory cytokines and release of IL-6 and TNF- α by U-937 cells. In contrast to literature evidence for glucuronides of quercetin,³⁸ the results of this study demonstrate that resveratrol glucuronides do not possess anti-inflammatory properties by an inhibition of IL-6 or TNF- α release from macrophages. Furthermore, resveratrol-3-sulfate inhibited the *E. coli*-LPS-stimulated gene expression of the pro-inflammatory genes IL-1 α , IL-1 β and IL-1R in a similar manner as resveratrol in plasma representative concentrations. Besides resveratrol, this metabolite was the most effective anti-inflammatory compound tested. These results are in accordance with the studies from Aires *et al.*,²¹ investigating the anti-proliferating activity of resveratrol metabolites, and Hoshino *et al.*²² who studied the anti-inflammatory activities of resveratrol sulfates in different test systems at higher concentrations. Both groups identified resveratrol-3-sulfate as the most active compound besides resveratrol. Aires *et al.*²¹ demonstrated that resveratrol-3-sulfate inhibits the proliferation of colon carcinoma cells (SW480, SW620) after 48 h in concentrations $\geq 10 \mu\text{M}$. Also a concentration of 30 μM resveratrol-3-sulfate, the most active metabolite in our test system, was found to induce apoptosis in the same manner as resveratrol.^{21,39} Furthermore, Hoshino *et al.*²² studied the anti-inflammatory potential of sulfated resveratrol metabolites in RAW 264.7 macrophages with regards to their impact on the eicosanoid pathway and iNOS expression. Here, 34 μM resveratrol-3-sulfate were found to reduce the iNOS protein level, induced by 1 $\mu\text{g ml}^{-1}$ LPS. In human intervention trials focusing on the metabolism of resveratrol, resveratrol sulfates and glucuronides have been identified in the plasma and urine of healthy volunteers, with resveratrol-3-sulfate being

the predominant metabolite.^{8,10,11} However, in the previously mentioned studies by Aires *et al.*²¹ and Hoshino *et al.*²² in which the biological effects of resveratrol metabolites were investigated, the applied concentrations exceeded the plasma levels for resveratrol-3-sulfate by up to 2.4 times, based on an intake of a high bolus dose of resveratrol.¹¹ Thus, for the first time, we demonstrated an anti-inflammatory activity for resveratrol metabolites in plasma representative concentrations.

In addition, we tested whether the plasma-representative concentrations of resveratrol, resveratrol-3-sulfate, the predominant metabolite, and a mixture of the metabolites exploit a preventive effect against a treatment with $1\ \mu\text{g ml}^{-1}$ *E. coli*-LPS. Here, we could demonstrate that resveratrol-3-sulfate and the mix of the tested metabolites attenuate the LPS-induced IL-6 and TNF- α release, after a 1 h treatment with the compound followed by 6 h LPS stimulation. Similar effects have been shown for lycopene⁴⁰ using RAW264-7 macrophages and stimulation with LPS. In the same cell model, a 30 min treatment with resveratrol ($\geq 25\ \mu\text{M}$) followed by an 8 h LPS stimulation resulted in a reduction of the IL-6 and TNF- α release compared to the LPS treatment solely.⁴¹ Our study shows, that the metabolites have a more pronounced effect under these conditions than the parent compound and exhibit their preventive potential against inflammation in plasma-representative concentrations.

Several studies focused on the identification of the mechanism of action. Resveratrol was found to block elements of the JAK/STAT-1 pathway in macrophages⁴² as well as the NF- κ B signalling and the Toll-like receptor 4,⁴¹ resulting in a decreased mRNA level of cytokines and chemokines. For example, resveratrol inhibited the expression of the IL-6 gene.⁴³ Additionally, an inhibition of the LPS-induced stimulation of the phosphatidylinositol 3-kinase pathway by resveratrol was recently shown, which led to a decreased expression of the genes encoding IL-1 β , TNF- α , iNOS and cyclooxygenase-2.¹³ We were able to show that resveratrol-3-sulfate and the resveratrol disulfates decreased the LPS-induced gene expression of IL-1 α , IL-1 β and IL-6. Thus, the mechanism described, might apply to explain how resveratrol-3-sulfate and the resveratrol disulfates act as anti-inflammatory agents. However, the anti-inflammatory control, used in the present study, dexamethasone is known to inhibit the translocation of NF- κ B to the nucleus and its binding to the DNA in macrophages leading to an attenuation of LPS-induced expression of pro-inflammatory cytokines.⁴⁴ This result was clearly shown for all tested mRNA expression levels, here, while the IL-1R expression was up-regulated in the presence of LPS and dexamethasone. In contrast, resveratrol showed different effects on the IL-1R, IL-6 and TNF- α mRNA expression compared to dexamethasone. This could indicate a different mechanism of action by resveratrol. While dexamethasone influences the main inflammatory NF- κ B pathway directly, resveratrol and resveratrol-3-sulfate act *via* different pathways (PI3K, JAK/STAT, NF- κ B), with a lower impact on the resulting expression of pro-inflammatory genes. With special regards to the preventive effects, the mechanism of action of the metabolites should be investigated in future studies.

Conclusion

In conclusion, plasma representative concentrations of resveratrol sulfates possess anti-inflammatory activities, showing that resveratrol-3-sulfate, found in higher plasma concentrations than the parent compound, might contribute to its anti-inflammatory effect. However, only a small number of resveratrol metabolites were tested here. Future studies should elucidate the anti-inflammatory potential of other metabolites.

Non-conflict of interest statement

The authors confirm that there is no conflict of interest with any financial organization regarding the results presented in this research manuscript.

Abbreviations

FITC	Fluorescein isothiocyanate
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MMP	Matrix metalloprotease
PMA	Phorbol-12-myristate-13-acetate
R3G	Resveratrol-3-glucuronide
R4'G	Resveratrol-4'-glucuronide
R3S	Resveratrol-3-sulfate
RDS	Resveratrol disulfates
RES	Resveratrol
TNF α	Tumor necrosis factor alpha

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3.5 Impact of Trans-Resveratrol-Sulfates and -Glucuronides on Endothelial Nitric Oxide Synthase Activity, Nitric Oxide Release and Intracellular Reactive Oxygen Species

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This manuscript assesses the impact of physiological Resveratrol metabolites on the enhancement of endothelial nitric oxide synthase, as already established for the parent compound, in endothelial cells. Furthermore, the ROS scavenging potential of metabolites was established in the same cell line.

I synthesized and purified the glucuronated metabolites used in this study. Additionally, I measured the DCF data on ROS production, did data analysis and preparation of figures. I also participated in data interpretation and writing the manuscript.

Article

Impact of Trans-Resveratrol-Sulfates and -Glucuronides on Endothelial Nitric Oxide Synthase Activity, Nitric Oxide Release and Intracellular Reactive Oxygen Species

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Abstract: Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic natural product mainly present in grape skin, berries and peanuts. In the vasculature resveratrol is thought to boost endothelial function by increasing endothelial nitric oxide synthase (eNOS) expression, by enhancing eNOS activity, and by reduction of reactive oxygen species (ROS) levels. Recent studies show that dietary resveratrol is metabolized in the liver and intestine into resveratrol-sulfate and -glucuronide derivatives questioning the relevance of multiple reported mechanistic *in vitro* data on resveratrol. In this study, we compare side by side different physiologically relevant resveratrol metabolites (resveratrol sulfates- and -glucuronides) and their parent compound in their influence on eNOS enzyme activity, endothelial NO release, and intracellular ROS levels. In contrast to resveratrol, none of the tested resveratrol metabolites elevated eNOS enzyme activity and endothelial NO release or affected intracellular ROS levels, leaving the possibility that not tested metabolites are active and able to explain *in vivo* findings.

Keywords: resveratrol metabolites; eNOS; NO; intracellular ROS levels; endothelial cells

1. Introduction

Endothelial nitric oxide (NO), the product of the enzyme endothelial nitric oxide synthase (eNOS), is considered a major anti-inflammatory and anti-atherogenic mediator in the cardiovascular system [1]. In particular, the impaired availability of endothelial NO, resulting in endothelial dysfunction, is regarded as the initial step in the development of cardiovascular diseases [2].

The polyphenol resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is found predominantly in grape skin, red wine, berries, and peanuts, is a natural product with well-known favorable effects on endothelial NO availability in the vasculature [3,4]. It has been shown that resveratrol promotes endothelial function by increasing eNOS expression, by enhancing eNOS activity [5]. Resveratrol has been reported to increase endothelial NO release via short and long-term effects. The rapid effect of resveratrol includes the phosphorylation of eNOS by AMPK, or ERK 1 and 2, and the deacetylation of eNOS by SIRT1 [6–9]. Long-term resveratrol increases eNOS mRNA and protein expression [10–13]. Resveratrol supplementation has been shown to be beneficial for endothelial function in several animal models [14,15]. However, an increase in eNOS expression upon resveratrol treatment could not be reproduced *in vivo* [15,16]. Apart from its cardiovascular effects, resveratrol supplementation was suggested to be beneficial for combating a wide range of diseases and conditions including inflammatory disorders, cancer, Alzheimer's disease and aging. Multiple cellular targets have been proposed to be responsible for the action of resveratrol [3,4,12].

In the last decades, many studies have been performed to elucidate the mechanism of action of resveratrol, with the aim to combine results obtained from cell culture systems with the data from *in vivo* observations. However, the relevance of *in vitro* data for the interpretation of *in vivo* effects of resveratrol is controversial since the oral bioavailability of resveratrol *in vivo* is rather limited due to its metabolism to resveratrol sulfates and glucuronides [17–19]. This sparked the issue whether resveratrol is in fact the active molecule *in vivo*. The oral intake of a dietary relevant dose of 25 mg of resveratrol was reported to lead to a plasma concentration of resveratrol in the nanomolar range, whereas the concentration of metabolites in plasma was in the micromolar range [20]. Additionally, the significant effects of resveratrol in *in vivo* experiments suggest that some resveratrol metabolites could be biologically relevant [21], either directly or by back conversion to resveratrol in target cells via glucuronidases and sulfatases [22].

Resveratrol metabolites have been reported to elicit a wide range of bioactivities similar to their parent compound resveratrol. For instance, resveratrol-4'-glucuronide, resveratrol-3-glucuronide, and resveratrol-3-sulfate reduced fat accumulation in adipocytes and influenced adipokine expression and secretion. Hence, they may be involved in anti-obesity effects after resveratrol consumption [23,24]. Resveratrol-3-sulfate and resveratrol disulfates showed anti-inflammatory activity by counteracting an inflammatory challenge in macrophages [25]. Furthermore, Resveratrol-4'-sulfate inhibited cyclooxygenase-1 and 2 (COX-1 and COX-2) with a potency similar to resveratrol, whereas resveratrol-3-sulfate and resveratrol-3-glucuronide were only weakly active on these enzymes [19].

Additionally, resveratrol-3-sulfate and resveratrol-4'-sulfate were able to increase sirtuin-1 activity to the same extent as resveratrol [26]. Sulfate and glucuronide metabolites were reported to inhibit colon cancer cell growth [22,27,28]. Resveratrol metabolites are therefore likely to be involved in the anti-cancer effects of resveratrol.

To interpret and understand results from *in vivo* studies, it is necessary to identify relevant resveratrol metabolites and to study their mode of action.

In this study, we determine the changes in eNOS activity and endothelial NO release as well as intracellular ROS levels, upon exposure of endothelial cells to several physiological resveratrol metabolites.

2. Results and Discussion

2.1. Impact of Resveratrol and Its Metabolites on eNOS Enzyme Activity

Since none of the metabolites could so far be identified as the major active compound, it is important to determine the activity of the physiologically relevant metabolites for every function and target, as in this study for the endothelial NO system.

Several *in vitro* studies reported beneficial effects of resveratrol on the eNOS system in micromolar [8,10,11,29] as well as in nanomolar concentrations [7,13,30]. Micromolar concentrations of resveratrol have been reported to elevate eNOS mRNA levels, activity and expression levels [8,10,11,29]. Additionally, an increase in eNOS expression was observed upon administration of 100 nM resveratrol [13]. A study performed by Klinge et al could demonstrate that nanomolar resveratrol induces ER α -Cav-1-c-SRC interaction, resulting in NO production through a G α -protein-coupled mechanism, suggesting an explanation for the effects seen with resveratrol *in vivo* [30].

