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Research paper

Optimization of *trans*-Resveratrol bioavailability for human therapy

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ABSTRACT

We have developed an innovative soluble galenic form to overcome the low absorption of *trans*-Resveratrol (t-Res) as a dry powder. We present here data on pharmacokinetics, bioavailability, and toxicity of t-Res in human volunteers treated with this soluble form, plus additional data on biological effects in rodents. Fifteen healthy volunteers of both sexes received 40 mg of t-Res in two forms, the soluble formulation (caplets) and the original powder (capsules), in a crossover design. Blood samples were collected at 15 min, 30 min, and every hour for 5 h. Plasma concentrations of t-Res and its metabolites were analyzed by liquid chromatography and mass spectrometry. The single dose (40 mg) of the soluble t-Res was well absorbed and elicited biologically efficient blood levels (0.1–6 μM) for several hours, despite metabolization into glucuronide and sulfate conjugates coupled to renal elimination. In contrast, t-Res administered as a dry powder barely elicited efficient blood levels for a short duration. The new formulation led to 8.8-fold higher t-Res levels in plasma versus the powder. t-Res metabolism was not modified and neither intolerance nor toxicity were observed during the study and the following week. The soluble formulation elicited a robust anti-inflammatory effect in various tissues of mice fed a high-fat diet, while dry powder t-Res was almost inactive. Our data suggest that significant improvements in t-Res bioavailability and efficiency can be obtained by this soluble galenic form, also allowing lower doses. The use of t-Res in human therapy is thus greatly facilitated and the toxicity risk is reduced.

1. Introduction

The beneficial properties of *trans*-Resveratrol (t-Res) are extensively depicted in the literature. They comprise antagonization of the arylhydrocarbon receptor, kinases inhibition, anti-inflammatory, analgesic and anti-tumoral activities. Protective effects against cardiovascular and neurodegenerative diseases as well as metabolic effects on bone and glucose metabolism homeostasis have also been described [1–7]. Conversely; the exact relation between t-Res and anti-aging mechanisms based on the AMPK/sirtuins pathway remains a matter of debate [8]. All animal and human studies concur on the poor bioavailability of t-Res through low uptake and extensive metabolization. In humans and rodents, t-Res rapidly undergoes sulfatation and glucuronidation resulting in 1% of the oral dose being observed as free t-Res in blood plasma [9–11]. The active concentration of t-Res lays in the micromolar range in most experimental models [12,13] and review in Saiko et al., [14]. The biological efficiency of t-Res will depend on the

Abbreviations: BMI, body mass index; C_{max}, maximal concentration; HPLC, high pressure liquid chromatography; LC–MS, liquid chromatography coupled to mass spectrometry; t-Res, *trans*-Resveratrol; SEM, standard error of the mean; HFD, high-fat diet; IL-10, interleukin-10; HO-1, heme oxygenase-1; PAI-1, endothelial plasminogen activator inhibitor or serpin E1; q-RT-PCR, quantitative real time polymerase chain reaction.

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amount of the active molecular species in the bloodstream and target tissues [14]. A gap between the properties of natural compounds found in foodstuffs and their potential clinical benefits is frequent and it is difficult to overcome their poor bioavailability. Different strategies have been designed to overcome this problem in the case of t-Res, as reviewed in Ref. [15]: (i) co-administration of inhibitors of t-Res metabolism (ii) search for analogs or (iii) elaboration of new t-Res delivery systems. We have increased t-Res total plasma concentration (native form and metabolites) about 10-fold by a novel soluble formulation. We consider this soluble state mimics t-Res natural condition in plants and is crucial for optimal oral absorption. The formulation consists in a complex dietary oil solution of 20 mg t-Res embedded in a caplet. A patent has been filed under the reference "WO: YVERY n° 2010/007252". We present here our investigations on the pharmacokinetics and toxicity of t-Res in human volunteers using this novel soluble form, with additional data on the anti-inflammatory effects of both t-Res forms in rodents.