For this study we decided to test resveratrol metabolites in micromolar concentrations since oral administration of 5 g of resveratrol led to a plasma concentration of metabolites in this concentration range [31]. The parent compound resveratrol itself was used in the same concentrations as previously as a control [10,29], although most likely physiologically not relevant.

It has been previously reported that resveratrol is able to enhance eNOS activity, among others, by stimulation of eNOS expression. This is achieved via stabilization of eNOS mRNA and direct increase in eNOS gene transcription [10]. Since effects on gene transcription are rather slow, we determined eNOS enzyme activity and endothelial NO release 24 h after treating endothelial EA.hy926 cells with resveratrol or resveratrol metabolites (Figure 1).

In a first experiment the [^{14} C]L-arginine/[^{14} C]L-citrulline conversion assay was utilized to measure eNOS enzyme activity (Figure 2). Treatment of endothelial cells with resveratrol concentration-dependently increased eNOS enzyme activity up to 1.64 fold at 100 μ M compared to control conditions, thereby confirming the previously reported eNOS stimulation by resveratrol [10]. As resveratrol is metabolized *in vivo*, we studied the effect of metabolites on eNOS activity. We used resveratrol-3-sulfate, resveratrol-4'-sulfate, resveratrol disulfates, resveratrol-3-glucuronide and resveratrol-4'-glucuronide in concentrations from 1 to 100 μ M in the same assay. However, none of the tested resveratrol sulfate or glucuronide metabolites was able to elicit a significant change in eNOS enzyme activity.

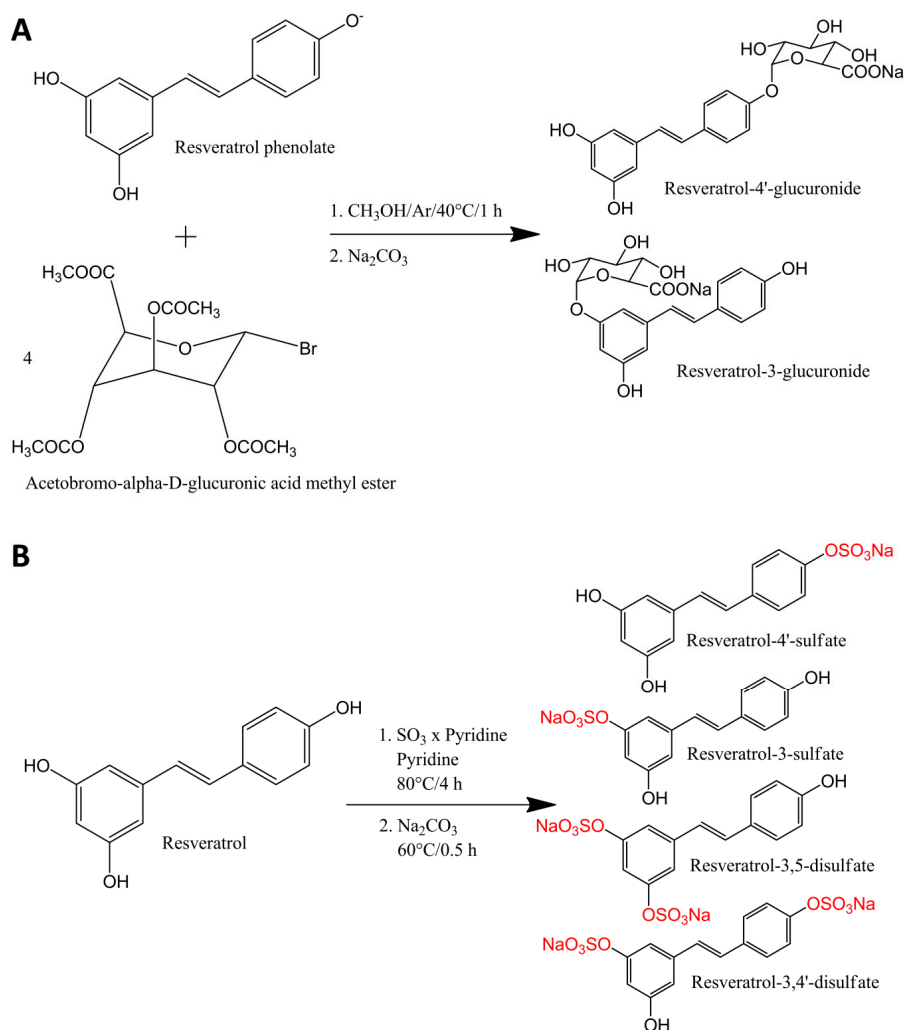
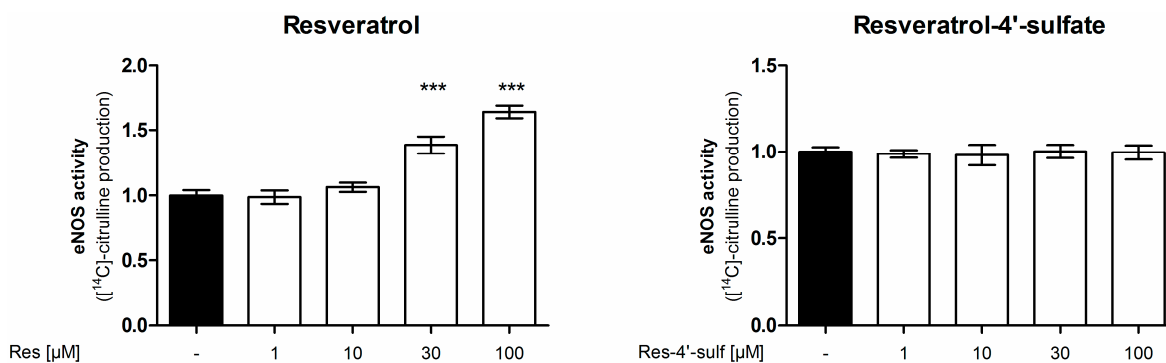
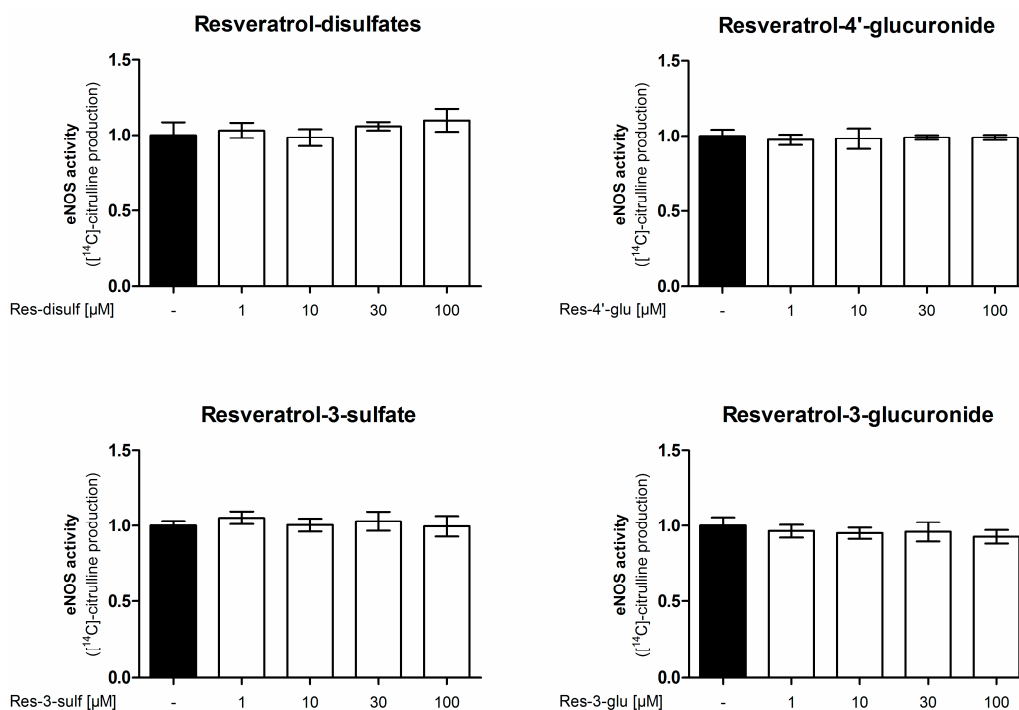
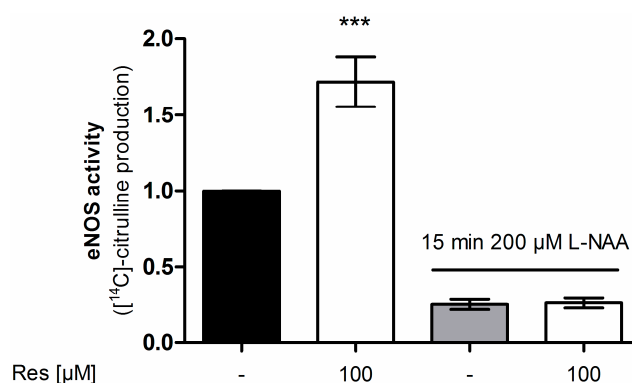
Figure 1. Synthesis of resveratrol glucuronides (A) and sulfates (B).**Figure 2.** Influence of resveratrol and its metabolites on eNOS enzyme activity. EA.hy926 cells were treated with the indicated concentrations of resveratrol or its metabolites for 24 h. Then an $[^{14}\text{C}]$ -L-arginine/ $[^{14}\text{C}]$ -L-citrulline conversion assay was performed as described. $[^{14}\text{C}]$ -L-citrulline production was normalized to the untreated control (***) $p < 0.001$; mean $\pm$ SD, $n = 3$).

Figure 2. Cont.



To demonstrate that ^{14}C -L-citrulline production upon resveratrol treatment derives from eNOS enzyme activity the experiment was repeated in the presence of the NOS-inhibitor L-NAA (N^{G} -Amino-L-arginine hydrochloride) (Figure 3). L-NAA reduced the basal and completely blocked the resveratrol-enhanced arginine conversion thereby confirming the NOS-specificity of the resveratrol effect.

Figure 3. Influence of the NOS inhibitor L-NAA on the effect of resveratrol on eNOS enzyme activity. EA.hy926 cells were treated with the indicated concentrations of resveratrol for 24 h. Then an ^{14}C -L-arginine/ ^{14}C -L-citrulline conversion assay was performed as described both in the presence and absence of the NOS inhibitor L-NAA. ^{14}C -L-citrulline production was normalized to the untreated control ($*** p < 0.001$; mean $\pm$ SD, $n = 3$).

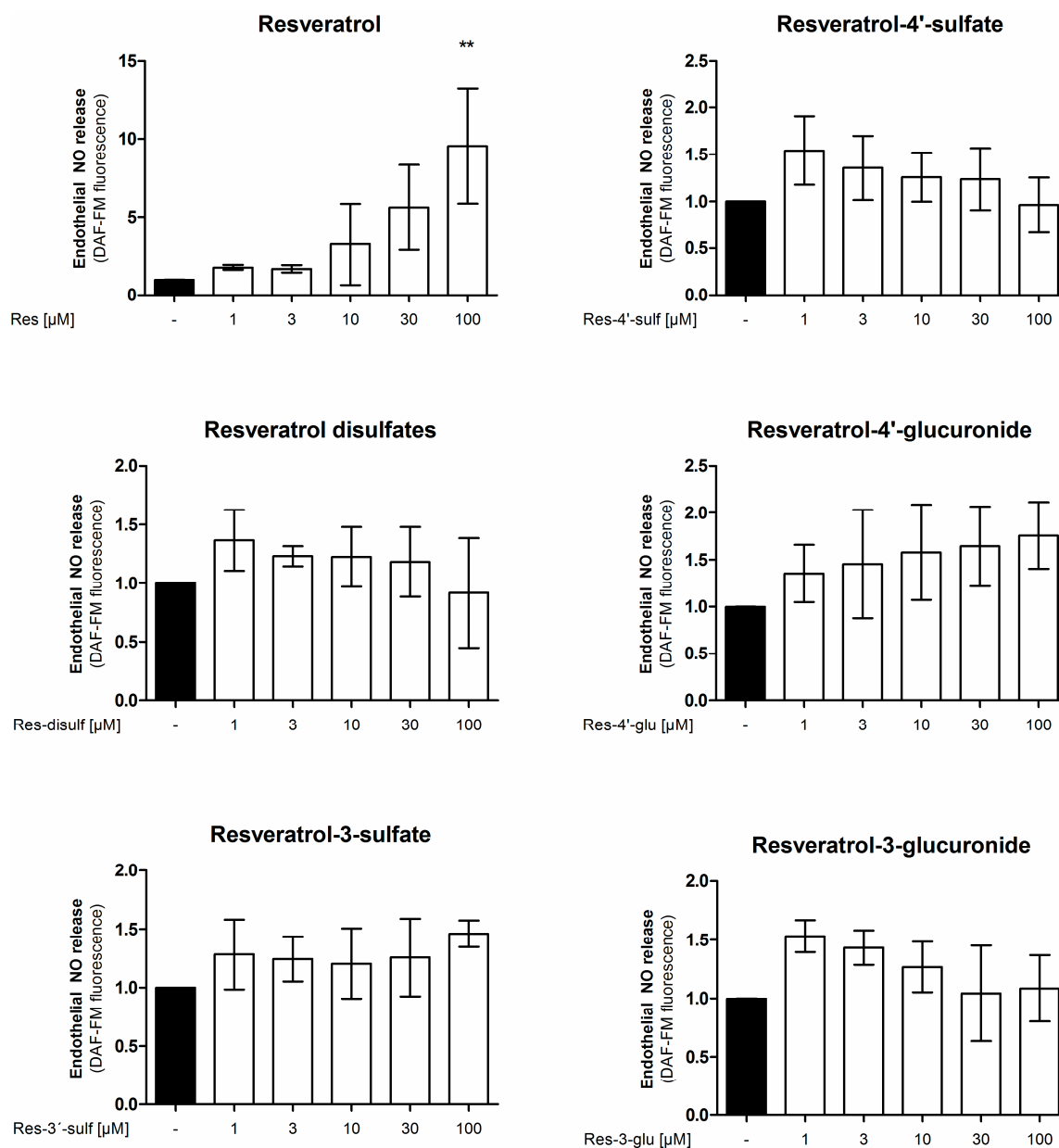


2.2. Impact of Resveratrol and Its Metabolites on Endothelial NO Release

It is possible that the tested resveratrol metabolites are not directly changing eNOS enzyme activity but enhance the bioavailability of endothelial NO for example through the inhibition of superoxide-mediated NO inactivation [5]. Therefore, we determined the levels of endothelial NO release

in EA.hy926 cells after treatment with resveratrol or resveratrol sulfate or glucuronide conjugates for 24 h (Figure 4). Resveratrol concentration-dependently increased endothelial NO release reaching significance and displaying a 5-fold increase at 30 μ M. However, none of the tested resveratrol sulfate or glucuronide metabolites was able to elicit a significant change in endothelial NO release.

Figure 4. Influence of resveratrol and its metabolites on endothelial NO release. EA.hy926 cells were treated with the indicated concentrations of compounds for 24 h. Endothelial NO release was measured by incubation with the NO-sensitive fluorescent probe diaminofluorescein-FM. Fluorescence values were normalized to the number of viable cells as determined by resazurin staining (** $p < 0.01$; mean $\pm$ SD, $n = 3$).



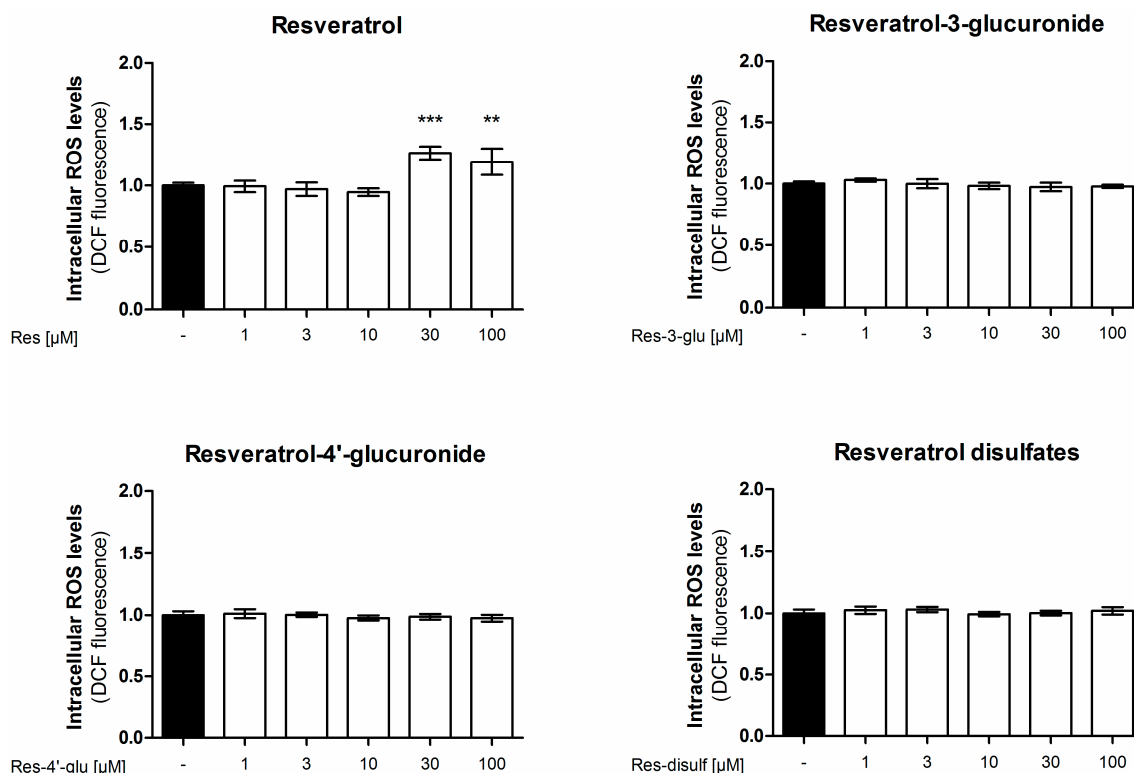
2.3. Impact of Resveratrol and Its Metabolites on Intracellular Reactive Oxygen Species (ROS) Levels

Increased ROS levels have been found to be a major contributor to the development of cardiovascular diseases. Resveratrol is well-known to reduce ROS levels in endothelial cells. It not only directly scavenges

ROS [32], but also activates expression of antioxidant enzymes such as superoxide dismutase 1, glutathione peroxidase or thioredoxin [33,34]. Under certain conditions resveratrol can, however, elicit pro-oxidant effects on the endothelium, suggesting a dual role of resveratrol in endothelial cells [35,36]. In a recent study 10 μM doses of resveratrol were shown to exert pro-oxidant effects whereas concentrations as low as 0.5 μM had an opposite effect [35]. Apart from the applied doses, the oxidative state of the cells seems to be important. Resveratrol has been shown to act as a pro-oxidant under low oxidative conditions and turning into an antioxidant under strong oxidative conditions [37].

To elucidate if the tested resveratrol metabolites affect the cellular ROS detoxification systems, we investigated their effect on intracellular ROS levels. We treated EA.hy926 cells with resveratrol or resveratrol sulfate or glucuronide derivatives for 24 h and determined their ROS load by DCF staining (Figure 5). However, apart from the parent compound resveratrol none of the tested resveratrol metabolites was able to significantly change intracellular ROS levels. The fact that resveratrol, starting at 30 μM , increased intracellular ROS levels is in agreement with an already published study [35].

Figure 5. Influence of resveratrol and its metabolites on intracellular reactive oxygen species levels. EA.hy926 cells were treated with the indicated concentrations of resveratrol or its metabolites for 24 h. Intracellular ROS was measured by incubation with the fluorescent probe 2',7'-dichloro-fluorescein-diacetate (DCF). (** $p < 0.01$, *** $p < 0.001$; mean $\pm$ SD, $n = 4$).



3. Experimental Section

3.1. Chemicals and Cell Culture Reagents

Dulbecco's modified Eagle's medium (DMEM) without phenol red containing 4.5 g/L glucose, glutamine, benzylpenicillin and streptomycin were purchased from Lonza (Verviers, Belgium), HAT

supplement (100 μ M hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine) from Biochrom (Berlin, Germany), and trypsin from Cambrex (Verviers, Belgium). Fetal bovine serum (FBS) was obtained from Gibco via Invitrogen (Paisley, UK). A23187 were bought from Alexis Biochemicals (Lausen, Switzerland) and [^{14} C]L-arginine (346 mCi/mmol) from New England Nuclear (Boston, MA, USA). N^G-Amino-L-arginine (hydrochloride) (L-NAA) was obtained from Cayman Chemicals via Biomol (Hamburg, Germany). All other chemicals, including trans-resveratrol, were bought from Sigma-Aldrich (Vienna, Austria). TLC plates were bought from Machery-Nagel (Markus Bruckner Analysentechnik, Linz, Austria).

3.2. Synthesis and Purification of Resveratrol Conjugates

Synthesis of resveratrol glucuronides (Figure 1A) was performed as described previously [18]. Synthesis and purification of resveratrol sulfates (Figure 1B) were modified according to Wenzel *et al.* [18]. Briefly, 4 mmol trans-resveratrol and 8 mmol sulfur trioxide pyridine complex were separately dissolved in anhydrous pyridine. The reaction was started by slowly adding resveratrol to sulfur trioxide pyridine complex. The mixture was kept under nitrogen atmosphere and was heated up to 80 °C under reflux for 4 h. To convert the synthesis products into sodium salts, 160 mmol Na₂CO₃ was added to the mixture and stirred at 60 °C for another 30 min. Prior to purification of the resveratrol sulfates, the solvent was removed by rotary evaporation. The purification of the resveratrol sulfates was performed by a Dionex Ultimate 3000 semi-preparative LC system (Thermo Fisher Scientific, Vienna, Austria) equipped with a UV detector set to 300 nm. The resveratrol sulfates were separated at a flow of 2.5 mL/min on a RP18 column (Synergi Fusion, 250 $\times$ 10 mm, 4 μ m, Phenomenex, Aschaffenburg, Germany) using a methanol gradient. The gradient started with 3% methanol and 97% ammonium formate (10 mM, pH 8.2) and reached 75% methanol after 45 min before 100% methanol was reached after another 2 min. To separate resveratrol-3-sulfate from resveratrol-4'-sulfate, the corresponding fraction from the first purification was further purified using the same column and mobile phase. The second purification step started with 25% methanol, which was kept constant for 35 min, and ended with 100% methanol after 40 min. After structure identification, purities of 91.5%, 98.4% and 98.6%, for resveratrol-3-sulfate, resveratrol-4'-sulfate and resveratrol disulfates (resveratrol-3,4'-disulfate:resveratrol-3,5-disulfate, 1:1) were determined by means of LCMS(+) and 2D-NMR, as described earlier [18].

3.3. Cell Culture

The human endothelial cell line EA.hy926 (kindly provided by Dr. C.-J.S. Edgell, University of North Carolina, Chapel Hill, NC, USA) [38] was grown in DMEM (without phenol red) supplemented with 2 mM glutamine, 100 U/mL benzylpenicillin, 100 μ g/mL streptomycin, HAT supplement, and 10% FBS up to passage 26. Resveratrol and resveratrol metabolites were dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C. Final DMSO concentrations did not exceed 0.1%. Control cells were always treated with an equal volume of solvent.

3.4. [^{14}C]L-Arginine/[^{14}C]L-Citrulline Conversion Assay

The enzymatic reaction catalyzed by eNOS converts the amino acid arginine into citrulline and NO. [^{14}C]L-citrulline production can thus serve as a surrogate marker of NO production. The assay was performed as previously described [39]. In short, EA.hy926 cells were seeded in six-well plates at a density of 5×10^5 cells/well and treated with test compounds at confluence, after approximately 72 h. Then the endothelial cells were equilibrated in HEPES buffer (HEPES 10 mM, NaCl 145 mM, KCl 5 mM, MgSO_4 2 mM, $\alpha\text{-D}(+)\text{-Glucose}$ 10 mM, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 1.5 mM, pH 7.4) for 10 min at 37 °C. In some experiments 200 μM of the irreversible eNOS inhibitor N^G -Amino-L-arginine (hydrochloride) (L-NAA) was added and the buffer was replaced after 15 min. Subsequently, 0.32 μM [^{14}C]L-arginine (346 mCi/mmol) and 1 μM of the calcium ionophore A23187 were added for 15 min. The reaction was stopped by lysing cells, followed by extraction with ethanol and ethanol/water. The extracts were dried under vacuum (SPD 1010 SpeedVac, Thermo Savant, Thermo Scientific, Langenselbold, Germany) and resolved in water/methanol (1:1). After separation of [^{14}C]L-arginine from [^{14}C]L-citrulline by thin layer chromatography (Polygram SIL N-HR, Machery-Nagel, Austria) in the solvent system water:chloroform:methanol:ammonium hydroxide 25% (2:1:9:4, v/v/v/v), [^{14}C]L-citrulline was quantified by autoradiography in a phosphorimager (BAS-1800II, Fujifilm, Düsseldorf, Germany). AIDA software (raytest, Langenselbold, Austria) was used for densitometric analysis.