2. Materials and methods

2.1. Chemicals

T-Res (t-Res, 99% pure) or [(*E*)-1-(4′-hydroxyphenyl)-2-(3,5-dihydroxyphenyl) ethene] was obtained from Yvery SARL, France, either as a natural compound from *Polygonum cuspidatum* (>98% pure) or as its galenic forms (dry powder and soluble lipid formulation). The lipid formulation consists of natural t-Res powder (40 mg) dissolved in a complex mixture notably containing polysorbate 20 and polyglyceryl-3-dioleate. This solution is embedded in a caplet as described in patent WO YVERY N° 2010/007252. Other chemicals were purchased from Sigma–Aldrich (France).

2.2. Patient recruitment and protocol

The human study was approved by the local Ethics committee of Can Tho Central General Hospital (315 National Highway 91B, Can Tho, Vietnam Republic). No extensive blood sampling was intended except for t-Res measurements. Only verbal consent was required from patients for this study, as stated by the French bioethics decree N° 2007–1220 published in the Official Journal of the French Republic. This work was carried out in accordance with the "Code of Ethics" of the "World Medical Association" (WMA Declaration of Helsinki, [16]) for experiments involving humans.

Fifteen healthy volunteers (11 men and 4 women) were anonymously recruited ($n = 15$, aged 25 to 68 years-old, male and female with a BMI = 20.68 kg/m^2 ± 2.01). The volunteers did not receive any other pharmaceutical a month before and during the study.

Both preparations (soluble formulation and dry powder caplets) were compared for t-Res bioavailability in humans. Volunteers received 40 mg (two 20 mg caplets) of t-Res in each form and blood samples were collected after 15 min, 30 min, and every hour for 5 h. A counter-analysis was performed with group inversion after a one-week washout period.

In parallel to the assay, subjects were monitored by clinical examination plus an oral questionnaire for possible side effects, aiming at the previously described gastrointestinal effects (abdominal pain, nausea and diarrhea), skin rash and headache [17,18]. Volunteer surveillance was pursued for a week after the test.

2.3. trans-Resveratrol analysis

Blood plasma t-Res levels in humans were measured by high performance liquid chromatography (HPLC) followed by mass spectrometry (LC–MS). The HPLC system consisted of a binary micro pump Series 200 (Perkin–Elmer, France). t-Res was extracted from plasma by methanol. Following centrifugation at 1700 g for 30 min, the supernatant was collected and dried under nitrogen. The sample was dissolved in 100 µl of phase A buffer (10% methanol/5 mM ammonium acetate), phase B was 95% methanol. t-Res and metabolites were separated by a [5 µm, 2.1 × 150 mm] C-18 Column (Waters, France). The elution gradient steps were: 40–80% B phase for 5 min, steady 1 min at 80%, back to 40% phase B in 1 min and 40% phase B for 4 min. Characterization of t-Res species was carried out by liquid chromatography coupled to mass spectrometry (LC–MS) taking in account the parameters described in literature for t-Res and its metabolites [19]. For LC–MS, an API 3000 triple quadrupole mass spectrometer was equipped with a Turbo Ionspray source (Applied Biosystems, France) with an Endplate offset at 500 V and capillary voltage settled at −4500 V. Dry gas pressure was set at 8 bars, and dry temperature was 180 °C. Detection limit was 1 ppb. Quantification at 306 nm used a calibration curve of t-Res. All analyses were performed in triplicate. Pharmacokinetics analysis was performed with Kinetic-Pro software. Plasma concentrations of t-Res and metabolites (in nM) are shown as mean (±SEM) of data from 15 individuals. Statistical differences (time to time) were evaluated by the Wilcoxon test using Graph Pad Prism 5.0 ($^*p < 0.05$). Differences in pharmacokinetic parameters (e.g. AUC, metabolization rates) were analyzed using Sigma-Stat software ($^{**}p < 0.01$).

2.4. Pharmacokinetics and statistical analysis

Plasma concentrations of t-Res and metabolites, expressed in nM are the means (±SEM) of 15 individual data. Statistical differences (time to time) were evaluated by the Wilcoxon test using Graph Pad Prism 5.0. Significance level was set at $p < 0.05$. Differences in pharmacokinetic parameters (e.g., Area Under the Curve (AUC), metabolization rates) were analyzed using Sigma-Stat software. A p value of 0.01 was considered statistically significant.