3.5. Quantification of NO Release by Diaminofluorescein-FM (DAF-FM)

Quantification of NO released from endothelial cells was performed using diaminofluorescein-FM (DAF-FM), a NO-sensitive fluorescent probe. EA.hy926 cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well and were treated with test compounds at confluence after approximately 72 h. Cells were washed two times with PBS + (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 0.5 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 0.68 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$) containing 100 μM arginine and equilibrated 10 min in this buffer. Then, A23187 was added to a final concentration of 1 μM and DAF-2 to a final concentration of 0.1 μM , and the cells were incubated for 1 h at 37 °C. Addition of L-NAME to a final concentration of 200 μM allowed correction for non-NO-specific fluorescence. The supernatant was transferred to a black 96-well plate and fluorescence was measured in a plate reader (Genios Pro, Tecan, Grödig, Austria) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence values were normalized to viable cells as determined by resazurin conversion method [40]. For this, the cells were incubated with 0.1 mg/mL resazurin in PBS for 30 min before measuring the fluorescence in a plate reader (Genios Pro, Tecan, Grödig, Austria) at an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

3.6. Measurement of Intracellular Reactive Oxygen Species (ROS) Levels

Intracellular ROS levels were measured with the 2',7'-dichloro-fluorescein-diacetate (DCF) probe. EA.hy926 cells were seeded in 12-well plates at a density of 1.6×10^5 cells/well and were treated with test compounds at confluence after approximately 72 h. Catalase (activity 21600 U/mg protein) was added to the cell culture medium at 50 U/mL and co-incubated with the test substances to inhibit formation of ROS in the cell culture supernatant [41]. After 24 h treatment, cells were washed once with

HBSS (140 mM NaCl, 5 mM KCl, 0.14 mM Na₂HPO₄, 0.37 mM KH₂PO₄, 0.80 mM MgSO₄, 1.2 mM CaCl₂ × 2H₂O, 5.6 mM glucose, 20 mM HEPES) and incubated with 20 µM DCF in HBSS for 25 min at 37 °C. After washing the cells once with HBSS, cells were trypsinized and resuspended in PBS containing 2% BSA. Samples were measured at a flow cytometer FACS Calibur™ (BD Biosciences, Franklin Lakes, NJ, USA) at the excitation/emission wavelength 488/530 nm (channel FL-1). Geometric means were calculated using CellQuest™ Pro.

3.7. Statistical Methods

Statistical analysis was done using GraphPad Prism software version 4.03 (GraphPad Software Inc., La Jolla, CA, USA). To determine statistical significance one-way analysis of variance (ANOVA) with Bonferroni post-test was performed. Asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Figures with bar graphs represent mean ± SD of at least three independent experiments.

4. Conclusions

To the best of our knowledge this study shows for the first time the absence of a direct role of the main physiological resveratrol metabolites on eNOS enzyme activity, NO release and intracellular ROS production in endothelial cells. Further studies are necessary to evaluate a possible influence of resveratrol metabolites not tested here, such as dihydro-resveratrol and its glucuronic and sulfated derivatives, which have recently been identified in rat liver and adipose tissue [42] and the potential synergy of combinations of resveratrol and the single metabolites. Back-conversion of resveratrol metabolites to resveratrol in the target tissue would also account for the lack of activity of metabolites observed in this study. Another explanation for the lack of activity of the resveratrol metabolites could be that endothelial function is influenced by a here not investigated pathway, or that the applied *in vitro* system is too artificial for the complex situation *in vivo*.

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Author Contributions

A.L. and D.S. designed and performed experiments as well as analyzed data; A.L. drafted the manuscript; K.S. synthesized resveratrol glucuronides and performed experiments; M.P. synthesized resveratrol sulfates; V.M.D., E.H.H. and V.S. conceived and supervised the study. All the authors read, commented on and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Not available.

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3.6 Concentration dependent effects of resveratrol and metabolites on the redox status of human erythrocytes in single dose studies

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This work establishes the concentration-dependent redox properties of Resveratrol and physiological metabolites in a cell free assay. The following aim was to implement the redox results into low (50 mg) and high (5 g) single dose oral Resveratrol human intervention studies. To this aim, plasma ROS levels were measured, and additionally erythrocytes were tested for antioxidant enzyme activities (SOD; CAT), as well as lipid peroxidation (conjugated dienes) and the occurring differences between low and high dose Resveratrol.

I synthesized and purified the glucuronated metabolites used in this study, and tested the redox capacities for Resveratrol and metabolites in a cell free assay. Additionally, I was involved in data interpretation, and writing the manuscript.



Concentration-dependent effects of resveratrol and metabolites on the redox status of human erythrocytes in single-dose studies

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Abstract

Dietary *trans*-resveratrol (RES) is rapidly metabolized into sulfated and glucuronated conjugates in humans. This study focused on the *in vitro* determination of the antioxidant capacity of RES and its main physiological metabolites and on its relevance *in vivo*. *In vitro*, RES, RES-3-O-sulfate (R3S) and 3-O-glucuronide (R3G) showed antioxidant activities at a concentration of 1 mM when compared to Trolox using an assay in which the antioxidant inhibits iron-induced linoleic acid oxidation: 0.87 ± 0.08 mM Trolox equivalents (TE) for RES, 0.52 ± 0.01 mM TE for R3S and 0.36 ± 0.02 mM TE for R3G. At a concentration of 1 μ M, compounds promoted linoleic acid peroxidation (RES -0.30 ± 0.09 mM TE, R3S -0.48 ± 0.05 mM TE and R3G -0.57 ± 0.07 mM TE). To elucidate whether these effects were reflected *in vivo*, total antioxidant capacity, reactive oxygen species (ROS), conjugated fatty acid dienes (CD), superoxide dismutase (SOD) and catalase (CAT) activities were determined in human plasma and erythrocytes over 24 h, after oral intake of either 0.05 g RES as piceid or 5 g RES. Oral administration of RES did not show an impact on total antioxidant capacity, ROS or CD. However, enzymatic activities of ROS scavenging SOD and CAT were significantly lower after high-dose compared to low-dose administration of RES ($P < .03$ and $P < .01$). In conclusion, in healthy subjects, neither 0.05 g nor 5 g RES changed blood oxidative state, although our *in vitro* data point to a prooxidative activity of low concentrations of RES and its metabolites, which could be important *in vivo* for individuals with compromised antioxidant defense capacity.

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Keywords: Resveratrol; Antioxidant capacity; ROS; SOD; CAT; Human

1. Introduction

trans-Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, RES) is predominantly found in red grapes, peanuts or products thereof [1]. Upon ingestion, RES and its predominant form in plant-derived foods, RES-3-O- β -D-glycoside, termed piceid, are metabolized rapidly, forming RES-sulfated and RES-glucuronated conjugates [2]. Depending on the administered dose, plasma concentrations of RES metabolites vary from nanomolar to micromolar range [2,3]. Quantitative data from a previously conducted pharmacokinetic pilot study showed maximum plasma concentrations ($c_{\max}$) of RES-3-O-sulfate (R3S), RES-disulfates (RdS) and RES-monoglucuronides and diglucuronides of 0.95 μ M, 1.27 μ M and 0.7 μ M, respectively, after a single oral dose of 85.5 mg

piceid (corresponding to 0.05 g RES) [2]. Dietary administration of a 100-fold higher bolus dose of 5 g RES revealed $c_{\max}$ values for the aforementioned metabolites of 81.3 μ M, 28.2 μ M and 67.6 μ M, respectively [3]. RES in plasma was below the limit of detection (0.02 μ M) after administration of 85.5 mg piceid and demonstrated a $c_{\max}$ value of only 0.90 μ M after administration of 5 g RES [2,3]. Both conducted pharmacokinetic studies showed a low bioavailability for RES in blood, in contrast to its metabolites, leading to the hypothesis that the conjugates carry the biological activity in humans or are reconverted into RES in target organs, as reported by Patel *et al.* [4]. Hence, reversion data are still limited, and it seems conceivable that observed health effects in humans can be attributed to RES conjugates [5]. The effects of the parent compound RES on oxidative and radical-scavenging mechanisms are controversially discussed [6]. Oxidative stress in form of reactive oxygen species (ROS) can lead to modification of proteins, lipids and carbohydrates and is hypothesized to be associated with the progression of various pathological states, e.g., cancer, age-related diseases and the development of type 2 diabetes [7,8]. In general, RES is known as a potent direct radical scavenger and has been shown to interact with redox systems *in vivo* by regulating antioxidant detoxification enzymes like superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase directly or

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via adjustment of redox-controlled gene transcription [9–11]. However, Bader *et al.* [12] investigated the degradation of RES by γ -radiation and hypothesized the formation of RES radicals in aqueous solution as a result of OH-radical attack. Accordingly, at a considerably low concentration of 8 μ M, Ahmad *et al.* [13] could show that RES induced a prooxidative state in human leukemia cells *in vitro* by increasing intracellular superoxide by 25%, indicating a promoting effect on radical formation. On the other hand, RES showed a direct radical-scavenging effect in rat macrophages *in vitro* at concentrations ranging from 0.65 to 1.3 mM [14]. Furthermore, in human breast cancer cells, Bader and Getoff [15] investigated the impact of 2 μ M and 50 μ M RES on the effectiveness of γ -ray irradiation on cell death *in vitro*, and they could show that, at 2 μ M, RES had a radical-scavenging protective effect on cancer cells, whereas cancer cell death was enhanced at 50 μ M RES. Besides direct radical-scavenging activity, another mechanism of action of RES is the up-regulation of ROS detoxification enzyme activity such as that of SOD and CAT. This was shown by Mokni *et al.* [16] in an *in vivo* study of healthy rats after intraperitoneal administration of 5–25 mg/kg body weight RES for 1 week, circumventing extensive metabolism in liver and digestive tract. Considering aforementioned controversial findings, studies in healthy human subjects after dietary relevant single dosage of RES could provide useful information on the influence of this natural compound and its metabolites on the natural balance of radical-scavenging systems and are, to our knowledge, not yet available. We therefore initially assessed the redox capacity of RES and its main physiological metabolites over a wide range of concentrations (1–1000 μ M) in a cell-free assay. Consecutively, the current study aimed to compare the impact of a RES-derivative piceid, present in plants, on the human blood antioxidative capacity after a single low oral dose of 85.5 mg (0.05 g RES) [2], representative for an average daily food intake from dietary sources [1], and a high oral amount of 5 g RES (10 capsules at 500 mg) [3], representing commercially available nutritional supplements. Plasma and erythrocyte samples were obtained from the pharmacokinetic studies referenced above, conducted and published earlier, by Burkon *et al.* [2] and Gualdoni *et al.* [3]. Formation of ROS, conjugated fatty acid dienes (as a marker for radical induced lipid peroxidation, CD) and ROS detoxifying enzyme activities (CAT and SOD) in human erythrocytes were compared after single, differentially dosed, oral administration of RES in healthy subjects to see if the obtained *in vitro* results were reflected in the redox status of human blood. Measurements were done in a timely manner after blood drawings, and as of now, we could show for the first time a dose-dependent impact of oral short-term administration of RES on antioxidative enzyme activities in erythrocytes of healthy subjects.

2. Materials and methods

2.1. Chemicals

The reagents and solvents used for the experiments were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Merck (Darmstadt, Germany). The SOD assay kits used for the activity measurements were acquired from Dojindo (Gaithersburg, MD, USA). Benzoyl-leucomethylene blue was purchased from TCI Germany (Eschborn, Germany).

2.2. Plasma and erythrocyte samples

The plasma and erythrocyte samples were obtained from two human intervention studies, as reported by Burkon *et al.* ($n=9$) [2] and Gualdoni *et al.* ($n=8$) [3] on the pharmacokinetics of RES and metabolites, both of which were performed and published earlier [2,3]. After drawing blood samples as references, a single oral dose of either 85.5 mg piceid (corresponding to 0.05 g *trans*-RES) per 70 kg body weight (Burkon *et al.* [2]) or an absolute amount of 5 g of RES (Gualdoni *et al.* [3]) was administered. After oral administration, blood samples were drawn over 24 h (piceid) and 48 h (5 g RES), immediately separated into plasma and erythrocytes by centrifugation, washed three times with 0.9% sodium chloride solution and stored at -80°C , shortly followed by further analyses.

2.3. Total antioxidative activity

Total antioxidative activity was determined following the procedure reported by Lindenmeier *et al.* that is based on the inhibition or promotion of the iron-catalyzed peroxidation of linoleic acid by added antioxidants [17]. Initially, the total antioxidant activity of RES and the RES metabolites was measured. R3S, RdS and 3-O-glucuronide (R3G) were synthesized and purified as published previously [2]. Solutions of each substance (1–1000 μ M in 15% aqueous ethanol) were prepared and aliquots (50 μ l) of these solutions were used for testing. For the determination of the antioxidative activity of plasma samples, these were spiked with ethanol for a final concentration of 15% ethanol and aliquots (50 μ l) were used for testing. The antioxidative activity was correlated with the degree of color development (conversion of benzoyl-leucomethylene blue to methylene blue), which was measured at 666 nm in disposable cuvettes on a TecanInfinite 200 (Groedig, Austria). Results were calculated using a seven-point-calibration curve with Trolox solutions as reference antioxidant (0–2 mM in 15% aqueous ethanol) and the total antioxidative activity was expressed as Trolox equivalents (TE) (mmol/L). Experiments were performed in triplicates, with two technical replicates.

2.4. Determination of total ROS in erythrocytes

Total ROS was determined by FACS measurement according to Amer *et al.* [18]. It is based on the uptake of nonfluorescent 2',7'-dichloro-fluorescein-diacetate (DCF-DA) into cells and its conversion to 2',7'-dichloro-fluorescein (DCFH) by endogenous esterases. ROS-containing cells convert DCFH to a fluorescent 2',7'-dichloro-fluorescein (DCF) radical, with absorption maximum at 488 nm. A 1:20 dilution of erythrocytes in PBS was incubated for 15 min (37°C , 5% CO_2) with 0.4 mM DCF-DA, centrifuged and washed once with 1 ml of PBS. Subsequently, an aliquot was incubated with 2 mM H_2O_2 for 30 min at room temperature. Measurement of 100,000 cells was performed with a FACSCalibur (Becton-Dickinson, New Jersey, USA). The difference between fluorescent values of H_2O_2 -stimulated and nonstimulated cells was normalized to hemoglobin content of the sample, and data were then calculated in percent as treated ($n=8$) over control ($n=2$).

2.5. Measurement of CD in erythrocytes

The lipid extraction from the erythrocytes was carried out by a modified method reported by Folch *et al.* [19], followed by saponification and methylation of the fatty acids according to the Association of Official Analytical Chemists method [20]. A volume of 10 μ l of this fatty acid composition was injected into an HPLC column and CD were quantified using diode array detection (DAD) at a wavelength $\lambda=234$ nm. Separation was performed on a Dionex Ultimate 3000 LC system (Thermo Fisher Scientific, Vienna, Austria), using a Nucleosil 100-5 C18 analytical HPLC column (250 $\times$ 4.6 mm ID, 5 μ m particle size; Macherey-Nagel, Düren, Germany) with a precolumn of the same material, at a flow rate of 0.8 ml/min at 25°C . Liquid chromatography was carried out using the following conditions: starting with 95% acidified H_2O bidest (0.1% formic acid) and 5% acidified acetonitrile (0.1% formic acid), the acetonitrile content was increased to 80% within 20 min then to 100% within 25 min and was maintained for 5 min. Thereafter, the column was reset to the initial conditions within 5 min and equilibrated for another 5 min. Each of the experiments was performed in triplicates. CD content was defined as the area under the curve (AUC) related to the content of total lipids extracted via the Folch extraction [19]. The total lipid content was determined by a photometric assay according to the manufacturers' protocol (Randox Laboratories, Krefeld, Germany).

2.6. Measurement of SOD activity in erythrocytes

The SOD activity in erythrocytes was determined by the SOD assay kit water-soluble tetrazolium salt, according to the manufacturers' protocol (Dojindo, Gaithersburg, MD, USA) with slight modifications. For sample preparation, 0.5 ml of erythrocytes was suspended with 2 ml of distilled water then 1 ml of ethanol and 0.6 ml of chloroform were added and the mixture was shaken vigorously with a shaker for 15 min at 4°C . Afterwards, the mixture was centrifuged at 600g for 10 min at 4°C , the upper water/ethanol phase was transferred into a new tube and diluted 1:8 with distilled water. To achieve the sample solution for the assay, a final dilution step was accomplished with 0.25% aqueous ethanol. In the SOD assay, a highly water soluble tetrazolium salt (WST-1) was used, which was converted to a water-soluble formazan dye through reduction by superoxide anions. The rate of reduction of the superoxide anions was linearly related to the xanthine oxidase activity and inhibited by SOD (sample solution). Thus, the SOD activity (50% inhibition) was determined colorimetrically in duplicates in 96-well microplates at a wavelength $\lambda=450$ nm. Finally, the SOD activity was related to the hemoglobin content of the sample.

2.7. Measurement of CAT activity in erythrocytes

The CAT activity in erythrocytes was determined following a photometric assay reported by Aebi [21]. For sample preparation, erythrocytes were diluted 1:5 with distilled water. A further 1:500 dilution step was performed with phosphate buffer immediately before the assay was started by adding H_2O_2 . The determination was performed in duplicates and the decrease of H_2O_2 absorbance was recorded at 240 nm for 30 s (20°C) in disposable cuvettes. Results were calculated and demonstrated as

the rate constant of a first-order reaction (k) [21]. Finally, CAT activity was related to the hemoglobin content of the sample.

2.8. Hemoglobin content in erythrocytes

The hemoglobin content in erythrocytes was determined following a method with Drabkin's reagent according to the manufacturers' protocol (Sigma-Aldrich, Taufkirchen, Germany) with slight modifications. For sample preparation, the erythrocytes were diluted 1:2 with distilled water and aliquots (20 μ l) were used in the colorimetric assay. The determination was performed in duplicates and the absorbance was recorded at a wavelength $\lambda=540$ nm. Finally, the results were calculated using a five-point-calibration curve with human hemoglobin solutions as standards (0.15–0.92 mg/ml in distilled water).

2.9. Statistical analysis

All data presented are expressed as means $\pm$ S.E.M. The differences in the enzyme activities and the oxidative exposure of erythrocytes (CD) between time point 0 h and the various time points after administration of piceid and RES were calculated using two-tailed Student's t test for nonpaired samples. The same test was used for calculation of differences between high-dose and low-dose administrations of RES, using AUC values. $P \leq 0.05$ was considered significant.

3. Results and Discussion

3.1. Total antioxidative capacity in vitro

The antioxidative activity of RES and its main physiological metabolites (R3S, RdS and R3G) was measured following a protocol by Lindenmeier *et al.* [17]. This method is based on the Fenton-type reaction, with linoleic acid as a substrate for iron-catalyzed radical attack by H_2O_2 -derived $OH^\cdot$ radicals. Resulting linoleic acid peroxides then catalyze the conversion of colorless benzoyl-leucomethylene blue to methylene blue, which absorbs at 666 nm. Addition of a given substance can either inhibit (antioxidative) or promote (prooxidative) linoleic acid peroxidation, resulting in differential color development [22,23]. Results were calculated using an eight-point Trolox calibration curve (0–2 mmol/L, $R^2=0.995$). RES, R3S, RdS and R3G at concentrations 1, 10 and 100 μ M showed a promoting effect on linoleic acid peroxidation, with lowest TE values (mM TE) of -0.30 ± 0.09 mM TE, -0.48 ± 0.05 mM TE, -0.36 ± 0.09 mM TE and -0.57 ± 0.07 mM TE, respectively, at a concentration of 1 μ M (Fig. 1). At 100 μ M, the parent compound showed an inhibiting effect on linoleic acid peroxide formation, compared to the metabolites, which still induced peroxidation. Whereas at 1000 μ M, the antioxidative activity of RES resulted in 0.87 ± 0.08 mM TE, values for the metabolites were lower with 0.52 ± 0.01 mM TE for R3S and 0.36 ± 0.02 mM TE for R3G. No effect with respect to total antioxidant capacity was observed for

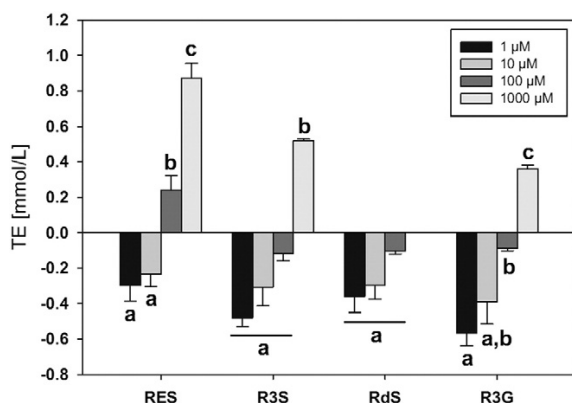


Fig. 1. *In vitro* total antioxidant activity of RES and its main physiological metabolites R3S, RdS and R3G in aqueous solution (in mM TE); concentration-dependent effects for individual compounds are indicated by lowercase letters as determined by ANOVA, followed by *post hoc* Holm-Sidak testing ($P \leq 0.05$); data are expressed as means $\pm$ S.E.M. ($n=3-6$).

RdS at 1000 μ M. These results clearly demonstrate that the redox capacity of RES and its conjugates varied dose dependently from 1 μ M to 1000 μ M from prooxidant to antioxidant in our cell-free assay. On the one hand, the linoleic acid peroxidation inducing effect of low concentrations of RES corresponds to *in vitro* findings by Martins *et al.*, where RES, at concentrations of 1–50 μ M, increased ROS levels in hepatic cells after a 24 h treatment [24]. On the other hand, a decrease in superoxide anion production could be shown at only 3 and 6 μ M RES *in vitro*, however in a model of hyperglycemic THP-1 monocytes to mimic diabetes, with higher levels of oxidative stress to begin with [25]. In our cell-free study, at a concentration of 100 μ M, RES is more antioxidatively active than its metabolites R3S, RdS and R3G (ANOVA, *post hoc* Holm-Sidak, $P \leq 0.007$). At 1000 μ M, RES inhibits linoleic acid peroxidation more effectively than R3S and R3G (ANOVA, *post hoc* Holm-Sidak, $P \leq 0.003$). This could be due to the conjugation of 3-hydroxy and 5-hydroxy groups, which was shown to impair the thermodynamic stabilization of the radical-scavenging system of RES [26]. The potential of free RES to inhibit linoleic acid peroxidation at concentrations of 100 and 1000 μ M, compared to RES conjugates, is further confirmed by Storniolo *et al.*, where at 50 μ M, activity of RES was more profound than that of R3S or R3G [27].

3.2. Oxidative status in vivo

3.2.1. Total antioxidant capacity in plasma

In a study by Olas *et al.* [28], a pronounced protective effect against the ROS species peroxynitrite could be shown in plasma of healthy volunteers for 100 μ M RES. Therefore, after determining the antioxidant effect of RES and its conjugates on the iron-induced linoleic acid peroxidation *in vitro*, we analyzed the redox status in plasma of healthy subjects after oral administration of 0.05 g RES, in form of 85.5 mg piceid by means of the *in vitro* applied test (Table 1) [17]. Contrary to our *in vitro* findings at 1 μ M, at c_{max} values of 0.95 μ M, 1.27 μ M and 0.7 μ M, for R3S, RdS and RES-glucuronides, respectively, as determined earlier by Burkon *et al.* [2] for the same study population, no changes compared to basal levels (t_0 , set at 100%) were observed *in vivo*. The absence of any measurable effect of RES and metabolites *in vivo* might be explained by the higher abundances of other antioxidants present, such as ascorbic acid, tocopherol, retinol or glutathione [29].