The total t-Res (parent and metabolite compounds) pharmacokinetic parameters from a single oral dose (approximately 0.18 mg/kg of body weight corresponding to two caplets of 20 mg each) to volunteers using two different galenic forms (original powder and soluble formulation) were derived from the individual plasma drug concentration–time profiles using a non-compartmental model with PK Solution™ software (version 2.0, Summit Research Services, Ashland, OH, USA). Parameters calculated were a maximum observed plasma t-Res concentration (C_{max}); time of occurrence of C_{max} (t_{max}); area under the plasma concentration–time curve from time zero AUC from time zero to infinity ($AUC_{0-\alpha}$), and apparent terminal half-life ($t_{1/2}$). These parameters were calculated for both formulations.

In the new formulation, as described in Section 2.1. "Chemicals", t-Res is encapsulated in a complex lipid mix. During the transfer to the gastrointestinal membrane, the lipid mix dissolves, and the drug, t-Res, corresponds to the same molecule as found in the original powder. The efficiency of the new soluble formulation was characterized by the ratio of plasmatic AUC's.

2.5. Rodent cytokines assays

Mice were treated with the two t-Res forms as previously described [20]. Briefly, eight week-old male C57Bl/6J wild-type mice (Charles River, L'Arbresle, France) were housed in pathogen-free conditions with a 12-/12-h light cycle and ad libitum access to water and food. Mice were maintained on a normal chow diet for five weeks (NC, 12% fat, 28% protein, and 60% carbohydrate), or a diabetogenic high-fat diet (HFD), containing 72% fat (corn oil, lard, 28% protein, 1% carbohydrate) (SAFE, Augy, France). Two subsets of

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HFD-fed mice (8 mice per group) were treated with t-Res in powder or soluble form by gavage. Mice blood and tissues were submitted to ARN extraction and analyzed for cytokine content by qPCR as previously described. Interleukin-10 (IL-10), Heme Oxygenase-1 (HO-1) and Plasminogen Activator Inhibitor-1 (PAI-1) mRNAs were determined and analyzed statistically by one-way ANOVA and Tukey test ($^{*}p < 0.05$; $^{**}p < 0.01$).

3. Results

3.1. Patients

Fifteen volunteers (11 males and 4 females) participated to this study. Clinical examinations and oral questionnaires searched possible side effects such as gastrointestinal tract effects (i.e., abdominal pain, nausea, and diarrhea), cutaneous rash, or headache. During this monitoring, no adverse effects of t-Res were discovered.

3.2. Pharmacokinetics

Following oral administration of two caplets (20 mg each) of t-Res (original powder and soluble caplets), seven samples were collected from all 15 volunteers and parameters calculation was performed (Table 1). A washout delay was observed between the two administrations. Pharmacokinetics parameters for individual volunteers are presented in Table 1. The C_{max} was approximately 10 times higher in the soluble caplet formulation compared to the original (powder) form. The T_{max} values observed were the same at 30 min. The half-life of the powder form was lower than soluble caplet at 49 and 78 min, respectively. The total $AUC_{0-\alpha}$ values for powder and soluble t-Res were 8.5 times lower for the powder caplet.

The comparative profiles of plasma t-Res kinetics between the soluble and powder formulations are shown in Fig. 1. Individual data from male and female volunteers showed no statistical difference. Gender differences were thus disregarded and data from all volunteers were combined. After powder intake, total t-Res (sum of all t-Res species) increased weakly during the first 30 min then decreased within the first hour. In contrast, following the administration of t-Res in the soluble formulation, plasma concentration reached a mean value in the μM range (2 μM after 15 min and 5.7 μM at 30 min), which was 8.79-fold higher than the with powder. Later, the plasma concentration of total t-Res remained around 500 nM between 2 and 5 h, similar to the peak maximum obtained at 30 min with the powder. All concentrations were significantly higher for the soluble formulation than the powder over the 5 h period ($^{*}p < 0.05$; $^{**}p < 0.01$). After 5 h, t-Res was undetectable when given in powder form. As shown in Fig. 1 insert, this resulted in significantly different kinetics for total t-Res over 300 min, represented as Area Under Curve (AUC) ratios ($^{**}p < 0.01$).