3.2.2. Total ROS in erythrocytes

The *in vitro* observed prooxidative effect of RES and its conjugates was not reflected *in vivo* by the total antioxidant activity in plasma after administration of 85.5 mg piceid (0.05 g RES). Therefore, the effects of RES and its metabolites on nonenzymatic and more specific markers of oxidative stress (ROS and CD) were determined in erythrocytes after a high oral load of 5 g. Levels of ROS in erythrocytes were determined by the intracellular conversion of DCFH to a DCF radical and were calculated as treated ($n=8$) over control ($n=2$), with time point t_0 set at 100% as baseline value, as depicted in Fig. 2. No

Table 1
Total antioxidant activity in human plasma after the administration of a single dose of 85.5 mg piceid per 70 kg body weight ($n=9$); time point 0 h (t_0) was set as 100%, and data represent means $\pm$ S.E.M.

Time points (t_x) (h)	Total antioxidant activity (t_x/t_0) (%)
0	100 $\pm$ 6.38
1	106 $\pm$ 5.00
2	105 $\pm$ 6.06
4	105 $\pm$ 5.05
6	109 $\pm$ 4.90
8	106 $\pm$ 6.00
10	109 $\pm$ 4.90
24	103 $\pm$ 3.09

differences to baseline level could be observed, suggesting no impact of metabolized RES on ROS formation in erythrocytes. After an amount of 5 g RES, $c_{\max}$ values in plasma were 0.09 μM , 81.3 μM , 28.2 μM and 67.6 μM , at time points ($t_{\max}$) 2.5 h, 5 h, 10 h and 2.5–5 h, for RES, R3S, RdS and RES-glucuronides, respectively, as reported by Gualdoni *et al.* [3]. According to our here-presented results on ROS formation in human erythrocytes, those concentrations are apparently not relevant for an antioxidant effect. On the other hand, we could not observe a prooxidant effect either, as suggested by our *in vitro* data (Fig. 1), although ROS values show an increase from 5 to 10 h (Fig. 2). This interval corresponds approximately to $c_{\max}$ for metabolites, namely R3S and the RES-4'-O-glucuronide with $t_{\max}$ at 5 h and RdS with $t_{\max}$ at 10 h after oral administration, as previously determined by Gualdoni *et al.* [3]. However, it should be mentioned that, *in vitro*, at a concentration of 100 μM , we could observe a radical inducing effect for RES conjugates, with values for predominating metabolites R3S and R3G of -0.12 ± 0.04 and -0.09 ± 0.01 mM TE, whereas in an *in vitro* study by Stornio *et al.* [27], 50 μM of the respective compounds showed a radical-scavenging effect of 0.11 ± 0.02 and 0.12 ± 0.03 mM TE. In the referenced study, results were also compared to TE but achieved by means of a commercially available kit, based on the inhibition of the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) to ABTS⁺ by present compounds [30]. As evaluated by Tabart *et al.* [31], total antioxidant values can vastly differ for individual compounds, depending on the applied test. Measuring the scavenging activity of the 2,2-diphenyl-1-picrylhydrazil radical and measuring the oxygen radical-scavenging capacity, for example, showed distinct differences in antioxidant capacities for selected flavonoids due to different reaction mechanisms [31]. We therefore estimate the impact of predominant RES conjugates on ROS levels in concentrations up to approximately 100 μM in human erythrocytes to be negligible, especially, as other antioxidants very likely cover their effect due to higher molar abundances [29].

3.2.3. CD in erythrocytes

To examine whether the metabolites formed *in vivo* after oral intake of 0.05 g or 5 g RES affected the oxidative exposure of erythrocytes, CD were quantified in erythrocytes by HPLC DAD (Fig. 3). As CD are more stable than ROS, these oxidized fatty acids can be used to determine changes in erythrocyte redox state that would be overseen due to short half-life of ROS, in combination with the time periods between blood drawings. In accordance with our data

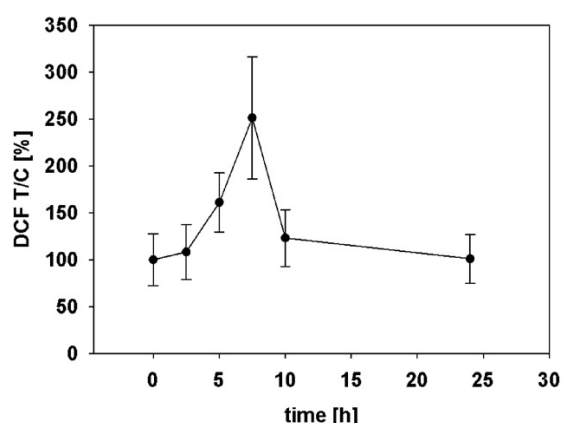


Fig. 2. ROS levels in erythrocytes, as determined by DCF fluorescence; values are normalized to hemoglobin content and calculated as treated ($n=8$) over control group ($n=2$) with baseline at time point t_0 set at 100%; data represent means $\pm$ S.E.M.; a two-tailed Student's t test for nonpaired samples was applied to calculate differences between time points, and $P \leq 0.05$ was considered significant.

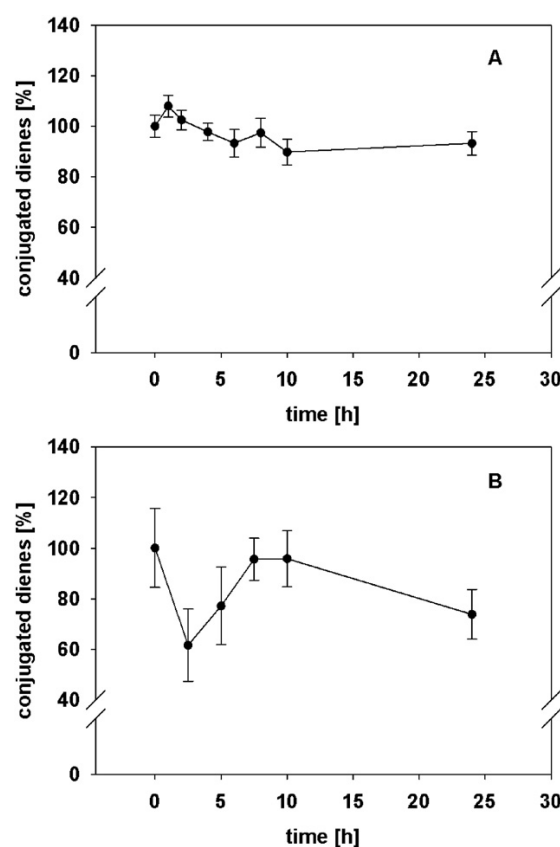


Fig. 3. CD in human erythrocytes after the administration of (A) single-dose piceid (0.05 g RES, $n=9$) and (B) single-amount RES (5 g, $n=8$); results are normalized to total lipid content and t_0 was set as 100%; data represent means $\pm$ S.E.M.; a two-tailed Student's t test for nonpaired samples was applied to calculate differences between time points, and $P \leq 0.05$ was considered significant.

on antioxidant capacity and ROS, no changes to baseline values (100%) could be determined at any measured time point. Comparison of AUCs (in arbitrary units, a.u.) between dietary low RES ($\text{AUC}_{0.05 \text{ g}} = 2260 \pm 76.8$ a.u.) and high RES, after a 5 g supplement ($\text{AUC}_5 \text{ g} = 2018 \pm 156$ a.u.) showed no significant difference for CD in erythrocytes, although Fig. 3B showed a decrease in CD levels in the first 5 h, which would indicate an antioxidant effect. This potential antioxidant effect is in agreement with other studies where incubation of erythrocytes with 0.1–100 μM RES attenuated oxidative-injured erythrocyte membrane *in vitro* [32,33]. A dose-dependent decrease of *tert*-butylhydroperoxide-induced formation of malondialdehyde was obtained after treatment of the erythrocytes with RES. However, in the current study, no changes in CD levels were determined after a dose of only 0.05 g RES (Fig. 3A).

3.2.4. Antioxidant enzyme activities in erythrocytes

The function of antioxidant enzymes (SOD, CAT, glutathione peroxidase) in blood and tissues is to protect from ROS-mediated damage [34]. Down-regulation of gene transcripts and the activity of those enzymes have been linked with increased amounts of ROS and cause increased susceptibility to oxidative damage [35,36]. As erythrocytes are not able to transcribe and translate genes, antioxidant enzyme activities were measured in red blood cells of participants, as a marker for oxidative stress.

3.2.4.1. SOD in erythrocytes. To elucidate the influence of RES on ROS detoxifying enzymes, SOD and CAT activity in erythrocytes was determined after oral administration of different amounts of RES over 24 h (Figs. 4 and 5). No differences compared to basal levels could be observed after administration of either 0.05 g or 5 g RES. However, by comparison of AUC values of the SOD activity in both studies, SOD activity was reduced after intake of 5 g RES compared to the low-dose intake of 0.05 g RES in form of piceid ($AUC_{0.05\text{ g}}=2365\pm43.0$ a.u. and $AUC_{5\text{ g}}=1829\pm216$ a.u., $P=.03$). This effect of the higher oral amount of RES might be at least partially explained by lower values at 2.5 h after administration of 5 g RES (Fig. 4B), corresponding to the time points for c_{max} of glucuronated metabolites (total of 67.6 μM), ranging from 2.5 to 5 h, as documented by Gualdoni *et al.* [3], and could indicate changes in erythrocyte oxidative state, which might not occur with low-dose RES, where c_{max} for glucuronides was only 0.7 μM with t_{max} at 6 h after ingestion [2].

3.2.4.2. CAT in erythrocytes. As for SOD, no differences to basal levels could be observed for CAT activity in either group, but again, a lower enzyme activity could be demonstrated for the high amount RES group by AUC comparison to the low-dose RES group ($AUC_{0.05\text{ g}}=2373\pm119$ a.u. and $AUC_{5\text{ g}}=1411\pm230$ a.u., $P=.01$, Fig. 5). For the parent compound RES, an enzyme inhibiting effect on CAT has been observed by Martins *et al.* [24], at 50 μM *in vitro* in liver cells. As the parent compound is not present in plasma in such high concentrations

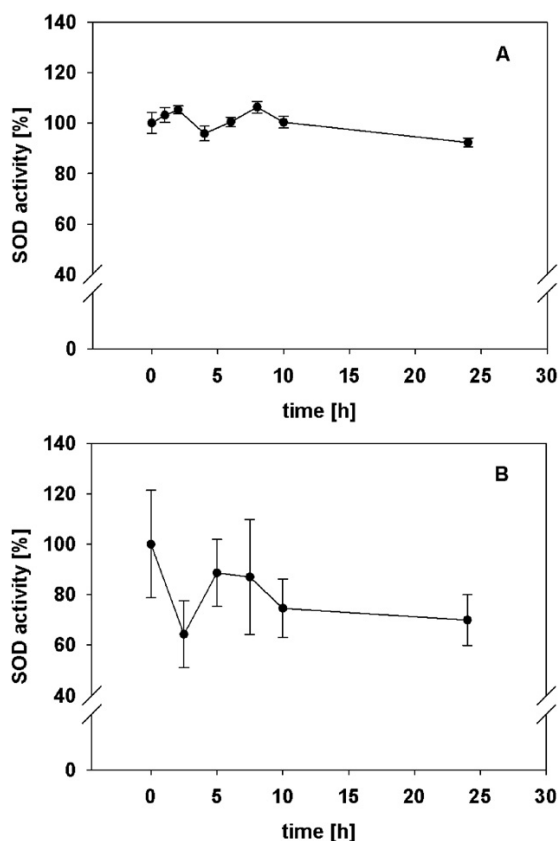


Fig. 4. SOD activity in human erythrocytes, normalized to hemoglobin content after the administration of (A) single-dose piceid (0.05 g RES, $n=9$) and (B) single-amount RES (5 g, $n=8$); t_0 was set as 100%; data represent means $\pm$ S.E.M.; a two-tailed Student's *t* test for nonpaired samples was applied to calculate differences between time points, and $P \leq .05$ was considered significant.