Table 1
Pharmacokinetics parameters: t-Resveratrol plasma concentrations observed after a single oral dose of 40 mg of the soluble formulation or the powder form given to 15 volunteers. C_{max}: maximal concentration. T_{max}: maximal time. $t_{1/2}$: apparent terminal half-life.

	Total t-Resveratrol	
	Powder (original)	Soluble form
Dose (mg t-Res/kg of body weight) (mean)	0.59 mg/kg bw (male) 0.80 mg/kg bw (female)	0.59 mg/kg bw (male) 0.80 mg/kg bw (female)
C_{max} (nM)	470	5707
T_{max} (minutes)	30	30
$t_{1/2}$ (minutes)	49	78
$AUC_{0-\alpha}$ (μmoL minute/L)	39.17	334.22

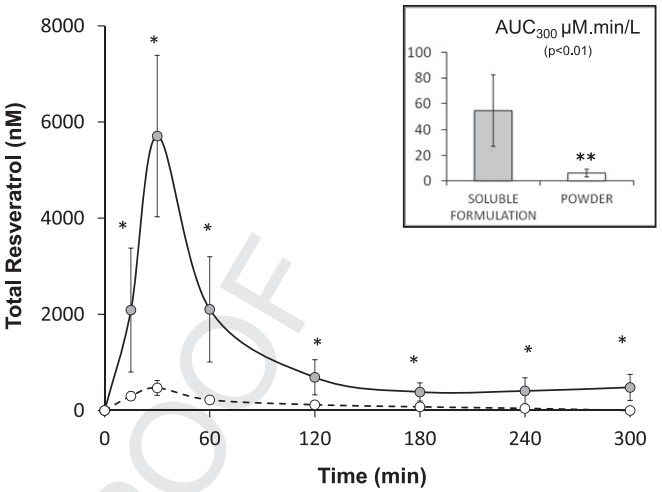

Fig. 1. Total plasma t-Res kinetics. Total t-Res values are expressed in nM and shown as the mean (±SEM). 15 volunteers were given the soluble formulation (gray circles) or powder (empty circles). Data were collected as shown during 5 h following intake. Statistical differences were evaluated by Student's *t*-test followed by the Wilcoxon test (Sigma Stat 2.03), $p < 0.05$ (*). Insert: total t-Res AUCs (300 min) for soluble formulation (gray) or powder (white), $p < 0.01$ (*).

AUC_{300} was 54.7 μM min/L for soluble formulation versus 6.1 μM min/L for the powder.

As shown in Fig. 2, the major conjugate of t-Res in plasma was monosulfate (Fig. 2A), with a maximum of 5.5 μM at 30 min. Besides monosulfate, two other conjugated forms were found and quantified: glucuronide (Fig. 2B) and disulfate (Fig. 2C) with maxima below 100 nM attained in 30 min–60 min. The kinetics of the three conjugates displayed comparable profiles, at variance with free t-Res. Free t-Res displayed a wave-shaped curve with three maxima observed at 15, 60 and 240 min for the soluble formulation. Maximal concentration (327 nM) was at 15 min. In contrast, the kinetic of free t-Res displayed a completely different pattern when the powder form was used. No maximum was found within 60 min. The plasma enrichment of free t-Res increased later and progressively until 180 min then decreased. The maximal concentration for powder was close to 75 nM.

Table 1 displays the differences in plasma concentrations observed after administration of the soluble form and the powder for free t-Res, sulfate-t-Res and total t-Res, represented as Area Under Curve (AUC) ratios. A significant difference was observed, the soluble formulation leading to a 1.7 up to 10.8-fold greater plasma concentration over the dry powder form. Table 2 displays the differential metabolization ratios of t-Res given as soluble formulation or powder. A higher conversion from the parent compound to active sulfate metabolite (93.1 versus 77.2%, $p < 0.01$) was observed with the soluble formulation while free t-Res concentration was lower with the soluble form (4.4% versus 21.3%) Table 3. Q2

In parallel, subjects were monitored by clinical examination plus an oral questionnaire for possible side effects on the day of the test and everyday of the following week. The survey focused on the previously described skin rashes, headache and gastrointestinal side effects (abdominal pain, nausea, and diarrhea) [17,18]. The survey did not reveal any adverse effect of t-Res.