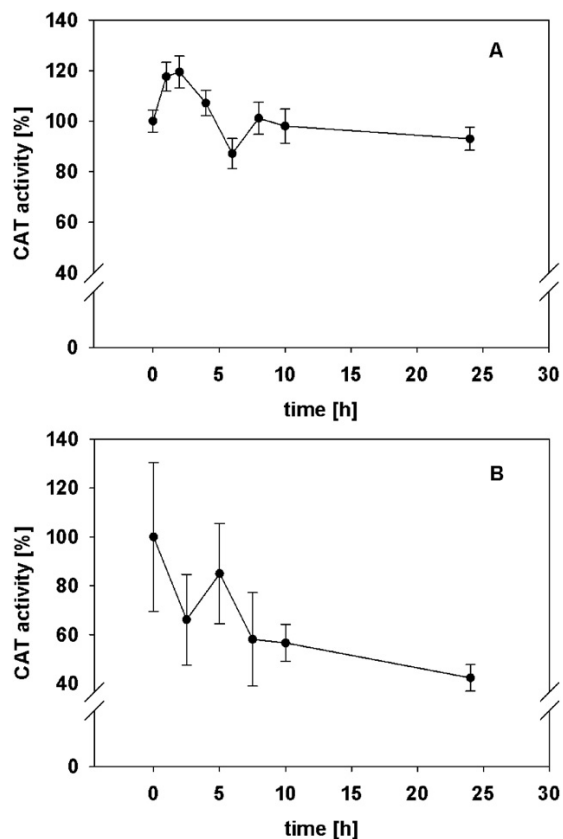


Fig. 5. CAT activity in human erythrocytes normalized to hemoglobin content after the administration of (A) single-dose piceid (0.05 g RES, $n=9$) and (B) single-amount RES (5 g, $n=8$); t_0 was set as 100%; data represent means $\pm$ S.E.M.; a two-tailed Student's *t* test for nonpaired samples was applied to calculate differences between time points, and $P \leq .05$ was considered significant.

($c_{\text{max}}=0.09\text{ }\mu\text{M}$) after oral administration of 5 g RES, those changes in enzyme activity could be attributed to the main conjugates. Fig. 5B shows a trend for lower enzyme activities, albeit not reaching the level of significance ($P=.063$), CAT activity at 2.5 h (t_{max} of glucuronide conjugates) and additionally from 5 h to 24 h, corresponding to t_{max} after oral administration for R3S at 5 h ($c_{\text{max}}=81.3\text{ }\mu\text{M}$) and for RdS at 10 h ($c_{\text{max}}=28.2\text{ }\mu\text{M}$), as determined by Gualdoni *et al.* [3].

4. Conclusions

Oral administration of RES did not affect the iron-catalyzed linoleic acid peroxidation and ROS formation in human erythrocytes, presumably due to the presence of more potent antioxidants [29] and the comparably low concentrations of RES metabolites (c_{max} from 0.27 to 1.27 μM after oral intake of 0.05 g RES and from 28.2 to 81.3 μM after 5 g RES) [2,3]. Moreover, the high-plasma-protein binding capacity of RES and its metabolites of over 93% for RES, 66% for R3S and 56% for RdS might also impede their effect on ROS formation [2]. However, our data suggest a prooxidative effect of orally ingested RES as demonstrated by decreased SOD and CAT activity in erythrocytes, after administration of a high bolus dose of 5 g RES, compared to low-dose administration as 85.5 mg piceid, especially at time points corresponding to maximum plasma concentrations of individual metabolites (c_{max} for glucuronides being 67.6 μM with t_{max} at 2.5 h and 81.3 μM for R3S with t_{max} at 5 h after 5 g RES intake). Down-

regulation of SOD and CAT activity has been associated with an increased ROS formation [35,36], which was most likely compensated by the presence of other antioxidants in our *in vivo* studies. In our *in vitro* assay, antioxidant activities were demonstrated for concentrations of 100 μM , which were not reached even after the bolus dose of 5 g RES, resulting in a mean maximum plasma concentration for the 3-O-sulfated metabolite of 81.3 μM , followed by RES glucuronides with 67.6 μM [3]. Additionally, plasma half lives in humans for RES conjugates were determined to be only 5–8 h, after an oral dose of 5 g, resulting in rapid elimination from the body [37]. In accordance with our *in vitro* data, at concentrations of 1 and 10 μM , Ahmad *et al.* [13] demonstrated that treatment with 4–8 μM RES for 24 h profoundly increased intracellular radical concentrations (up to 25%) in human leukemia cells and even showed an interference with chemotherapeutic-induced apoptosis. Our *in vitro* data indicate that the major RES metabolites have a similar potential on radical formation. Considering the antioxidant effect of higher concentrations of RES and its metabolites, blood or tissue accumulation resulting in higher steady-state concentrations of RES could have not been reported in healthy human subjects so far, as demonstrated by Almeida *et al.* [38]. Here, a mean c_{max} value in plasma was only 0.28 μM and half-life of RES was determined to be 2–5 h after intake of 4 $\times$ 150 mg RES over 24 h. Accordingly, after oral intake of 1 g RES administered twice a day for a week to healthy human subjects, a mean c_{max} value in plasma was 5.58 μM , and half-life was 2.4 h [39]. In one of our conducted pharmacokinetic studies, intake of 85.5 mg piceid led to plasma metabolite concentrations below 1.27 μM (RdS), and in line with their short elimination half-lives and our herein obtained data, we conclude that dietary ingested amounts of RES or piceid do not significantly influence the redox balance in blood samples of healthy subjects [2]. Taken together, even with single oral administration of RES in a dose corresponding to a high-dose nutritional supplementation, it seems unlikely to achieve plasma concentrations high enough for antioxidative effects in healthy subjects as observed in our *in vitro* experiments. However, Brasnyó *et al.* [40] showed a decrease of oxidative stress in urine of type 2 diabetic patients, after only 5 mg oral RES twice daily for 4 weeks. It has to be considered, though, that type 2 diabetics have higher basal systemic oxidative stress levels than metabolically healthy subjects and endogenous cellular stress response is attenuated, possibly featuring the effects of administered xenobiotics [41,42]. In conclusion, further investigations on the short-term and long-term dose-dependent effects of RES on blood and tissue redox mechanisms and enzymes especially in patients with compromised antioxidant defense capacity are needed to determine its antioxidant potential in disease.

Disclosure statement

The authors declare no conflict of interest.

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4 Conclusion and Perspectives

In this thesis, the bioactivity of selected Resveratrol metabolites has been shown in various pathologies in *in vitro* models. In a recent review, Joven and colleagues [109] comprehensively discussed the multifunctional effects of polyphenols in a wide range of diseases. As evident from the diversity of cell models and assays used in the publications this PhD thesis is based on, this also applies to the here investigated group of *in vivo* formed Resveratrol metabolites. Considering the high metabolic transformation rate of oral Resveratrol, much more attention should be directed to the properties, activities, synergies and direct interactions of naturally formed metabolites, and the possible consequences thereof. This is especially important, since substantial effort is made to increase bioavailability of the parent stilbene using novel drug delivery systems, which in turn would also increase metabolite concentrations [128]. Not only could elevated metabolite concentrations result in unexpected effects, but the impact of increased circulating Resveratrol should also be considered. Nutrient-drug interactions can have deleterious effects on drug levels, due to competition for receptors, drug metabolizing cytochromes, or transporters [69, 129]. In *(1) Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells*, sulfated metabolites have been identified as OATP substrates. Resveratrol itself was not only identified as a substrate for various transport proteins, but is also known as a natural modulator of drug uptake, for example increasing the accumulation and effectiveness of doxorubicin in breast cancer tissue at 50 μM , by downregulating BCRP and MRP1 levels [130, 131]. This modulation of drug uptake could also be a way to circumvent drug resistance in the future. On the other hand, in another study on colon cancer cells, Schroeter and Marko [132] demonstrated that the combination of Resveratrol (200 μM) and doxorubicin affected long-term cell growth less than Resveratrol, thus showing no synergistic effects at higher Resveratrol concentrations. These controversial effects should be assessed for metabolites as well, as they are the major circulating agents in human plasma. Another point to consider in nutrient-drug interactions is substrate overlap at cellular transporters. As already mentioned

in chapter 1.3.3, the consumption of orange juice, for example, is associated with lower systemic absorption of the anti-allergic drug fexofenadine [69]. Also, Mandery and colleagues [133] could show the inhibition of hepatic OATP transporters by the flavonoids quercetin, kaempferol, apigenin, rutin, naringin and naringenin. As Resveratrol and metabolites are substrates for OATPs, drug absorption and distribution could be impaired in the presence of Resveratrol and metabolites, due to their possibly higher affinity to the transporters or a direct inhibition of other OATP transporter subtypes. The uptake data presented in *(1) Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells*, *(2) Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells* and *(3) Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages* shows that Resveratrol and sulfated Resveratrol conjugates were preferably taken up by colon cancer cells, breast cancer cells and immune cells in *in vitro* culture, whereas glucuronated metabolites were not taken up into the latter two.

Besides uptake, the TOP2 inhibition shown in a cell free assay in *(2) Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells*, aimed at one aspect of molecular mechanisms of the metabolites' differential anti-cancer properties. Although growth inhibition was not significantly altered in colon cancer cells, TOP2 inhibition seemed to be structurally related, with metabolites conjugated in C3-position inhibiting the enzyme already at $\geq 100 \mu\text{M}$ (Resveratrol-3-glucuronide, Resveratrol-disulfate) as compared to Resveratrol (inhibition at $\geq 200 \mu\text{M}$), whereas the tested C4'-substituted sulfate and glucuronide conjugates did not inhibit TOP2. The structure-activity relationships of substitution patterns combined with a possible structure dependent uptake into cells would be an important step in identifying the most active structural principle in the future.

Considering the chemokine and cytokine network, the measured uptake does not completely fit the bioactivity pattern. Whereas the bioactivity of Resveratrol and Resveratrol-3-sulfate at 1 and 10 μM could be dependent of their uptake into macrophages, Resveratrol-4'-glucuronide was not taken up, but also showed bioactivity for some factors, as described in *(3) Sulfated and Glucuronated trans-*

Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages.

According to the data presented for Sirt1, glucuronated metabolites show an effect on gene expression at 1 μ M, without a measurable uptake. On the other hand, no such effect was seen for sulfated metabolites at 1 μ M for Sirt1, despite being taken up. So far, no extracellular receptor has been identified for Resveratrol or its conjugates but it is possible that glucuronides exert bioactivity via cell surface receptors, independent of uptake into cells.

The effects on cytokines and chemokines in activated macrophages in publications (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages* and (4) *Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations* are promising, due to the dietary achievable concentrations used (1 – 10 μ M), as well as the fact that macrophages are involved in a multitude of chronic diseases, many of them worldwide health issues.

In contrast to other bioactivities, metabolites did not have an effect on the endothelial nitric oxide system, as discussed in (5) *Impact of Trans-Resveratrol-Sulfates and -Glucuronides on Endothelial Nitric Oxide Synthase Activity, Nitric Oxide Release and Intracellular Reactive Oxygen Species*, whereas the unconjugated compound did. In this case, as opposed to TOPII inhibition, conjugation seemed to have a deleterious effect on bioactivity.

As reviewed by Carrizzo and colleagues in 2013 [12], repeated doses of oral Resveratrol (5 to 500 mg) showed an alleviating effect on plasma ROS in healthy humans. In contrast, after single low (50 mg) and high (5 g) dose dietary Resveratrol, presented in (6) *Concentration dependent effects of resveratrol and metabolites on the redox status of human erythrocytes in single dose studies*, no impact on blood oxidative state was seen in either intervention, but an effect on the activity of detoxifying enzymes in erythrocytes was determined by comparison of low to high dose. As oral Resveratrol is antioxidatively active in repeat-dose studies, but not single dose studies, accumulation of the parent compound could elicit these

positive effects on ROS, but due to conjugation, a loss of effect could occur after only a single dose.

As demonstrated in the publications in this thesis, there is an obvious difference in active concentrations of Resveratrol and its metabolites. To influence cytokines and chemokine levels, concentrations of 1 – 10 μM have shown activity, whereas for TOP2 inhibition or the growth inhibition of breast cancer cells, concentrations of up to 250 μM are required. This opens up the questions of drug delivery, of systemic versus local administration. With dietary supplementation, one could reach plasma levels required for altering cytokine and chemokine levels, as detailed in 1.2. As for tissue accumulation, a very high mean concentration of about 670 μM Resveratrol has been found in proximity to colon cancer tissue, after oral Resveratrol supplementation for 8 days ($n = 10$), exceeding concentrations needed for cancer growth inhibition [46]. Such high concentrations of active metabolites reached in target tissues could also prove effective in cancer therapy. Novel drug delivery systems based on nanoformulations with an affinity to cancer tissues could present a future perspective not only for Resveratrol but for targeted transport of active metabolites [128].

Not all assays in this thesis could be conducted with all known metabolites, due to limited availability. Metabolites that were not included in the here presented studies were, for example, Resveratrol-diglucuronides [45], Resveratrol-glucuronide-sulfate [134], or the dihydrated form of Resveratrol, a metabolite formed by gut bacteria [48]. One major perspective would be to complete data for all known metabolites, to better be able to compare bioactivity profiles and estimate metabolites' *in vivo* impact. A prospect for the future would also be the further testing of combinations of metabolites, and the exploration of possible synergies in effects, as physiological metabolites occur at roughly the same time in plasma (also see 1.2).

Equally important would be studies of molecular targets of metabolites, not only to look into the underlying mechanisms of bioactivity of metabolites, but also for predicting competition and interaction with drugs or other nutrients at targets like Sirt1, NF- κB , or OATPs.

Conclusion and Perspectives

Another focus could be the effects of Resveratrol and metabolites on chemokine and cytokine receptors (also see 3.3 and 3.4). The importance of chemokine and cytokine receptors has been shown in animal receptor knockout models or humans with altered allele function [105]. Receptors seem to play a role in allergic airway and intestinal diseases, cancer, HIV and West Nile virus resistance, malaria, arteriosclerosis, immunosuppression and transplant rejection, and could provide valuable drug targets [105]. The effects of macrophage treatment on receptor mRNA and proteins with Resveratrol and metabolites presented in (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages* and (4) *Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations* are not easy to interpret, especially since the used anti-inflammatory control dexamethasone is already acting differentially in activated macrophages, as determined in (3) *Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages*. To better be able to interpret the impact of substances on receptor levels, a detailed characterization of changes in receptor transcription, protein localization and function (transcript variants) in the respective cell culture model would be of advantage.

For the parent compound Resveratrol many indirect and some direct mechanisms of action have already been determined. Resveratrol has been shown to indirectly interfere with pathways underlying inflammation, nitric oxide synthesis, apoptosis, post-transcriptional mRNA modulation and cytochrome activity [129, 135, 136]. Resveratrol was shown to directly interact with various enzymes of inflammation, metabolism, cell signaling and cell cycle regulation [137]. For Resveratrol metabolites, no such data are available yet, making the interpretation of differential outcomes for Resveratrol and its metabolites difficult and so far, only suggestive.

As a very interesting future perspective, albeit not directly related to this thesis, Resveratrol has also been proven effective in various skin diseases, and local drug delivery systems for with high skin penetration and photostabilization have been established [138]. To my knowledge, there are no studies on the effects of oral Resveratrol on skin condition, but based on the results presented in this thesis, it

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might be hypothesized that metabolites or combinations thereof could be as or even more effective than the parent stilbenoid, if locally applied.

It might also be worth looking at the ongoing metabolism of Resveratrol metabolites, as demonstrated in the *in vitro* publications (1) *Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells* and (2) *Inhibition of topoisomerase II by phase II metabolites of resveratrol in human colon cancer cells*, and the currently unknown extent and cellular specificity of formation, as well as the bioactivity of those *in situ* formed metabolites.

To sum up, in this thesis, physiologically formed Resveratrol metabolites were profiled for their bioactivity in various *in vitro* cell assays, but also *in vivo* in the context of free radical balance in human blood. The publications this thesis is based on show that all tested metabolites are in some way bioactive, but also bring up a lot of unanswered questions, to be addressed in the near future.

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6 Abstract

Resveratrol is extensively metabolized in the human body after oral ingestion. This thesis aimed at the bioactivity profiling of the most abundant physiological sulfated and glucuronated metabolites Resveratrol-3-sulfate, Resveratrol-disulfates, Resveratrol-3-glucuronide and Resveratrol-4'-glucuronide. For this purpose, *in vitro* cell culture models were established for uptake, cancer cell growth and topoisomerase II inhibition, atherosclerosis, immunity and inflammation.

Uptake experiments (12.4 - 400 μ M) showed that Resveratrol-3-sulfate was exclusively transported by OATP1B3 and Resveratrol-3,4'-disulfate was transported into cells by OATP1B1 and OATP1B3. Glucuronides showed no OATP-dependent transport. Additional cytotoxicity tests in the breast cancer cell line ZR-75-1 showed only a maximum reduction of viable cells to about 60% after incubation with the disulfate (10 - 400 μ M) whereas comparable concentrations of Resveratrol decreased viable cells to less than 20%.

In colon cancer cells, Resveratrol, Resveratrol-3-sulfate, Resveratrol-disulfates and Resveratrol-3-glucuronide showed inhibition of Topoisomerase II at concentrations between 100 and 200 μ M, whereas the 4' conjugated compounds did not. The minor cytotoxic effect of Resveratrol-3-sulfate in colon cancer cell lines CaCo2 and HT29 was in accordance with its four times lower uptake into cells as compared to Resveratrol.

In cell based assays of immune-modulation and inflammation in activated macrophages, especially sulfated conjugates (Resveratrol-3-sulfate, Resveratrol-disulfates) showed alleviating effects on chemokine and cytokine release at 1 μ M (IL-8, MIP-1 β , MCP-1, IL-6, TNF- α) and glucuronated compounds up-regulated Sirt1 mRNA at 1 μ M.

Resveratrol metabolites showed no bioactivity regarding endothelial nitric oxide synthase, NO release or intracellular ROS, whereas the parent compound did ($\geq$ 30 μ M).

After oral intake of high (5 g) and low (50 mg) single dose Resveratrol, no effect on plasma antioxidant capacity and plasma ROS could be determined in humans, but enzyme activity of detoxifying SOD and CAT in erythrocytes was blunted after the higher dose as compared to the lower dose.

In conclusion, physiologically formed metabolites exert bioactivity and could contribute to the health effects of the parent compound *in vivo*.

7 Zusammenfassung

Resveratrol wird nach oraler Aufnahme in den menschlichen Körper fast vollständig metabolisiert. Diese Dissertation untersuchte die Bioaktivität der gebildeten Metabolite Resveratrol-3-sulfat, Resveratrol-disulfate, Resveratrol-3-glukuronid und Resveratrol-4'-glukuronid. Zellkultursysteme für Transporter-abhängige Aufnahme, Krebszellwachstum und Topoisomerase II Hemmung, Arteriosklerose, Immunabwehr und Entzündung wurden etabliert. Resveratrol-3-sulfat wurde nur über den Transporter OATP1B3 aufgenommen und Resveratrol-3,4'-disulfat über OATP1B1 und OATP1B3 (12.4 - 400 μ M). Glukuronide zeigten keinen OATP-abhängigen Transport. Zusätzliche Zytotoxizitätstests in der Brustkrebs-Zelllinie ZR-75-1 mit dem Disulfat (10 - 400 μ M) zeigten eine Reduktion der lebenden Zellen auf ca. 60%, während vergleichbare Konzentrationen von Resveratrol die lebenden Zellen auf unter 20% reduzierten. In Darmkrebszellen zeigten das 3-Sulfat, Disulfat und das 3-Glukuronid eine Topoisomerase II-Hemmung zwischen 100 und 200 μ M, während an 4'-Position konjugierte Derivate keinen Effekt hatten. Die geringe Zytotoxizität des 3-Sulfates in den Darmkrebszellen CaCo2 und HT29 spiegelte sich auch in dessen, verglichen mit Resveratrol, viermal geringerer Aufnahme in die Zellen wieder. In einem zellbasierten Versuch zu Immunmodulation und Entzündung in aktivierten Makrophagen konnten speziell die sulfatierten Konjugate eine Verminderung von Chemokinen und Zytokinen (IL-8, MIP-1 β , MCP-1, IL-6, TNF- α) bei 1 μ M und eine Hochregulierung von Sirt1 mRNA durch glukuronisierte Substanzen bei 1 μ M zeigen.

Metabolite zeigten im Gegensatz zu Resveratrol (≥ 30 μ M) keine Bioaktivität im Bezug auf endotheliale Stickstoffmonoxid Synthase, Stickstoffmonoxid (NO) Freisetzung oder intrazellulären reaktiven Sauerstoffspezies (ROS). Nach oraler Aufnahme einer hohen (5 g) und einer niedrigen (50 mg) Dosis Resveratrol konnte kein Einfluss auf die antioxidative Kapazität und ROS in humanem Plasma festgestellt werden, aber die Enzymaktivität von SOD und CAT in Erythrozyten wurde nach Aufnahme der höheren Dosis verringert, im Vergleich zur niedrigeren Dosis.

Zusammenfassend kann festgestellt werden, dass physiologisch gebildete Metabolite bioaktiv sind und zu den positiven Effekten von Resveratrol auf die Gesundheit beitragen könnten.

8 Curriculum Vitae

EDUCATION AND TRAINING

30.09.2011	Austrian Pharmacist's diploma , with degree of excellence
01.07.2010	Diploma Exam , Pharmacy, with degree of excellence
2005-2010	Universität Wien , Diploma studies Pharmacy
2004-2005	Universität Wien , Diploma studies History of Art
2004	General Qualification for University , GRG13 Wenzgasse, 1130 Vienna, with degree of excellence

PUBLICATIONS (Peer-reviewed)

2013	<i>Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations</i> Walker, J., Schueller, K. , Schaefer, L.-M., Pignitter, M., Esefelder, L. and Somoza, V., Food Funct. 2013 , 5 (1):74-84, DOI:10.1039
2014	<i>Resveratrol and its major sulfated conjugates are substrates of organic anion transporting polypeptides (OATPs): impact on growth of ZR-75-1 breast cancer cells</i> Riha, J., Brenner, S., Böhmendorfer M., Giessrigl, B., Pignitter, M., Schueller, K. , Thalhammer, T., Stieger, B., Somoza, V., Szekeres, T. and Jäger, W., Mol. Nutr. Food Res. 2014 , 00,1–13, DOI: 10.1002
2014	<i>Impact of Trans-Resveratrol-Sulfates and -Glucuronides on Endothelial Nitric Oxide Synthase Activity, Nitric Oxide Release and Intracellular Reactive Oxygen Species</i> Ladurner, A ; Pignitter, M ; Schueller, K ; Heiss, E ; Schachner, D ; Somoza, V ; Dirsch, V :, Molecules 2014 , 19(10), 16724-16736; doi:10.3390
2015	<i>Sulfated and Glucuronated trans-Resveratrol Metabolites Regulate Chemokines and Sirtuin-1 expression in U-937 macrophages</i> Schueller, K ; Pignitter, M ; Somoza, V : J. Agric. Food Chem. 2015 , 63, 6535-6545; DOI: 10.1021/acs.jafc.5b01830
2015	<i>Inhibition of topoisomerase II by phase II metabolites of Resveratrol in human colon cancer cells</i> Schroeter, A; Groh, I; Del Favero, G; Pignitter, M;

Schueller, K; Somoza, V; Marko, D : **Mol. Nutr. Food Res.** **2015**, DOI: 10.1002/mnfr.201500352

2015 *Concentration dependent effects of Resveratrol and metabolites on the redox status of human erythrocytes in single dose studies*

Pignitter, M ; **Schueller, K** ; Burkon, A ; Knorr, V ; Esefelder, L ; Doberer, D ; Zlabinger, G.J. ; Somoza, V : **J. Nutr. Biochem.** **2015**, DOI:10.1016/j.jnutbio.2015.08.032

SYMPOSIA

- | | |
|---------------------------|--|
| 03/2014 | DGE-Meeting Paderborn, Poster: |
| (Poster Award)
Germany | „Identification of t-Res-3-sulfate as antiinflammatory active immune-modulatory metabolite of t-Resveratrol in immune-competent U-937 macrophages“ |
| 11/2015 | Resveratrol Regional Meeting 2015 , Dijon |
| France | Invited Lecture on behalf of V. Somoza: “Resveratrol Bioavailability and Metabolism” |

PUBLICATIONS IN PREPARATION

- | | |
|-------------|---|
| 2016 | <i>Identification of interleukin 8 reducing lead compounds based on SAR studies on dihydrochalcone related compounds</i> |
| | Schueller, K ; Hans, J ; Pfeiffer, S ; Walker, J ; Ley, J P ; Somoza, V : Med. Chem. Letters |
| 2016 | <i>Indicator operational taxonomic units (OTUs) in the oral cavity are related to IL-8 release from lipopolysaccharide challenged gingival epithelial cells in a cohort of healthy volunteers</i> |
| | Schueller, K ; Riva, A ; Pfeiffer, S ; Berry, D ; Somoza, V: Mol. Nutr. Food Res. |

REVIEWING ACTIVITIES

Journal of Agricultural and Food Chemistry