3.3. Solubilization increases anti-inflammatory markers production in rodents

We used our established model of HFD-fed C57Bl/6J wild-type mice [20] to analyze the impact of t-Res on anti-inflammatory markers production in three different organs at the level of

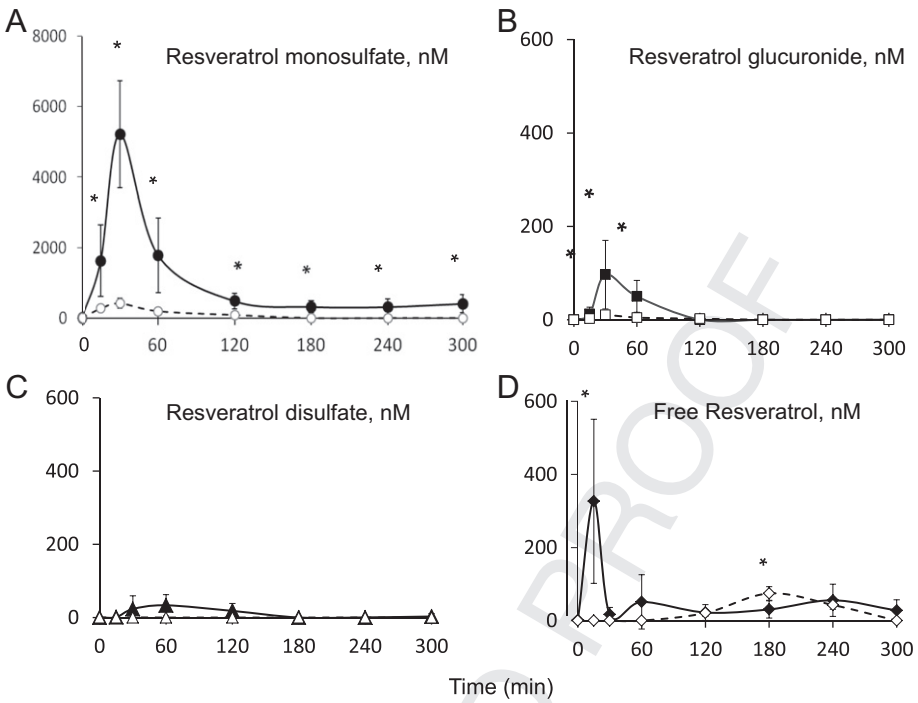

Fig. 2. Plasma free t-Res and metabolites kinetics. Values in nM are shown for t-Res monosulfate (A), glucuronide (B), disulfate (C) and free t-Res (D). Values are shown as the mean (±SEM) of data from 15 individuals, as in Fig. 1. Statistical differences were evaluated by Student's *t*-test followed by Wilcoxon test (Sigma Stat 2.03), ($p < 0.05$ (*)).

mRNA expression measured by qPCR (Fig. 3). Data are presented as mean ± S.E.M and analyzed statistically by one-way ANOVA and Tukey test (**$p < 0.01$). Liver IL-10 and colon HO-1 were robustly increased by soluble t-Res in HFD-fed mice while the powder form was inactive. Similarly, the increase in hypothalamus PAI-1 observed in HFD-fed mice was counteracted by soluble t-Res while the powder form was almost inactive.

4. Discussion

We have developed an efficient formulation (lipid caplets) to significantly improve the oral absorption of t-Res. This was performed by solubilizing natural t-Res from *P. cuspidatum* in a complex lipid solution without any modification of the molecule. The present study shows a significant improvement in total t-Res bioavailability (+780%) when given as a soluble lipid formulation compared to the dry powder. The maximum concentration was 5.7 μM at 30 min for the soluble formulation. This is superior to previously reported data using comparable or higher amounts of dry powder t-Res in capsules.

The increase in bioavailability enabled us to use doses well below those reported in the literature (10–100-fold) [21]. A single dose in two lipid caplets (40 mg) of soluble t-Res was able to increase t-Res plasma concentrations around 10-fold, depending of the molecular species, compared to the dry powder form. In these conditions, t-Res is well absorbed and remains at biologically efficient blood levels (0.1–6 μM) for several hours (up to 5 h) despite rapid metabolism and renal elimination. In contrast, the dry powder form was unable to elicit efficient blood levels at any time: Maximal concentrations ranged from 7 to 73 ng/mL. As shown in Fig. 2D, free t-Res kinetics was different between powder and soluble formulation: the powder curve showed a single peak at 180 min. Differences in plasma free t-Res were also significant but to a lesser extent (1.73-fold increase, $p = 0.009$).

The free/total ratio represented 21% with the standard powder and 4% only with the soluble formulation (Table 2). This may be due to a galenic-dependent variance in free t-Res cellular uptake and/or liver metabolism [22,23]. Further studies are required to validate these hypotheses but the important point is that the maximal t-Res concentration remained below 75 nM at all times for the powder form. Walle et al., report t-Res bioavailability values (maximum and duration) comparable to ours, but they used an ethanol solution, not an option for drugs or nutraceuticals [11].

Table 2
t-Resveratrol plasma concentrations reached after administration of the soluble formulation and powder form. Data are shown as Area Under Curve (AUC) of total t-Resveratrol, monosulfate t-Resveratrol and free t-Resveratrol. SEM: standard error of the mean.

	Total t-Resveratrol		Sulfate t-Resveratrol		Free t-Resveratrol	
	Soluble form	Powder	Soluble form	Powder	Soluble form	Powder
AUC (0–5 h) in μM min/L	343.7	39.1	320.8	30.2	14.2	8.2
SEM	93.8	4.6	89.9	4.2	7.5	1.3
Inter-individual variability (%)	27.3	11.8	28	14	52	15.7
AUC ratio: soluble form/powder	8.79-fold		10.6-fold		1.73-fold	
Paired *t*-test	$p < 0.001$		$p < 0.001$		$p < 0.009$	

Table 3
Metabolization of t-Resveratrol. Data are shown as Area Under Curve (AUC) ratios calculated from plasma concentrations of sulfate and free t-Resveratrol relative to total t-Resveratrol.

	AUC (0–5 h) ratios in μM min/L	
	Sulfate/total t-Resveratrol	Free/total t-Resveratrol
Soluble form	93.1 ± 2.3%	4.4 ± 2.6%
Powder	77.2 ± 3.2%	21 ± 3.3%
Paired *t*-test	$p < 0.01$	$p < 0.01$

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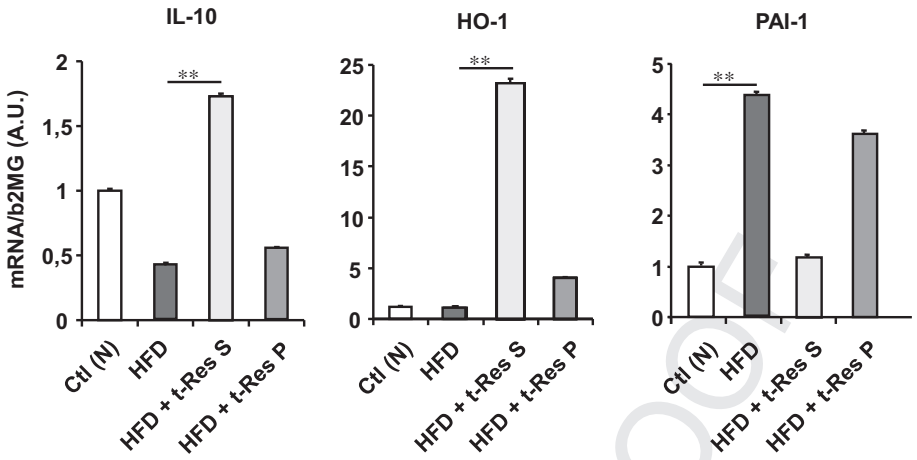

Fig. 3. t-Res decreases inflammation markers in HFD-fed mice. Liver IL-10, colon HO-1 and hypothalamus PAI-1 were measured by Q-RT-PCR with β2-Microglobulin (b2MG) as standard. The fold-induction of treatments versus control conditions is shown as arbitrary units (mRNA/b2MG (A.U.)). IL-10: linterleukin-10 mRNA levels in liver. HO-1: Heme Oxygenase-1 in colon. PAI-1: Plasminogen Activator Inhibitor-1 in hypothalamus. Control conditions: normally fed mice, Ctl (N), white bars. HFD: high fat diet-fed mice treated with vehicle, HFD, dark gray bars or t-Res soluble form, HFD + t-Res S, light gray bars or powder, HFD = t-Res P, medium gray bars. Data are presented as mean ± S.E.M, $n = 8$ mice per group and analyzed with one-way ANOVA followed by Tukey test, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

A greater inter-patient variability was observed in plasma concentrations when t-Res was given in soluble formulation. This variability was below 30% for total t-Res, a value that matches that of most oral forms [24]. In the specific case of polyphenols, these concentrations vary between chemical structures but remain generally well below micromolarity [25,26].

Genetic differences in metabolism between Asians and Caucasians are frequent, especially in phase I and phase II enzymes, which functionalize and conjugate drugs. Pharmacokinetics variability may cause differential results in drug metabolism, therapeutic or adverse effects and toxicity in persons of different ethnic origin as reviewed in Ref. [27]. A potential difference in t-Res metabolism in caucasian population is therefore a possibility, but its impact on t-Res efficiency should be largely superseded by the major absorption increase brought about by our formulation. The question remains of the potential pro-drug ability of the monosulfate, the major t-Res circulating form, after hydrolysis into active t-Res in target tissues [28].

We initially demonstrated the inhibitory effect of t-Res on Interleukin-1β production *in vitro* [12]. Since then, numerous works have demonstrated other anti-inflammatory properties of t-Res as reviewed in Refs. [3,6]. In the present study, we have taken advantage of the ability of t-Res to elicit tissular anti-inflammatory responses. Liver IL-10 and colon HO-1 levels were robustly increased by soluble t-Res in HFD-fed C57Bl/6J wild-type mice while hypothalamus PAI-1 was inhibited (Fig. 3). The powder form was practically inactive, showing that bioavailability is the major determinant for biological activity.

We previously observed that t-Res levels of 50 μM and above were required to elicit an estrogenic effect in a model of breast cancer cells transfected with an estrogen-responsive gene construct (J.F. Savouret, unpublished data). As a weak estrogen, t-Res might stimulate breast cancer growth. However, several studies reported that t-Res actually impedes the growth of breast cancer cells and promotes their apoptosis [29–32].

Another benefit of the low doses used in the soluble form is the improved tolerance for t-Res. Previous dose-escalation studies in humans volunteers showed that a 1 g dose of t-Res (as dry powder) caused no significant adverse effects, only minor signs, which were not clearly related to t-Res administration [10]. Higher doses (5 g) elicited benign gastrointestinal effects in a few volunteers [18,33]. A study using 400 mg reported minor side effects such as skin rash, headache and nasopharyngitis [17]. These high doses of t-Res in powder form are in sharp contrast with the 40 mg used in the present study. t-Res was well tolerated and no toxicity was reported for the duration of the test and its immediate aftermath despite the significant increase in t-Res plasma levels.

5. Conclusion

We have developed an efficient formulation (lipid caplets) for t-Res therapeutical use in humans. This was performed by solubilizing natural t-Res from *P. cuspidatum* in a complex lipid solution without any modification of the molecule. This bioavailability increase enabled us to use doses well below those previously reported in the literature and still increase t-Res absorption around 10-fold over the dry powder form. In these conditions, t-Res was well absorbed and remained at biologically efficient blood levels for several hours despite rapid metabolism and renal elimination. In contrast, the dry powder form was unable to elicit efficient blood levels at any time. As a benefit of the low dose used, t-Res was well tolerated during the test and its immediate aftermath. Our data bring a significant improvement for t-Res use in human nutrition and therapeutics.

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